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LACTIC ACID BACTERIA AND THEIR USES IN ANIMAL FEEDING TO IMPROVE FOOD SAFETY

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I. MICROBIAL ANTAGONISM

In 1908 Elie Metchnikoff was awarded the Nobel Prize for his work with the lactic acid bacteria (LAB). He reported that populations that ingested soured milk such as the Bulgarians were known for their longevity. He studied intestinal microflora and reported that LAB were beneficial to human health, making him the first scientist to report benefits of these organisms. Since then, numerous scientists have studied not only the health benefits associated with the LAB, but also the concept of microbial antagonism of the LAB toward food-borne pathogens.

The concept of microbial antagonism or microbial interference has been known for several decades. This concept refers to the inhibition of undesired or pathogenic microorganisms, caused by competition for nutrients, by the production of antimicrobial metabolites (Gombas, 1989; Holzapfel *et al.*, 1995; Hugas, 1998; Hurst, 1973; Jay, 1996; Stiles, 1996) or by various other mechanisms depending on the situation in which the LAB are used.

Microbial antagonism was first observed in food products as one of the earliest means of preservation. Pure cultures of LAB have been used since the beginning of the twentieth century as starter cultures in fermented food products. Metabolism of these cultures may contribute in a number of ways to the control of pathogens and the extension of the shelf life in addition to the modification of the sensory attributes of the food product (Gombas, 1989; Holzapfel *et al.*, 1995; Hurst, 1973; Jay, 1996). Antagonism between two species or genera of microorganisms takes place when they compete for a common niche, or one of the microorganisms may produce an antagonistic extracellular agent or modify the environment so the other is inhibited (Hugas, 1998; Lindgren and Dobrogosz, 1990; Vandenbergh, 1993).

The use of LAB as "protective cultures" rather than starter cultures has gained importance. This biopreservation approach refers to the extended storage life and enhanced safety of food using their natural or controlled microflora and their antibacterial products (Gombas, 1989; Holzapfel *et al.*, 1995; Jay, 1996; Stiles, 1996). Scientists have discovered the protective effects of the LAB that can inhibit food-borne pathogens in the live animal before slaughter. Because many animals are reservoirs for food-borne pathogens, inhibition of the pathogen in the animal can protect the food supply from

pathogen contamination. The use of LAB to inhibit pathogens in live animals is the focus of this chapter.

II. LACTIC ACID BACTERIA

The LAB constitute a group of gram-positive bacteria that share similar morphologic, metabolic, and physiologic characteristics. They are non–spore-forming rods and cocci that ferment carbohydrates forming lactic acid as the major end-product (Aguirre and Collins, 1993), hence, the denomination *lactic acid bacteria.* Depending on the metabolic pathways they use to ferment carbohydrates and the resulting end-products, LAB are divided into two major groups: homofermentative or heterofermentative. They are generally catalase negative, anaerobic in nature, and nonmotile and do not reduce nitrate (Salminen *et al.*, 1996). LAB have complex nutritional requirements for their growth, such as carbohydrates, amino acids, peptides, nucleic acid derivatives, fatty acids, salts, and vitamins (Hardie *et al.*, 1986). They are generally acid tolerant with different species having adapted to grow under widely different environmental conditions. They are widespread, and their distribution is related to wherever high concentrations of soluble carbohydrates, protein breakdown products, vitamins, and a low oxygen tension occur (Sandine, 1979). Consequently they are common in milk and dairy products, other fermented foods, intact and rotting vegetable material, silage and intestinal tracts, and mucous membranes of humans and animals. Phylogenetically, the LAB belong to the clostridial branch of the gram-positive bacteria, which also includes genera such as *Clostridium*, *Bacillus*, *Listeria*, and *Staphylococcu*s and is characterized by a low G + C DNA content (Aguirre and Collins, 1993). However, the term *lactic acid bacteria* has become commonly associated with the genera *Lactobacillus*, *Lactococcus*, *Leuconostoc*, *Pediococcus*, *Enterococcus*, and *Streptococcus.* Members of this bacterial group are known to provide considerable benefits to humans, some as natural inhabitants of the intestinal tract and others as fermentative bacteria that impart flavor and texture to a multitude of fermented foods. For many years, there has also been widespread interest in the use of LAB in the biological preservation of foods. These organisms are particularly suitable as antagonistic microorganisms in food because of their ability to inhibit other food-borne bacteria by a variety of means including production of organic acids, hydrogen peroxide, or bacteriocins. Their use as direct-fed microbials for humans and animals has received increased attention (Fuller, 1989; Juven *et al.*, 1991).

Their favorable effect on growth and health is thought to be due to the modulation of other bacterial growth through one or more of these antagonistic factors.

III. ANTIMICROBIAL SUBSTANCES PRODUCED BY LACTIC ACID BACTERIA

Traditionally, reduction of pH and removal of large amounts of carbohydrates by fermentation were considered the primary actions by which LAB inhibit food-borne pathogens. However, it has also been recognized that LAB are capable of producing inhibitory substances other than organic acids with inhibitory activity to different microorganisms. Additionally, these substances may be produced at refrigeration temperatures with no growth (Amezquita and Brashears, 2000). These substances include hydrogen peroxide (Dahiya and Speck, 1968; Juven and Pierson, 1996; Price and Lee, 1970; Rodriguez *et al.*, 1997; Villegas and Gilliland, 1998; Whittenbury, 1964), diacetyl (Jay, 1982; Ouwehand, 1998), reuterin (Axelsson *et al.*, 1989; El-Ziney *et al.*, 1999), bacteriocins (Bruno *et al.*,1992; Christensen and Hutkins, 1992; Kanatani *et al.*, 1995; Okerke and Montville, 1992; Stiles and Hastings, 1991; Tahara *et al.*, 1996), and other low-molecular-weight metabolites (Niku-Paavola *et al.*, 1999).

A. HYDROGEN PEROXIDE

Many fermentative bacteria, including LAB, produce hydrogen peroxide (H_2O_2) as a mechanism for protecting themselves against oxygen toxicity. Lactobacilli, as well as other lactic acid–producing bacteria, lack heme and thus do not use the cytochrome system (which reduces oxygen to water in respiratory metabolism) for terminal oxidation. Lactobacilli use flavoproteins, which generally convert oxygen to H_2O_2. This mechanism, together with the absence of the heme protein catalase, generally results in the formation of H_2O_2 in amounts that are in excess of the capacity of the organism to degrade it. The H_2O_2 formed may inhibit or kill other members of the microbiota. Hydrogen peroxide is an effective antimicrobial because of its strong oxidizing effect on the bacterial cell; sulfhydryl groups of cell proteins and membrane lipids can be oxidized (Condon, 1987; Juven and Pierson, 1996; Kandler and Weiss, 1986; Lindgren and Dobrogosz, 1990; Villegas and Gilliland, 1998; Whittenbury, 1964). The formation and accumulation of H_2O_2 in growth media with a subsequent antagonistic effect was shown with *Staphylococcus aureus* (Dahiya and Speck, 1968) and *Pseudomonas* species (Price and Lee, 1970). Hydrogen peroxide can react

with other components to form inhibitory substances. In raw milk, for instance, hydrogen peroxide generated by LAB can react with endogenous thiocyanate, which is catalyzed by lactoperoxidase to form intermediary oxidation products inhibitory to microorganisms. This mechanism, also known as the *lactoperoxidase antibacterial system*, has been well documented (Condon, 1987).

B. WEAK ORGANIC ACIDS

LAB are non-respiring microorganisms, principally generating ATP by fermentation of carbohydrates coupled to substrate-level phosphorylation. The two major pathways for the metabolism of hexoses are homofermentative or glycolysis (Embden-Meyerhof pathway), in which lactic acid is virtually the only end-product, and heterofermentative (phosphoketolase pathway), in which other end-products such as acetic acid, CO_2, and ethanol are produced in addition to lactic acid (Axelsson *et al.*, 1989; Kandler, 1983; Zourari *et al.*, 1992).

Weak organic acids are known to have strong antimicrobial activity. In solution, these acids exist in a pH-dependent equilibrium between the undissociated and the dissociated state. The effectiveness as antimicrobials is greater at low pH levels because this favors the uncharged undissociated state of the molecule, which is freely permeable across the cell membrane because they are lipid soluble (Cramer and Prestegard, 1977). Subsequently, upon encountering the higher pH level inside the cell, the molecule will dissociate, resulting in the release and accumulation of charged anions and protons that cannot cross the cell membrane (Booth and Kroll, 1989; Eklund, 1983; Ouwehand, 1998).

Of the two major weak organic acids produced by LAB (acetic and lactic acid), acetic acid is the strongest inhibitor because of its higher dissociation constant ($pK_a = 4.75$) as compared to lactic acid ($pK_a = 3.08$) at a given molar concentration and pH level (Eklund, 1983; Holzapfel *et al.*, 1995; Ouwehand, 1998).

C. REUTERIN

Reuterin is a neutral broad-spectrum antimicrobial substance formed during anaerobic growth of *Lactobacillus reuteri* in the presence of glycerol (Axelsson *et al.*, 1989). Reuterin is an equilibrium mixture of monomeric, hydrated monomeric, and cyclic dimeric forms of β-hydroxypropionaldehyde. The inhibitory effect of reuterin has been associated with its action on DNA synthesis by acting as an inhibitor of the substrate binding subunit of ribonucleotide reductase.

D. DIACETYL

Diacetyl is the compound responsible for the characteristic aroma and flavor of butter. It is produced by species of the genera *Lactobacillus*, *Leuconostoc*, *Pediococcus*, and *Lactococcus* (Jay, 1982). Diacetyl is formed from the metabolism of citrate via pyruvate (Axelsson *et al.*, 1989; Lindgren and Dobrogosz, 1990). Jay (1982) reported that this compound was more effective when the pH level was lower than 7.0, but it was progressively ineffective at pH values higher than 7.0. This study was particularly extensive because it evaluated the antimicrobial effects of diacetyl against 40 cultures, including 10 of LAB, 12 of gram-positive non-LAB, 14 of gram-negative bacteria, and 4 of yeasts. Diacetyl was found to be more active against gram-negative bacteria, yeasts, and molds than against gram-positive bacteria.

E. BACTERIOCINS

The biosynthesis, classification, mode of action, and characterization of bacteriocins has been well reviewed and published (Barefoot and Nettles, 1993; Jack *et al.*, 1995; Klaenhammer, 1988, 1993; Montville and Bruno, 1994; Nes *et al.*, 1996; Nettles and Barefoot, 1993; Sahl *et al.*, 1995; Stiles and Hastings, 1991; Vandenbergh, 1993; Yang and Ray, 1994). Bacteriocins are low-molecular-weight, heat stable, ribosomally synthesized, and cationic proteinaceous compounds, produced by gram-positive organisms, with antibiotic-like functionality against closely related species, mainly gram-positive bacteria, by adsorption to receptors on the target cells (Jack *et al.*, 1995; Klaenhammer, 1993). Klaenhammer (1993) proposed that bacteriocins be classified into four major groups based on their biochemical properties. Class I or lantibiotics are small peptides (<5 kDa) and membrane-active bacteriocins, containing the unusual dehydro-amino acids and thioether-amino acids lanthionine and 3-methyllanthionine, respectively. Nisin is the most widely studied class I bacteriocin, and it is produced by *Lactococcus lactis* subspecies *lactis*. Class II bacteriocins are subdivided into three subclasses and in general are small, non–lanthionine-containing, heat-stable, membrane-active peptides. Further subdivisions of class II bacteriocins include class IIa or *Listeria*-active bacteriocins, class IIb that are poration complexes requiring two peptides, and class IIc that are thiol-activated peptides requiring cysteine residues. Examples of class II bacteriocins are pediocin JD (IIa) produced by *Pediococcus acidilactici*, lactacin F (IIb), produced by *Lactobacillus johnsonii*, and lactococcin B (IIc) produced by *L. lactis* subspecies *cremoris*. Class III bacteriocins are defined as large heat-labile proteins, and class IV includes complex bacteriocins, in which lipids and carbohydrates appear to be necessary for activity (Klaenhammer, 1993;

Ouwehand, 1998). In cases in which the mode of action has been investigated, the cell membrane appears to be the site of action. There is enough evidence to conclude that bacteriocins produced by LAB act by the common mechanism of depleting proton motive force (PMF) (Bruno *et al.*, 1992).

Nisin is the best-characterized LAB bacteriocin. The structure of nisin was first elucidated by Gross and Morell (1971). Nisin dissipates the membrane potential in cells of sensitive organisms (Ruhr and Sahl, 1985) and causes PMF depletion of whole cells of *L. monocytogenes* (Bruno *et al.*, 1992) and *Clostridium sporogenes* (Okereke and Montville, 1992). Other LAB bacteriocins such as lactococcin A, lactococcin B (Venema *et al.*, 1993), pediocin JD, (Christensen and Hutkins, 1992), and others share this mechanism of action, which is the dissipation of the PMF in sensitive cells.

F. LOW-MOLECULAR-WEIGHT METABOLITES

Some species of LAB have been reported to produce some metabolites of low molecular weight, such as benzoic acid, mevalonolactone, and methylhydantoin, which exhibit inhibitory activity toward gram-negative bacteria and some fungi (Niku-Paavola *et al.*, 1999). Niku-Paavola *et al.* (1999) reported the production of compounds with a molecular weight lower than 700 Da that inhibited the growth of the gram-negative *Pantoea agglomerans* when these substances were used in combination at a level of 10 ppm. Addition of 1% lactic acid produced a synergistic effect, inhibiting the growth of *P. agglomerans* by 100%.

Although antimicrobial substances produced by the lactobacilli likely contribute to inhibition of food-borne pathogens *in vivo*, the exact mechanisms associated with inhibition in the animal are not well defined. Other factors such as competition for nutrients, inhibition of attachment to the gastrointestinal (GI) tract, and various other properties can occur in the animal. It is likely that a combination of factors are responsible for the ability of LAB to inhibit food-borne pathogens when fed to animals.

IV. INTERACTIONS OF LAB IN THE GASTROINTESTINAL TRACT

Although it is impossible to describe all microbial interactions in the GI tract, some information has been discovered about the interactions of LAB supplementation and the impact on the GI tract microflora and inhibition of food-borne pathogens. We know that the carbohydrate concentration of a diet fed to an animal is changed as it passes through the GI tract, making an *in vitro* model inadequate to predict the behavior of LAB in a live animal (Fuller, 1992). Model systems have been developed but may not exactly

mimic the metabolism encountered *in vivo.* Use of gnotobiotic animals (germ free), combined with model systems, has given us some insight into the behavior of the LAB in the gut of the animal and the mechanisms associated with reduction of pathogens by direct-fed microbial (DFM) supplementation, but the exact mode(s) of inhibition are not known.

After ingestion of any microorganism by a host animal, it can become established in the animal or eliminated. When the microorganism becomes established, it can be at high or low population levels (Fuller, 1992). Ducluzeau *et al.* (1970) described the "barrier effect," which is a condition in the intestinal tract that protects the host from colonization by outside microorganisms. The barrier is established after birth with a small number of bacterial species, and over time, new species will be established. Some of the species involved in colonization have been well documented and are not discussed in this chapter. The barrier can be very important with respect to the administration of a DFM. The DFM must be administered daily to become established as one of the organisms in the natural barrier if it is to continuously provide the desired effects (i.e., suppression of a food-borne pathogen).

Work in gnotobiotic animals has suggested that production of inhibitory substances is at least partially responsible for microbial antagonism, so it is important to select strains that produce antimicrobial products. It is essential to select LAB that have the most potential to produce the desired effect in the animal and then test the LAB *in vivo* to verify that the strain is effective in the animal.

A. EFFECT ON IMMUNE RESPONSE

The effect of LAB on the host immune response has been studied to some extent, and it is postulated that both mucosal and systemic immune responses can be affected by DFM. Bealmer *et al.* (1984) demonstrated that conventional animals with complete gut flora have higher immunoglobulin levels and phagocytic activity compared to germ-free animals. Roach and Tannock (1980) suggested that a systemic effect was exerted by *Enterococcus faecium,* that was established as a monoassociate in germ-free mice and was able to reduce *Salmonella typhimurium* counts in the spleen. Similarly, *Lactobacillus casei* was involved in the stimulation of phagocytic activity when administered perorally to mice in a study by Perdigon *et al.* (1986). For a microorganism to affect systemic immunity, it may be necessary for it to enter the systemic circulation. Bloksma *et al.* (1981) showed that *Lactobacillus* organisms were able to survive in the spleen, liver, and lungs for several days. Saito *et al.* (1981) showed that *L. casei*, given parenterally, stimulated phagocytic activity in mice. Serum immunoglobulin

A (IgA) and immunoglobulin G (IgG) levels have been shown to be increased with administration of *Lactobacillus* in piglets and mice (Lessard and Brisson, 1987; Perdigon *et al.*, 1990). These findings suggest that DFMs have the potential to modulate immunity, and their effect on systemic immune response can be used to overcome infections caused by pathogens such as *Salmonella* that occur in tissues away from the intestinal tract.

V. USE OF LACTIC ACID BACTERIA FOR *IN VIVO* REDUCTION OF FOOD-BORNE PATHOGENS

A. DEFINITION OF *PROBIOTIC* AND DIRECT-FED MICROBIAL

The literal translation of the word *probiotic* is "for life," but for the purposes of animal feeding, it has been given several definitions (Fuller, 1992). Definitions have been defined and modified over the years by Lilly and Stillwell (1965), Parker (1974), and Fuller (1989), and in 1991 Huis in't Veld and Havenaar (1991) defined *probiotics* as "a mono- or mixed culture of live microorganisms, which applied to man or animal (e.g., as dried cells or as a fermented product) affects beneficially the host by improving the properties of the indigenous microflora." The definition was further modified in 1993 when Kmet *et al.* defined *ruminal probiotics* as "live cultures of microorganisms that are deliberately introduced into the rumen with the aim of improving animal health or nutrition." Based on the various definitions, *probiotic* could possibly refer not only to microbial cultures, but also extracts and enzyme preparations. The Office of Regulatory Affairs of the Food and Drug Administration (FDA), as well as the Association of American Feed Control Officials, has recommended the term *direct-fed microbials* be used to describe feed products that contain a source of live naturally occurring microorganisms. Therefore, the term *DFM* is used in this text to describe live cultures that are fed to animals to reduce food-borne pathogens.

VI. SELECTION CRITERIA FOR LACTIC ACID BACTERIA TO BE USED AS DIRECT-FED MICROBIALS

Although some LAB used as DFMs have been shown to have a positive effect *in vitro* and *in vivo,* the responses attained with some studies have been variable. One of the reasons for controversial results is the selection of strains for DFM use. An organism must possess certain attributes to be functional or desirable as a microorganism that will make a good candidate

for a DFM. Following is a discussion of the selection criteria that are considered to produce suitable DFM bacteria.

A. SURVIVAL IN THE GASTROINTESTINAL TRACT

For a DFM to produce desirable effects, it must be able to survive and metabolize in the intestine. This means that the strain must be resistant to conditions encountered in the GI tract.

Although most LAB are somewhat acid tolerant, many may not survive well at low pH values and the acidic conditions of the GI tract could have an adverse effect on the microorganisms. Therefor, it is suggested that microbial cultures to be used as DFMs should be screened for their resistance to acidity. In a study by Conway *et al.* (1987) on the survival of LAB in the human stomach, strains showed variable survival at different pH conditions, acknowledging the importance of screening the strains for acid tolerance.

Similarly, resistance of LAB to bile is an important characteristic that enables them to survive and grow in the intestinal tract (Gilliland, 1979; Gilliland *et al.*, 1984). Bile entering the duodenal section of the small intestine has been found to reduce survival of bacteria, probably because all bacteria have cell membranes consisting of lipids and fatty acid, which are very susceptible to destruction by bile salts. Gilliland *et al.* (1984) reported that when a diet supplemented with a more bile-resistant strain of *L. acidophilus* was fed to newborn dairy calves, greater numbers of facultative lactobacilli were detected in the upper part of small intestine (jejunum) than when a strain with lower bile resistance was used. In some studies, it has been suggested that the ability of *L. acidophilus* to cause a significant increase in numbers of *Lactobacillus* in the intestinal tract may be critical for controlling growth of intestinal pathogens. Hence, the success of a DFM also depends on the selected strain possessing bile-resistant qualities (Gilliland and Speck, 1977).

Acid and bile tolerance are not the only factors affecting survival in the GI tract, but they are the most important. If the DFM fails to survive after ingestion, then the desired effects will not be observed *in vivo*.

B. ADHESION TO INTESTINAL EPITHELIUM

Selection of new DFM strains often involves screening the LAB for adhesion to intestinal cells, which would enhance colonization and reduce the need for daily feeding of the DFM (Salminen *et al.*, 1996). It is speculated that by attachment to the gut wall, the DFM may occupy colonization sites and make them unavailable to other microorganisms, including pathogens. The

role of adhering bacteria in protection against enteric pathogens was recognized by Fuller (1973) who suggested treating newly hatched chicks with pure cultures of adhering lactobacilli. Adhesion is also considered necessary for the microorganism to resist being washed away by contents of the stomach and intestine and by peristalsis (Fuller, 1999). For the DFM to manifest its effect, the ability to remain in the gut for a maximum amount of time is important (Fuller, 1989). This is especially true if the DFM is fed only once or intermittently. However, if the DFM is fed daily, attachment to the intestinal wall and establishment as a part of the natural flora may not be as important because new cultures of the DFM are introduced daily.

The ability of LAB to attach to the cell wall and become colonized varies with strains. Mayra-Makinen *et al.* (1983) reported that the degree of adherence varied greatly among the 13 strains of *Lactobacillus* that showed adherence to columnar epithelium of pigs and calves.

C. HOST SPECIFICITY

In selecting strains for use as DFM supplements, one must consider the source of the organism, which is important because most of these organisms exhibit host specificity. For example, lactobacilli isolated from a specific site of a specific animal source can colonize only epithelium of the same kind. Host specificity of bacterial strains is well recognized and documented (Barrow *et al.*, 1980; Fuller, 1975). Barrow *et al.* (1980) tested the attachment of LAB to gastric epithelium of pigs *in vitro* and found that with the exception of two strains of *Lactobacillus* isolated from the chicken gut, no isolates from animals other than domestic pigs and closely related wild boar were able to adhere to pig squamous epithelium. Similarly, Fuller (1973) demonstrated that *Lactobacillus* obtained from fowl crop adhered only to squamous epithelial cells of chicken intestine but not mouse, rat, or pigs. Host specificity is at least somewhat related to adherence to the GI tract. Again, if a DFM is fed daily, host specificity may not be as important if the organism can survive passage through the GI tract. Daily feeding of the DFM can provide new live cells daily, so the antimicrobial impact can be achieved without adherence or establishment as a part of the normal flora.

D. PRODUCTION OF ANTIMICROBIAL COMPOUNDS

LAB produce a wide variety of antimicrobial compounds such as bacteriocins, organic acids, hydrogen peroxide, and other low-molecular-weight metabolites (discussed previously). Production of these substances may be the primary mechanism for reduction of the food-borne pathogens *in vivo*.

However, several DFM strains of LAB have been shown to exert beneficial effects in the intestinal tract without possessing this property, so other mechanisms such as colonization of adherence sites could be involved with reduction of the pathogen in the animal.

E. ANTIBIOTIC SUSCEPTIBILITY

Although susceptibility to antibiotics does not likely affect the ability of a DFM to exert antagonistic action against a food-borne pathogen, antibiotic resistance in DFM bacteria is an area of growing concern. According to a report, the FDA blocked the introduction of two DFM products for use in chickens on grounds that some of the microorganisms in the products were possibly antibiotic resistant, which could lead to contraction of diseases in humans not treatable by drugs (Philip Brasher, 2000). It is speculated, but not proven, that antimicrobial drugs used in food animals can promote emergence of resistant bacteria that may not necessarily be pathogenic to the animal but may cause severe infections in humans (Philip Brasher, 2000). The use of antibiotics in food animals can also cause nonpathogenic bacteria to become resistant, which may directly or indirectly cause infections in humans.

In the past few years, the LAB most commonly associated with antibiotic resistance have been strains of the genera *Enterococcus,* especially *Enterococcus faecalis* and *E. faecium* (Franz *et al.*, 1999). Enterococci have been used as DFMs to maintain intestinal microbial balance and as a treatment for gastroenteritis in humans and animals. However, the fact that these bacteria have acquired resistance toward clinically used antibiotics, including the glycopeptide antibiotics vancomycin and teicoplanin, increases their threat as opportunistic pathogens (Franz *et al.*, 1999). Resistance is acquired by gene transfer systems such as conjugative or nonconjugative plasmids or transposons (Perreten *et al.*, 1997). Several antibiotic resistance plasmids from *Lactobacillus* species have also been detected. Ishiwa and Iwata (1980) indicated plasmid linkage of tetracycline and erythromycin resistance in human isolates of *L. fermentum*. Morelli *et al.* (1983) observed plasmid-linked resistance for chloramphenicol in *L. acidophilus* and *L. reuteri* isolated from poultry.

Plasmid-associated antibiotic resistance is of special concern because of the possibility of resistance spreading to other more harmful species and genera. Resistance can be transferred from nonpathogenic bacteria to pathogenic bacteria and from bacteria that are normally present in the intestinal tract of animals to those that cause infections in humans. *In vitro* studies have demonstrated that vancomycin resistance is transferable to other

gram-positive bacteria including *Listeria monocytogenes* and *S. aureus* (Leclercq *et al.*, 1989). A chloramphenicol resistance plasmid from an *L. plantarum* strain isolated from raw ground pork (Ahn *et al.*, 1992) was shown to be transferred to other gram-positive bacteria by the help of a wide host range (Clewell *et al.*, 1974). Of other concern is the potential risk of transfer of antibiotic resistance and associated virulence traits to other LAB in foods that could eventually lead to emergence of opportunistic pathogens, especially in immunocompromised individuals. Although streptococci and enterococci are the predominating LAB associated with human infections, other LAB have also been implicated in human infections despite their traditional "generally regarded as safe" (GRAS) status (Gasser, 1994). New species and more specific strains of bacteria that may not share the same historical safety of traditional strains are constantly being sought. It, therefore, becomes important to carefully assess antibiotic resistance in new strains before incorporating them into DFM products that are commercially available.

F. TECHNOLOGICAL PROPERTIES

It was not until recently that technological properties were established as a selection criteria for DFM strains because they largely dictate the successful production and delivery of DFMs. DFMs are fed to the animals directly or through their food in the form of pellets, capsules, paste, powder, or granules. Therefore, at the industrial level, DFM strains that are produced in large quantities have to undergo several processing steps before their use as feed supplements. The processing steps may involve separation by centrifugation or filtration, freezing, or freeze-drying (Klaenhammer, 1998). DFM bacteria should, therefore, be able to withstand stresses such as freezing, high pressure, and temperatures (60–80 °C for 5–10 minutes during pelleting) and should have a high growth rate and achievable cell mass. They should have growth characteristics that make them easy and economical to grow under commercial conditions. They should also be able to retain their viability under storage conditions.

The stability of DFM strains in continuous industrial culturing provides challenge for the industry (Lee and Salminen, 1995). According to Nousiainen and Setälä (1993), most *Lactobacillus* strains do not tolerate pelletizing in an economically feasible way (Klaenhammer, 1998), and they showed a dramatic decrease in the viability of *L. acidophilus* in dried pellets held under refrigeration or at room temperature for 12 months. Stability during culture propagation and storage may have an impact on the *in vivo* response toward the probiotic. Therefore, during the selection of a probiotic culture, one must consider the aforementioned production and stability criteria. If a

live culture is not delivered to the animal, then the beneficial effects will not be observed.

VII. USE OF DIRECT-FED MICROBIALS IN FARM ANIMALS

A. CONCEPT OF DIRECT-FED MICROBIALS

The GI tract microflora, which is established immediately after birth, is considered very important for the performance of farm animals (Fuller, 1989; Nousiainen and Setälä, 1993). The fetus' digestive tract *in utero* is sterile, but on passage through the reproductive tract during birth, it acquires microorganisms, which are rapidly added to the gut (Fuller, 1989). Similarly, chicks are colonized soon after hatching. The gut microflora is obtained from the immediate environment and from nursing from the mother (Bryant and Small, 1960; Ratcliffe, 1985). The final natural gut microflora develops over time and is very complex. It is influenced by initial colonization and the diet of the animal (Fuller, 1989). This stable flora helps the animal to resist infections (Freter, 1956; Lloyd *et al.*, 1977) and aids in digestion, especially in ruminants in which the metabolism of fibrous components in the diet is dependent on the fermentative action of bacteria in the rumen (Savage, 1977, 1986).

According to the FDA (Fuller, 1999), DFMs that have been included in food for many years without any adverse effects are GRAS and have been shown to have beneficial effects in the animal (Fuller, 1989; Juven *et al.*, 1991; Lee *et al.*, 1999). The concept of DFMs is now universally accepted, and a substantial amount of research is being directed toward formulation of mixtures of DFM strains that would have potential beneficial effects in the animal, including improvement of animal performance and inhibition of food-borne pathogens.

B. MICROORGANISMS TRADITIONALLY USED IN DIRECT-FED MICROBIALS

Intestinal strains of LAB and bifidobacteria are most widely used as DFMs, although yeasts such as *Saccharomyces, Aspergillus,* and *Torulopsis,* as well as microorganisms belonging to the genera *Bacillus* and *Clostridium,* have also been used (Fuller, 1999; Macfarlane and Cummings, 1999; Tannock, 1995, 1997). Among the LAB, strains of *Lactobacillus* species are most common and include *Lactobacillus acidophilus*, *Lactobacillus bulgaricus*, *Lactobacillus helveticus*, *L. casei*, *Lactobacillus lactis*, *Lactobacillus plantarum*, *Lactobacillus rhamnosus*, *L. reuteri*, *Lactobacillus fermentum*, *Lactobacillus*

brevis, and *Lactobacillus salivarius* (Fuller, 1989, 1999; Macfarlane and Cummings, 1999). Other strains of LAB include *E. faecalis, E. faecium*, *Streptococcus salivarius* ss. *thermophilus, S. lactis,* and *Pediococcus pentosaceus.* Most frequently used strains of *Bifidobacterium* include *Bifidobacterium bifidum*, *Bifidobacterium breve*, *Bifidobacterium thermophilus*, and *Bifidobacterium pseudolongum* (Fuller, 1989; Macfarlane and Cummings, 1999). The composition of DFM preparations may vary from those containing a single strain of microorganisms to those containing multiple strains of bacteria (Fuller, 1989, 1999).

C. DIRECT-FED MICROBIALS AND MICROBIAL ANTOGONISM IN ANIMALS

Since the first scientific explanation of the favorable effects of soured milk products in humans by Metchnikoff (1907) at the beginning of the twentieth century, the most beneficial part of the intestinal flora is suggested to be LAB. LAB are also the most common organisms used for commercial DFM preparations (Anonymous, 1990; Tuschy, 1986). The emphasis on the LAB stems from evidence that LAB play a central role in the gut flora that enables them to influence the composition of the flora to the benefits of the host. The stomach of the neonatal pigs is shown to be colonized by *Lactobacillus* and *Streptococci* within 48 hours after birth (Dulcuzeau, 1985). Similarly, in newborn calves one of the first groups of microorganisms in the rumen is LAB (Nousiainen and Setälä, 1993). Studies show that when the gut flora develops after birth, as the lactobacilli increase, other components of the flora decrease (Smith, 1965). The claims made for DFM effects of LAB in farm animals are many and varied.

The potential benefits of DFM LAB can be placed into three broad categories: (1) reduction of food-borne pathogens, (2) improved animal performance, and (3) stimulation of immune response. Reduction of food-borne pathogens in the live animal to prevent subsequent contamination of the food supply is the focus of this discussion.

D. PATHOGEN REDUCTION

The most commonly identified beneficial effect of LAB as live feed supplements is their role in resistance to infection, particularly in the GI tract. It has been proposed that these organisms can prevent infection through competitive exclusion (CE) or other mechanisms against pathogenic bacteria in the animal intestine (Bailey, 1987; Pivnick and Nurmi, 1982). According to Bailey (1987), "competitive exclusion" implies the prevention of entry of

one entity into a given environment because that space is already occupied, the competing entity is better suited to establish and maintain itself in that environment, or the competing entity is producing a product hostile (toxic) to its competition." The phenomenon of CE was first described by Nurmi and Rantala (1973) when they demonstrated that *Salmonella* colonization in the gut of a newly hatched chicken could be prevented by dosing it with a suspension prepared from gut contents of healthy adult chickens. The efficacy of the CE concept has since been demonstrated in several laboratories around the world (Barnes *et al.*, 1980).

E. INHIBITION OF FOOD-BORNE PATHOGENS IN POULTRY

The GI tract of poultry is complex. A newly hatched chick will become colonized rapidly with facultative aerobes, but lactobacilli will eventually become the primary organisms present in the crop and small intestine of the chick (Fuller, 1992). Modern poultry production prevents contact of the chick with the parent. Therefore, DFMs have been administered soon after hatching to increase the likelihood of the organisms in the DFM to become a part of the natural microflora of the bird. More research has been done in the area of DFM and inhibition in poultry than in any other species.

Much of the work involving reduction of pathogens by LAB has been done in poultry and has focused on the reduction of *Salmonella,* but some work has also been done on inhibition of *Campylobacter.* The most commonly studied defined organisms are strains of *Lactobacillus,* especially *L. acidophilus.* However, the results obtained from the studies to demonstrate the efficacy of DFMs have been controversial. Additionally, work done on undefined cultures also indicates that pathogen reductions can occur using species other than lactobacilli.

The original work by Nurmi and Rantala (1973) was done with *Salmonella enteritidis,* and subsequent studies have shown that DFM can work against other strains of *Salmonella* such as *S. typhimurium, Salmonella pullorum, Salmonella salivarius,* and *Salmonella blockley*. Fuller (1977) reported that the crop microflora could influence the intestinal microflora. They were able to mimic the crop microflora *in vitro* and in gnotobiotic chicks to demonstrate that a reduction of *Escherichia coli* (nonpathogenic) in the crop was also seen in the ileum.

Watkins *et al.* (1982) administered *L. acidophilus* (unknown orgin) to 2-day-old chicks and subsequently challenged the chicks with pathogenic *E. coli.* The mortality rate of chicks not administered the *L. acidophilus* was 66.7%, whereas that of the ones fed the DFM was only 3.7%. A follow-up

study using the same *L. acidophilus* strains was performed by Watkins and Miller (1983) to determine the impact on *S. typhimurium* and *S. aureus*. When *L. acidophilus* was given 2 days before the pathogen challenge, there were significant reductions in both mortality and pathogen shedding associated with the chicks given the DFM.

Jin *et al.* (1996) demonstrated that a combination of *Lactobacillus* strains isolated from chicken intestine were able to inhibit growth of five strains of *Salmonella,* including *S. enteritidis* 94/448, *S. typhimurium, S. pullorum, S. blockley,* and *S. enteritidis* 935/79, and three serotypes of *E. coli*, viz. *E. coli* O1:K1, O2:K1, and O78:K88.

The mechanisms associated with the reduction of the pathogens have not been fully explained. Although pH reduction appears to be partially responsible for reductions, other mechanism appear to be involved.

On the other hand, some studies have raised questions regarding the efficacy of DFM microorganisms in reducing colonization of pathogenic bacteria. It is important to note that the strains of LAB used in the studies, the age of the chicks, the timing of administration of the DFM, and various other factors varied from study to study. In experiments conducted by Hinton and Mead (1991), results showed that DFM products containing strains of *Lactobacillus* or *Enterococcus* administered to day-old chicks in feed or drinking water or by spraying on bird's feathers did not reduce *Salmonella* in the caeca. In a small study with only five chicks, Adler and Da Massa (1980) reported no reductions of *S. infantis* after feeding lactobacilli after hatching. Similarly, Adler and Da Massa (1980) found no protection by *Lactobacillus* against *Salmonella* or *E. coli* colonization in the caeca of newly hatched chicks. Soerjadi *et al.* (1983) administered a mixed culture of lactobacilli to newly hatched chicks and challenged them with *S. typhimurium.* Initially, there were no reductions, but 2–3 days after the challenge, significant reductions in the number of *Salmonella* shed in the feces were observed, but no reduction in the numbers of chicks shedding the pathogen.

The controversy regarding the effectiveness of the DFM microorganisms can be explained partially by the use of "defined" and "undefined" cultures and by using various strains/species to get the desired effects. "Defined" product comprises of a known mixture of pure bacterial cultures derived from fecal and caecal contents of the bird, whereas "undefined" product consists of a homogenous mixture of known aerobic microorganisms and unknown mainly anaerobic microorganisms derived from the caeca of the bird (Mulder *et al.*, 1997).

The focus of this chapter is on LAB, but a brief overview of undefined cultures is given. According to a review by Mulder *et al.* (1997), results from studies on the effect of microflora consisting of 50 pure cultures were less

promising than those obtained after the administration of undefined microflora. Similarly, in a study by Stavric *et al.* (1987), results with mixtures of pure cultures of *Lactobacillus* showed that the preparations were ineffective in reducing *Salmonella* carriage in chicks. On the other hand, undefined anaerobic culture prepared from feces of adult birds showed a significant reduction in the number of *S. typhimurium* in chicks.

The variation in results from study to study likely can be explained by variations in strains of LAB used, experimental design, age of the animal, type of challenge given to the animal, and the timing of sample collection. Also likely is that some strains of LAB are beneficial and can inhibit food-borne pathogens in poultry when administered correctly.

F. INHIBITION OF FOOD-BORNE PATHOGENS IN SWINE

The work associated with the use of DFM in pigs to reduce pathogen loads is very limited; however, the effect of LAB has been growing in the past few decades. The most commonly tested LAB are the strains of *Lactobacillus* and *Enterococcus,* and most studies involve starter pigs based on the assumption that adult pigs are more resistant to intestinal disorders. It has been demonstrated in several feeding trials that selected strains of LAB can be beneficial in reducing the pathogenic bacterial count (Barrow *et al.*, 1980; Deprez *et al.*, 1989; Ozawa *et al.*, 1983). A study by Barrow *et al.* (1980) demonstrated that when 2-day-old piglets weaned to a sow's milk–substitute diet were given *L. fermentum* alone or in combination with *S. salivarius* in their milk, there was a significant decrease in the *E. coli* counts in the stomach and duodenum. Similarly, fecal coliform counts and hemolytic *E.coli* O141:K85ab were reduced in piglets when treated with *E. faecalis* and *E.faecium,* respectively (Deprez *et al.*, 1989; Ozawa *et al.*, 1983). Underdahl *et al.* (1982) also demonstrated that *E. faecium* reduced the number of pathogenic *E. coli* and the severity of illness associated with it in gnotobiotic piglets. However, contrasting results have also been reported by some researchers regarding the efficacy of DFM LAB in pigs. One such study involving the interaction between *Lactobacillus* species and *E. coli* K88 in gnotobiotic pigs showed that *Lactobacillus* species was unable to prevent the adherence of *E. coli* to the intestinal mucosa (Bomba *et al.*, 1996). In spite of some negative results, the use of LAB in pigs holds considerable potential. The selection criteria for probiotic microorganisms is a big factor influencing the efficacy of a particular strain or mixture of strains, combined with the need for appropriate *in vitro* and animal models, sensitive and reproducible techniques, and repeated experimentation to validate the efficiency of probiotic LAB.

G. INHIBITION OF FOOD-BORNE PATHOGENS IN CATTLE

Since the recognition of cattle as the principal reservoir of *E. coli* O157:H7, the use of DFMs to reduce the carriage of pathogen in the animal has received tremendous attention. Unfortunately, the literature on the use of LAB as DFM in cattle is limited. Many of the studies examining the effects of LAB have been limited to calves and not to adult cattle (Nousiainen and Setälä, 1993). Because the pathogen is a concern at slaughter, it is important to test the impact on adult animals. Additionally, the impact on naturally infected and artificially challenged animals seems to vary. New methodology on the isolation of *E. coli* O157 from cattle makes it possible to examine populations of naturally infected animals to determine the impact in a commercial setting.

The number of studies done in feedlot animals is growing. As with other species, there is also inconsistency in the results obtained from studies involving use of DFMs in cattle. Nonetheless, use of DFM microorganisms in cattle is increasing, and several studies have been conducted to understand the specific role of LAB in reducing the carriage of pathogenic bacteria in cattle.

One of the first studies done by Ellinger *et al.* (1978) reported a decrease in fecal coliforms when liquid diet of newborn calves was supplemented with *Lactobacillus* cultures. In a similar study, Gilliland *et al.* (1980) demonstrated that use of *Lactobacillus* strain isolated from the cow was more effective in reducing commensal *E. coli* than those isolated from pigs, suggesting the importance of host specificity of the strains. Ozawa *et al.* (1983) tested the effect of *E. faecalis* BIO4R on intestinal flora of calves and found that the strain had an antagonistic effect on *Salmonella.* The early studies were conduced on pathogen-challenged animals and were limited in that the number of animals used in the studies was small.

Studies by Brashears *et al.* (2003a,b) indicate that *E. coli* O157:H7 can be reduced in feedlot-age cattle. The DFMs were isolated from cattle. From more than 600 candidate strains of LAB, only 19 were chosen based on acid and bile tolerance, inhibition of *E. coli* O157 in laboratory media, and antibiotic resistance characteristics (Brashears *et al.*, 2003a,b). The 19 strains were subsequently screened for the inhibition of the pathogen in manure and ruminal fluid, and 2 candidate strains were chosen as the best candidates along with 2 commercially available strains to be used in cattle feeding trials. A small trial was conducted with five artificially inoculated finishing calves, which indicated that the numbers of *E. coli* O157 present in cattle fed two of the DFM strains—one isolated from the previous study and one commercially available—reduced shedding by 80% (unpublished data; Brashears and Moxley, 2000). These two strains were selected for use in large-scale

feeding trials. The commercially available strain was originally isolated from a calf and identified as *L. acidophilus* and is referred to as NP 51. The newly isolated culture was identified through biochemical and genetic testing as *Lactobacillus crispatus* NP 35.

The large-scale trials at Texas Tech University, the University of Nebraska, and Colorado State University indicate that use of DFMs has been effective in significantly reducing the amount of *E. coli* O157:H7 detected in the feces and on the hides of beef feedlot cattle. Three separate large-scale studies were conducted at Texas Tech University (Table I). In the first study, 180 beef feedlot cattle were separated into three treatment groups. One group received NP 51, one received NP 35, and one received a carrier of the DFM and served as a control. NP 51 and NP 35 contained two separate strains of *L. acidophilus* as the DFM. The cultures were fed at a level of 1×10^9 cells/head/day for the last 60 days of the feeding period. Overall, the reduction in the shedding was 50% for the animals fed NP 51 compared to the control group. There were no significant reductions in those fed NP 35. At slaughter, the prevalence on the hides was reduced from 20% in the controls to 1.7% and 0% in NP 51 and NP 35, respectively. In a follow-up study conducted the following summer, the treated animals were fed NP 51 and a combination of other commercially available DFM cultures for the entire duration of the feeding period (Younts-Dahl *et al.*, 2003). At 7 days before slaughter and at slaughter, 27% of the fecal samples in the control

TABLE I

REDUCTION OF *E. COLI* O157 IN BEEF FEEDLOT CATTLE FED 10^9/HEAD/DAY OF *LACTOBACILLUS ACIDOPHILUS* NP 51

Study	Fecal[a] *E. coli* O157 reduction	Hide[b] *E. coli* O157 reduction	Duration of feeding
Brashears *et al.*, 2003a	54%	92%	60 days
Younts-Dahl, 2004	57%	64%	Entire feedlot feeding period
Younts-Dahl (in review)	80%	62%	Entire feedlot feeding period
Moxley *et al.* (2003)	28.5%	Not tested	Entire feedlot feeding period
Moxley *et al.* (unpublished observations)	35%	Not tested	Entire feedlot feeding period
Ranson and Belk, 2003	70.9%	43%	74 days

[a]Fecal samples collected directly from the rectum of the animal.
[b]Hide samples collected by swabbing the hide with hydrated sterile sponges.

animals tested positive for *E. coli* O157:H7, whereas the treated animals contained significantly fewer detectable numbers, with only 13% being positive. Again, there were significant reductions in the number of animals testing positive for *E. coli* O157:H7 on the hides, with 14% of the control samples testing positive and only 5% of the treated samples testing positive. In 2003, a dose-titration study was conducted to pinpoint the most effective dose to reduce *E. coli* O157 in the animal during the feeding period. The DFM was supplemented in the diet throughout the entire feeding period (in the feedlot). Doses of 1×10^7, 1×10^8, and 1×10^9 lactobacilli/head/day were administered daily throughout the entire feedlot feeding period. Fecal grab samples were collected from the rectum of each animal every 28 days. While all three doses significantly reduced the number of animals shedding the pathogens by slaughter, the 10^9 dose resulted in the fastest and most dramatic reduction, with significant reductions occurring after 28 days of feeding. In the control group, 31.7% of the animals tested positive for the pathogen at slaughter, whereas only 8.3% of those fed the 10^9 dose were positive. Significant reductions in the number of hides that were positive were also observed with the 10^9 dose, with 8.7% testing positive in the control group and 3.4% testing positive in the treated group.

The reductions of *E. coli* O157 have been confirmed not only in studies at Texas Tech, but also in studies at the University of Nebraska and Colorado State University. In a study at the University of Nebraska, 21.3% of the control animals tested positive for *E. coli* O157 at slaughter in the control group, whereas those receiving a 10^9/head/day dose of NP 51 throughout the feeding period only had 13.3% animals testing positive. A separate study found that there was a 38% reduction in the number of animals testing positive for *E. coli* O157 after continuous feeding with *E. coli* O157 (Moxley, 2003).

At Colorado State University, NP 51 was fed for the last 74 days in the feeding period. They reported that 45.8% of control animals tested positive for *E. coli* O157 while only 13.3% of animals fed NP 51 were positive. Significant reductions were also observed on hide samples with 40.3% of the controls testing positive and 22.7% of the animals fed NP 51 testing positive (Ranson and Belk, 2003).

VIII. POSSIBLE *IN VIVO* MECHANISMS OF ACTION

As previously discussed, one of the reasons for the beneficial effects exhibited by LAB is a direct antagonistic action against harmful microorganisms. However, the exact mechanisms by which LAB affect the microflora of the intestinal tract are not clearly understood.

The inhibition of pathogenic bacteria *in vitro* by production of organic acids is well documented, but the evidence for *in vivo* inhibition is not very convincing. In a study by Jin *et al.* (1996), inhibition shown by *Lactobacillus* strains against pathogenic strains of *Salmonella* and *E. coli* was suggested to be due to the production of organic acids by *Lactobacillus* isolates. It is assumed that the primary reason for antagonism by lactic acid production is the reduction in pH, which inhibits the growth of many bacteria including gram-negative pathogenic organisms (Burnett and Hanna, 1963; Sorrells and Speck, 1970). Although pH is the main factor in antagonism, it has also been demonstrated that lower pH values govern the activity of organic acids because the undissociated forms are most bactericidal (Acheson, 1999; Sorrells and Speck, 1970). The undissociated acid is easily diffused through the bacterial cell wall, thereby reducing the intracellular pH level and slowing metabolic activities of the bacteria (Holzapfel *et al.*, 1995). *E. coli* is shown to be inhibited by lactic acid at a pH value of 5.1 (Gudkow, 1987). Tramer (1966) also showed that the inhibition of *E. coli* by *L. acidophilus* was due to the strong bactericidal effect of lactic acid at low pH levels. However, because of its higher dissociation constant, acetic acid shows stronger inhibition than lactic acid at a given molar concentration and pH value (Holzapfel *et al.*, 1995). These volatile acids are especially antimicrobial under the low oxidation-reduction potential (Saito *et al.*, 1981), which LAB help maintain in the intestine.

Hydrogen peroxide, which is produced in the presence of molecular oxygen together with lactate, pyruvate, and NADH by flavin enzymes (Condon, 1987; Gilliland and Speck, 1969; Gotz *et al.*, 1980; Kandler, 1983), is one of the primary metabolites of LAB that may contribute to antagonism. It inhibits the growth of pathogens through its cytotoxic effect on the bacterial cells by generating highly reactive and toxic oxygen species such as the hydroxyl radical that initiates oxidation of biomolecules (Juven and Pierson, 1996). The antimicrobial activity of hydrogen peroxide is well recognized and documented. Bacteriocin production by LAB is also recognized as one of the mechanisms used for antagonism against other microorganisms. Within the LAB group, *Lactobacillus* organisms have been extensively studied for production of bacteriocins as antagonists. The antibacterial effect for most bacteriocins seems to be bactericidal, although some studies have also reported bacteriostatic effects. The inhibitory spectrum of bacteriocins is restricted to closely related organisms, which implies that bacteriocins produced by LAB may not be active against gram-negative pathogens. A number of gram-positive toxinogenic and pathogenic bacteria have been found to be inhibited by bacteriocins of certain LAB.

Competition for adhesion sites on the intestinal epithelium, thereby preventing colonization of pathogens, is another mechanism involved in CE by LAB. A prerequisite for invasion by enteropathogens, including *E. coli,* is for the pathogen to have access to receptors on the host tissue (Krogfelt, 1991; Smith, 1992). It is, therefore, believed that occupation of the receptor or attachment site by the native or protective intestinal flora is part of their protective role. The ability to adhere to mucosal surfaces has been suggested to be an important property of the bacterial strains used in probiotic products (Lopez *et al.*, 1989). LAB, particularly *Lactobacillus* and *Streptococcus,* are known to be intimately associated with the nonsecretory squamous epithelial cells of pig stomach (Barrow *et al.*, 1980; Fuller, 1978) and chicken crop (Fuller and Turvey, 1971). Mayra-Makinen *et al.* (1983) demonstrated the adhesive capacity of *Lactobacillus* strains to columnar epithelial cells of calves and pigs *in vitro.* The role of adhering LAB in protection against enteric pathogens has been studied to some extent with mixed results. Barrow *et al.* (1980) reported a statistically significant reduction in the numbers of *E. coli* in the stomach when strains of *Lactobacillus* and *Streptococci* were fed, alone or in combination, to artificially reared pigs. Stavric *et al.* (1987) demonstrated that the microflora that remained attached to the cecal wall of chickens after four successive washes in buffered saline had a protective effect against *Salmonella.* On the other hand, in a trial by Spencer and Chesson (1994), strongly adherent strains of *Lactobacillus* did not have any effect on the attachment of enterotoxigenic *E. coli* to porcine enterocytes under conditions of exclusion (*Lactobacillus* added to enterocytes before *E. coli*), competition (simultaneous addition of *Lactobacillus* and *E. coli*), and displacement (*E. coli* added before *Lactobacillus*). The inconsistencies in the results reported so far have made the mechanism of CE difficult to understand because too many generalizations have been made about CE being able to work regardless of attention to individual pathogens' mechanism of adherence to host cells. Some researchers believe that the principle of exclusion by occupation of receptor sites is only applicable with both LAB and pathogens having the same attachment sites. It will not work with gram-negative pathogens because the mechanisms of attachment by LAB and gram-negative bacteria are different. For example, adhesion of *E. coli* usually takes place by an interaction between the glycan component of host glycolipids and glycoproteins, which act as receptors for bacterial proteinaceous projections (fimbrial lectins) (Ofek and Sharon, 1990). The adherence of LAB, on the other hand, is a process mediated by extracellular components including carbohydrate, protein, or lipoteichoic acid polymers (Vandervoorde *et al.*, 1992). However, another pool of scientists believe that adherent strains can mask pathogen and toxin receptors

without necessarily binding to the same epitope and, thus, limit the ability of a pathogen to colonize and infect (Spencer and Chesson, 1994). In view of the controversy associated with the exact mechanism that makes an adhering strain effective, it becomes necessary that the importance of adhesion in CE be evaluated thoroughly.

A. EFFECT ON IMMUNE RESPONSE

The effect of probiotic LAB on the host immune response has been studied to some extent, and it is postulated that both mucosal and systemic immune responses can be affected by DFMs. Bealmer *et al.* (1984) demonstrated that conventional animals with complete gut flora have higher immunoglobulin levels and phagocytic activity compared to germ-free animals. Roach and Tannock (1980) suggested that a systemic effect was exerted by *E. faecium* that was established as a monoassociate in germ-free mice and was able to reduce *S. typhimurium* counts in the spleen. Similarly, *L. casei* was involved in the stimulation of phagocytic activity when administered perorally to mice in a study by Perdigon *et al.* (1986). For a microorganism to affect systemic immunity, it may have to enter the systemic circulation. Bloksma *et al.* (1981) showed that *Lactobacillus* were able to survive in the spleen, liver, and lungs for several days. Saito *et al.* (1981) showed that *L. casei*, given parenterally, stimulated phagocytic activity in mice. Serum IgA and IgG levels have been shown to be increased with administration of *Lactobacillus* in piglets and mice (Lessard and Brisson, 1987; Perdigon *et al.*, 1990). These findings suggest that DFMs have the potential to modulate immunity, and their effect on systemic immune response can be used to overcome infections caused by pathogens such as *Salmonella* that occur in tissues away from the intestinal tract.

IX. CONCLUSION

Although the concept of microbial antagonism by LAB is not new, the application to farm animals has gained interest only in the past few decades. Inhibition of *Salmonella* in poultry was the first research area of interest, and LAB have been proven to reduce *E. coli* O157 in cattle before slaughter. Reports in the literature vary with respect to the efficacy of LAB in reducing food-borne pathogens in farm animals. It is important to consider the source of the DFM, application of the product, and methods used to evaluate the

efficacy of the product. Although not all LAB will give reductions of food-borne pathogens in farm animals, carefully selected strains administered under appropriate conditions are effective at reducing *E. coli* O157 in cattle and *Salmonella* in poultry.

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MYCOTOXINS IN FRUITS: MICROBIOLOGY, OCCURRENCE, AND CHANGES DURING FRUIT PROCESSING

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I. INTRODUCTION

Mycotoxins are secondary metabolites of molds (primarily deuteromycetes), which are generally produced under optimum conditions at the end of the exponential growth phase. The term *mycotoxin* combines the terms "mykes," the Greek word for molds, and "toxicum," the Latin word for toxic or poisonous. Accordingly, mycotoxins occasionally also are defined as

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secondary metabolites of molds that exert toxic effects on animals and humans (1999). The physiological function of mycotoxins is not understood fully. Metabolic control mechanisms within the fungus, as well as defense mechanisms against other organisms, are discussed. Molds are characterized by a ruderal strategy of life, filamentous growth, intensive sporulation, primarily vegetative propagation, a parasexual life cycle, and a marked metabolic diversity, and they are distributed ubiquitously because the spores are disseminated with wind and water. About 300 fungal species are considered to fall into the group of molds that constitute an evolutionary relatively young branch of the Eumycota (Weidenbörner, 2000). Most species of the Eumycota have not been described, and conservative estimations are that at least 10^4 species occur on earth. Two unique secondary metabolites are assumed to be synthesized per species, and about 10% of the known secondary metabolites of fungi are mycotoxins, leading to the estimation of 2×10^3 mycotoxins, of which more than 300 have been characterized (Riley, 1998).

Only a few of these mycotoxins are regularly found in foods, with the predominant ones including aflatoxins, ochratoxin A, patulin, and different toxins produced by *Fusarium* species. The latter are only briefly discussed in this chapter, because their occurrence is mainly limited to grains and seeds (Parry *et al.*, 1995). In the last decade, another group of toxins produced by *Alternaria* species including alternariol, alternariol methyl ether, tenuazonic acid, altenuene, altertoxin I, and altertoxin II has attracted attention (Scott, 2001; Scott and Kanhere, 2001; Serdani *et al.*, 2002; Singh *et al.*, 2001; Tournas and Stack, 2001).

The history of mycotoxin research is closely related to research on fungal antibiotics; for example, patulin was first isolated from *Penicillium patulum* during the search for antibiotics in 1941 (Weidenbörner, 2001). After isolation of citrinin in 1931, it was first considered a highly effective antibiotic, but its toxic effects were discovered during antibiotic testing. Intensive research on aflatoxins and ochratoxin A started in the 1960s after some severe acute mycotoxicoses had occurred in domestic animals and humans in Japan, the Balkan region, the USSR, and England. Mycotoxins are toxic for vertebrates at low concentrations when ingested or inhaled (via spores) under natural conditions. Physicochemically, the mycotoxins are thermostable and in most cases aromatic and nonantigenic low-molecular-mass metabolites. Mycotoxins exert a diverse range of toxic effects because their chemical structures are very heterogeneous (Figure 1).

Apart from their acute and chronic toxicity, mycotoxins may possess carcinogenic, mutagenic, and teratogenic properties. They may act primarily on the liver (hepatotoxicity), kidney (nephrotoxicity), nervous (neurotoxicity), and immune systems (immunotoxicity or immunosuppression), on the uterus (uterotropism), and on the skin (dermatotoxicity), or they may act as

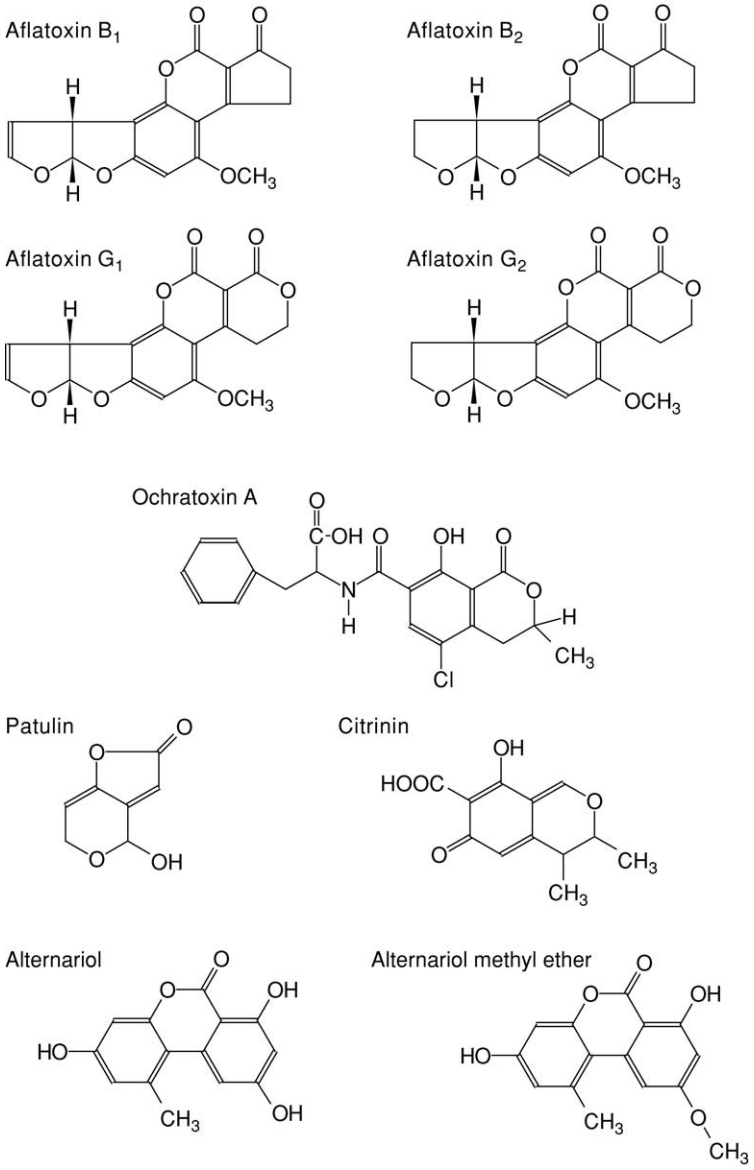

FIG. 1 Chemical structures of different mycotoxins.

general cytotoxins (Weidenbörner, 2000). Aflatoxins are acutely toxic, immunosuppressive, mutagenic, teratogenic, and carcinogenic. The main target organ is the liver (Peraica *et al.*, 1999), and there is sufficient evidence for their hepatocarcinogenicity to humans (Dirheimer, 2000). The main target organ of ochratoxin A is the kidney. Apart from nephrotoxic effects, immunosuppressive, carcinogenic, and teratogenic effects are described. Ochratoxin A has been discussed as a main factor in the etiology of a kidney disease in Bulgaria, the former Yugoslavia, and Romania, which was first described in the 1950s and was named *Balkan endemic nephropathy*. Pfohl-Leszkowicz *et al.* (2002) reviewed scientific research on this topic and concluded that Balkan endemic nephropathy is of multifactorial origin, but the authors strongly believe that mycotoxins including ochratoxin A are among these factors. Toxicity of patulin is controversial. As summarized by Rychlik (2003), symptoms of acute and chronic toxicity have been observed in different feeding trials in rats in addition to teratogenic and immunotoxic effects. The author also cites different studies, in which orally administered patulin resulted in no signs of carcinogenicity, whereas subcutaneous injection produced sarcoma at the site of injection. Rychlik (2003) showed that patulin is rapidly degraded in humans in the blood before reaching other tissues outside the gastrointestinal tract.

Whereas endotoxins are restricted to the fungal mycelium, mycotoxins covered in this chapter exert their effects on other organisms as exotoxins. They diffuse into the environment and can be found in food and feed areas, which do not show any sign of mycelial growth. Therefore, the absence of molds does not guarantee freedom from mycotoxins, and conversely, the presence of a toxin-producing mold does not automatically imply the presence of mycotoxins in foods and feeds. Generally, three causes for contamination of foods are distinguished: a primary contamination of agricultural commodities in the field and upon postharvest storage, a secondary contamination during processing as a consequence of poor hygienic processing conditions, and finally, a carryover effect may occur with residues in animal-derived food via mycotoxin-contaminated feed.

Mycotoxin contamination of foods may cause considerable economic losses. On a global perspective, aflatoxins in tree nuts, dry fruits, and spices, *Fusarium* toxins in cereals (particularly maize, wheat, and barley), and ochratoxin A in cereals and coffee are of major importance (Bhat and Vasanthi, 1999). Regional problems also may arise from mycotoxins in fruits such as patulin in apples, ochratoxin A in grapes and dried vine fruits, or aflatoxins in different dried fruits.

The aim of this chapter is to summarize and critically discuss scientific data on mycotoxins in fruits. After giving an introduction on mold spoilage of fruits in general and factors affecting growth and mycotoxin formation by

molds, emphasis is placed on mycotoxin contamination of fruit products destined for human consumption. Strategies for preventing a possible post-harvest mycotoxin contamination and decontamination strategies for fruit products are then described. Based on data on the occurrence of mycotoxins in fruits and fruit products, the impact of mycotoxins in fruits on human health is also discussed.

II. MOLD SPOILAGE OF FRUITS

The food technological term *spoilage* covers "any chemical or physical alteration of food that makes it unfit or unsafe to eat" (Morris, 1992). *Plant-rot diseases* are characterized by "the softening, discoloration, and often disintegration of a succulent plant tissue as a result of fungal or bacterial infection" (Arneson and Hodge, 2004). Fruit rots are usually subdivided into field rots, which damage the plants before harvest, and storage rots, which occur after harvest. Rots are caused by multiple interacting factors, including those from bacterial or fungal pathogens or a complex of several pathogens under a diverse range of environmental factors (McManus, 2004). The most common rot pathogens are summarized in Table I.

Because most fruits contain the seeds of permanent crops like bushes and trees, which grow in orchards and yards for several years to decades, crop rotation, a feasible measure to control toxigenic fungi in agricultural and vegetable crops, is not applicable in fruit orchards and yards. Tillage and other soil cultivation systems that play an important role in the reduction of the infective potential of toxigenic fungi in agricultural soils are only applicable between the rows of fruit bushes and trees and, thus, are significantly less effective in orchards. The most important method to control the growth of mycotoxigenic fungi on fruits in the field and in storage devices is the application of fungicides (mostly synthetic) that more or less specifically inhibit mold growth and, thus, the production of mycotoxins. Fungicides are sprayed rarely to control the synthesis of mycotoxins and, thus, are an important determinant of yield quality directly, but they are applied in most cases to improve the yield or visible quality of fruits.

A. *ASPERGILLUS*

One of the most ubiquitous fungal genera is the genus *Aspergillus. Aspergillus* species grow like the other molds saprophytically on a wide range of organic substrates. The many species of the genus are arranged in sections, and the taxonomic situation of the sections, especially *Circumdati* and *Nigri,*

TABLE I
COMMON MOLD ROT DISEASES OF FRUITS AND THE MOLDS ASSOCIATED WITH THEM[a]

Commodity	Disease	Most important mold species
Citrus fruits	Blue rot	*Penicillium italicum (Penicillium ulaiense)*
	Green rot	*Penicillium digitatum*
	Sour rot	*Geotrichum candidum*
	Grey rot	*Alternaria alternata*
	Black rot	*A. alternata*
Apples	Brown rot	*A. alternata, Aspergillus niger, Aspergillus flavus*
Pome fruits	Blue rot	*Penicillium expansum*
	Grey rot	*Botrytis cinerea*
Stone fruits	Brown rot	*Monilia fructicola*
	Transit rot	*Rhizopus stolonifer, Rhizopus oryzae*
Melons	Black rot	*A. alternata*
	Pink rot	*Trichothecium roseum*
Figs	pink rot/soft rot (endosepsis)	*Fusarium moniliforme*
	Smut	*A. niger*
Grapes	Grey rot	*B. cinerea*
	Black rot/sour rot	*A. niger, Aspergillus carbonarius*
Berries		*B. cinerea, R. stolonifer, Mucor piriformis*

[a]Data from Logrieco *et al.* (2003) and Pitt and Hocking (1997).

is in a constant state of flux (Kusters van Someren *et al.*, 1991; Varga *et al.*, 2000; Yokoyama *et al.*, 2001). Many of the species mentioned in this chapter may have had different names or will have different names in the future.

Most fruits grown in the tropical and subtropical regions of the earth seem to be contaminated with *Aspergillus* species because they are ideally adapted to the climatic conditions prevailing in these regions. The growth of *Aspergillus* species of the section *Nigri,* for instance, starts above 10°C. The *A. niger* growth optimum is higher than 37°C, whereas that of *Aspergillus carbonarius* is consistently lower (Battilani *et al.*, 2003b). *A. carbonarius* optimum growth occurred at 35°C for all isolates tested by Mitchell *et al.* (2003). No growth occurred below 15°C. The optimum water activity (a_w) of the isolates varied between 0.93 and 0.987, with the widest a_w tolerance occurring at 25°C.

A wide spectrum of toxigenic molds, primarily belonging to the genera *Aspergillus* and *Penicillium,* were isolated from Egyptian strawberries, apricots, plums, peaches, grapes, dates, figs, apples, pears, and mulberries (Table II).

TABLE II

ASPERGILLUS SPECIES IDENTIFIED FROM FRESH FRUITS

Mold	Commodity	Region	Reference
A. flavus, A. parasiticus, A. tamarii, and *A. alliaceus*	Figs	California	Doster *et al.*, 1998
A. ochraceus, A. melleus, A. sclerotiorum, and *A. alliaceus*	Figs	California	Bayman *et al.*, 2002
A. niger var. niger, A. flavus, A. niger var. awamori, A. foetidus, and *A. candidus*	Grapes	Argentinia	Magnoli *et al.*, 2003
A. niger	Grapes	Brazil	Rosa *et al.*, 2002
A. carbonarius, A. section *Nigri, A. aculeatus, A. fumigatus, A. terreus*, and *A. ustus*	Grapes	France	Sage *et al.*, 2002
A. section *Nigri* and *A. carbonarius*	Grapes	Portugal	Serra *et al.*, 2003
A. niger, A. tubigensis, A. carbonarius, A. aculeatus, A. japonicus, A. ochraceus, and *A. fumigatus*	Grapes	Italy	Battilani *et al.*, 2003a
A. niger var. *niger* and *A. carbonarius*	Grapes	Spain	Belli *et al.*, 2002
A. niger, A. flavus, and *A. terreus*	Apples	Egypt	Hasan, 2000
A. flavus	Quinces	India	Sharma and Sumbali, 1999
A. niger, A. flavus	Dates	Egypt	Ragab *et al.*, 2001
A. candidus, A. flavus, A. niger, A. ochraceus, A. sclerotiorum, and *A. terreus*	Strawberries, apricots, plums, peaches, grapes, dates, figs, apples, pears, mulberries	Egypt	Aziz and Moussa, 2002

Besides nontoxigenic species, the toxigenic fungi *Aspergillus candidus, Aspergillus flavus, A. niger, Aspergillus ochraceus, Aspergillus sclerotiorum,* and *Aspergillus terreus* frequently were found (Aziz and Moussa, 2002). Mold isolates from brown-rot lesions of Egyptian apples included, besides fungi from other genera, the following *Aspergillus* species: *A. niger*, *A. flavus,* and *A. terreus* (Hasan, 2000). Belli *et al.* (2002) isolated the *A. niger* aggregates (formerly *A. niger* var. *niger*) and *A. carbonarius* from Spanish grapes. *A. flavus* also usually infects quinces (*Cydonia oblonga*) in the State of Jammu and Kashmir, and according to Sharma and Sumbali (1999), about 23% of the tested isolates were aflatoxigenic.

Several *Aspergillus* species infect figs. Doster and Michailidis (1998) detected aflatoxin-producing L and S strains of *A. flavus* and *A. parasiticus,* as well as the nonproducing species *A. tamarii* and *A. alliaceus* in Californian figs, whereas Bayman *et al.* (2002) detected *A. ochraceus, A. melleus,* and *A. sclerotiorum* from the section *Circumdati* and *A. alliaceus,* which seems to be more closely related to aspergilli from the section *Flavi*, on figs from California. *A. alliaceus*–infected figs contained ochratoxin A, whereas figs infected with isolates from the section *Circumdati* contained little or no toxin (Bayman *et al.*, 2002). The section *Circumdati* has taxonomic problems for *Aspergillus* species, because several species are poorly defined or may be synonymous, so a taxonomic revision of the section is needed (Varga *et al.*, 2000). Toxigenic strains of *A. flavus* are often found in molded fresh and dried fig products in the Mediterranean region (Logrieco *et al.*, 2003).

Grapes from tropical and subtropical regions also are regularly contaminated with *Aspergillus* species, whereas *Alternaria* was isolated most frequently from Argentinian grapes (80% of the samples), followed by *Aspergillus* (70%). In grapes, *Alternaria alternata* was the only species identified in the genus, followed by *A. niger* var. *niger* and *A. flavus, A. niger* var. *awamori, A. foetidus,* and *A. candidus* (Magnoli *et al.*, 2003). Grapes from Argentina and Brazil had *Aspergillus, Penicillium,* and *Botrytis* species isolated from them (Rosa *et al.*, 2002). Other genera identified (in decreasing order) were *Phytophthora, Moniliella, Alternaria,* and *Cladosporium.* Rosa *et al.* (2002) isolated 48 *A. niger* strains from Argentinian and 53 from Brazilian grape samples. They additionally identified *A. flavus* and *A. ustus* in Argentinian and *A. carbonarius, A. flavus, A. ochraceus,* and *A. terreus* in Brazilian grape samples. *A. flavus* and *A. terreus* did not produce ochratoxin A when cultured on nutrient medium, and of the remaining species, the percentage of toxigenic strains and the ochratoxin A levels produced were significantly lower than in *A. niger.* In 11 vineyards from four wine-making Portuguese regions, *Aspergillus* species from the section *Nigri* were predominant. Most of the *A. carbonarius* (97%) and 4% of the *A. niger* isolates tested produced ochratoxin A on nutrient agar plates (Serra *et al.*, 2003), confirming the hypothesis

that *A. carbonarius* is the main producer of ochratoxin A in grapes. Fifty samples of currants, raisins, and sultanas from the Spanish market were analyzed by Abarca *et al.* (2003) for mold contamination and 98% of the dried fruits had mainly black aspergilli and species from the order *Mucorales.* Among the *Aspergillus* section *Nigri*, *A. niger* var. *niger* was detected in 49 (98%) and *A. carbonarius* in 29 (58%) of the samples. Other species from the genus found in this study were *A. flavus* and *A. versicolor.* According to Sage *et al.* (2002), four of 11 French grape samples were contaminated by potentially ochratoxigenic strains of *A. carbonarius.* Other grape-infecting species from the genus were species from the *Aspergillus* section *Nigri, A. aculeatus, A. fumigatus, A. terreus,* and *A. ustus* in descending prevalence. Battilani *et al.* (2003b) collected 508 mold isolates from Italian grapes, with 477 isolates belonging to the genus *Aspergillus* and 31 to the genus *Penicillium.* Among the genus *Aspergillus,* species from the section *Nigri* largely predominated (464 isolates) and species from the sections *Circumdati* (*A. ochraceus*) and *Fumigati* (*A. fumigatus*) were found only once or twice, respectively. Among the species of the section *Nigri*, the biseriate species *A. niger* and *A. tubingensis* dominated, followed by *A. carbonarius* and the uniseriate species *A. aculeatus* and *A. japonicus*. Logrieco *et al.* (2003) concluded that toxigenic strains of *A. carbonarius* are often associated with the Mediterranean region and black rot of grapes. Greek grapes from Corinth raisin and wine-producing regions regularly were infected with *Aspergillus* species. Species from the *A. niger* section *Nigri* prevailed as causal agents of the sour rot of grapes, and particularly, *A. carbonarius* caused concern as the dominating ochratoxin A producer on grapes (Tjamos *et al.*, 2004).

Compared to studies on effects of fungicides on growth of mycotoxigenic molds in cereals, however, relatively little is known about fungicidal effects on molds that infect fruits. The effect of several fungicides on toxigenic *Aspergillus* species of the section *Nigri* that infect grapes in Greek Corinth raisin and wine-producing regions has been tested. According to Tjamos *et al.* (2004), fungicides with the active ingredients fludioxonil and cyprodinil showed the most efficient growth-reducing effect on *Aspergillus* section *Nigri* fungi, whereas cyprodinil and carbendazim were largely ineffective.

B. *PENICILLIUM*

The climatic requirements of *Penicillium* species are quite different from those of the genus *Aspergillus.* Whereas *Penicillium* species grow over a temperature range of 4–31°C, *Aspergillus* species require temperatures of 12–39°C (International Programme on Chemical Safety, 1990). *Penicillium* species frequently is found outside tropical and subtropical regions of the Earth.

Sage *et al.* (2002), for instance, detected the potential ochratoxin A producer *P. chrysogenum* in three of 11 French grape samples. Other grape-infecting *Penicillium* species detected by these authors were (in decreasing incidence) *P. brevicompactum, P. expansum, P. thomii, P. glabrum, P. purpurogenum, P. chrysogenum, P. miczinskii, P. atramentosum, P. griseofulvum, P. minioluteum,* and single isolates of *P. canescens, P. citreonigrum, P. citrinum, P. echinulatum, P. oxalicum, P. paxilli, P. rugulosum,* and *P. spinulosum. P. chrysogenum* was isolated from 22% of Argentinian grape samples collected in the Mendoza province by Magnoli *et al.* (2003), whereas *P. glabrum* and *P. crustosum* were detected occasionally. Apart from a wide spectrum of molds from other genera, *P. brevicompactum, P. chrysogenum, P. citrinum, P. aurantiogriseum, P. commune, P. expansum, P. oxalicum, P. griseofulvum, P. islandicum,* and *P. verrucosum* were isolated by Aziz and Moussa (2002) from Egyptian strawberries, apricots, plums, peaches, grapes, dates, figs, apples, pears, and mulberries. Pianzzola *et al.* (2004) detected 11 *P. expansum* and three *P. solitum* strains, which cause blue mold of apple, the most important postharvest disease in Uruguay. Brown rot of apple also is caused by *P. expansum* besides other fungal species (Hasan, 2000). According to Logrieco *et al.* (2003), molded fig products in the Mediterranean region are often contaminated by *P. expansum,* among other fungi.

C. *ALTERNARIA* AND OTHER MYCOTOXIGENIC GENERA

The worldwide occurrence of *Alternaria* and other more rarely detected toxigenic species is less well documented than the case for *Aspergillus* and *Penicillium* species. According to Magnoli *et al.* (2003), about 80% of grape samples from a winery in the Argentinian Mendoza province were contaminated with *A. alternata,* but the authors did not detect any other species of the genus. *A. alternata* may also cause brown rot of apple (Hasan, 2000). *A. alternata* was identified by this author as the predominate species in the pathogenic complex causing brown rot of apples in Egypt. *A. alternata* has been isolated by Aziz and Moussa (2002) from strawberries, apricots, plums, peaches, grapes, dates, figs, apples, pears, and mulberries purchased in Egyptian grocery stores. In addition to the predominant *A. alternata,* unidentified species of the genus *Fusarium* were isolated by Hasan (2000), among other molds as components of the pathogenic complex causing brown rot of apples in Egypt.

In conclusion, the most common mycotoxin-producing fruit-rot pathogens belong to the genera *Aspergillus, Penicillium, Alternaria,* and *Fusarium.* Their impact on human health is based primarily on their occurrence, and the toxicity profiles of their mycotoxins are thought to decrease in this order as well. Several species of the genus *Aspergillus* infect fruits in tropical and

subtropical orchards, whereas *Penicillium* species and *A. alternata* infect fruits in nearly every fruit-growing region on earth. Fruit rots are caused by a pathogenic complex, and the causal agents cannot be identified reliably with the naked eye.

III. POTENTIAL FOR MYCOTOXIN FORMATION AND OCCURRENCE OF MYCOTOXINS IN FRUITS

Toxin production is, more or less, a metabolic burden. Non–mycotoxin-producing mold strains may thus be favored in specific cases and may displace mycotoxin-producing strains through competition (Desjardins *et al.*, 1993). However, growth of mycotoxigenic molds does not necessarily mean the formation and presence of mycotoxins in fruits. It is well described that minimum a_w values for mold growth and mycotoxin formation differ for several species. Table III summarizes these differences for the most important mycotoxigenic fungi.

Furthermore, differences in optimum temperatures for growth and mycotoxin formation exist. For *Aspergillus* species, temperature optimum for mycotoxin formation is at 25–28°C, whereas optimum temperature for mold growth is between 30 and 35°C. In the field, factors such as temperature or humidity (a_w values) may influence production of a mycotoxin more than application of fungicides, which usually are not designed to directly inhibit or prevent the production of mycotoxins (Scientific

TABLE III
ENVIRONMENTAL CONDITIONS FOR GROWTH AND MYCOTOXIN PRODUCTION BY DIFFERENT TOXIGENIC MOLDS[a]

	Mold growth		Mycotoxin production
Fungus	Optimum temperature [°C]	Minimum a_w value	Minimum a_w value
Aspergillus flavus	35–37	0.80	0.84
A. parasiticus	30	0.84	0.87
A. ochraceus	28–35	0.77	0.85
A. carbonarius	35	—[b]	—[b]
Penicillium expansum	25–26	0.84	0.99
Alternaria alternata	25–28	0.85–0.88	—[b]

[a]Data from Corry, 1987; Mitchell *et al.*, 2003; Weidenbörner, 2000.
[b]Not determined.

Committee on Plants of the European Commission, 1999). In a summary, the committee concluded in 1999 that "there is no sufficient evidence that pesticides play a prominent and consistent role in preventing or inhibiting the production of mycotoxins by toxigenic fungi. However, it cannot be excluded that, in the future fungicides will be selected on the basis that they effectively can inhibit the production of mycotoxins."

A. AFLATOXINS

As reviewed by Logrieco (2003), aflatoxin B_1 and B_2 are mainly produced by *A. flavus* and *A. parasiticus,* with the latter also producing aflatoxins G_1 and G_2. Because of the aforementioned optimum conditions for mold growth and mycotoxin production, plant products from tropical or subtropical regions are particularly at risk for aflatoxin contamination resulting from infection with *Aspergillus* species in the field. Accordingly, scientific papers on aflatoxin contamination in fruits focus on fruits cultivated in warm climates like figs, dates, and citrus fruits. However, under adverse storage conditions, postharvest infection and mycotoxin contamination of other fruits also may occur.

The susceptibility of maturing figs to decay by aflatoxin-producing fungi has been investigated by Doster *et al.* (1998). Figs became more susceptible as they matured through the four developmental stages: green with eye (ostiole) closed, green with eye open, yellow, and brown. The mature brown figs were the most susceptible to decay by *A. flavus.* Aflatoxin analysis showed that brown figs had more than six times the aflatoxin of yellow figs and more than 30 times that of green figs. Wounding did not result in a significant increase in infections or aflatoxin concentration in mature brown figs compared to nonwounded figs. The authors suggested that damage to mature brown figs does not favor aflatoxin production, which might explain why insect damage to mature figs did not result in increased aflatoxin contamination in figs.

Twenty-five varieties of dates were examined at different maturation stages for total microbial counts, aflatoxins, and aflatoxigenic *Aspergillus* species and lactic acid bacteria by Shenasi *et al.* (2002). Microbial counts were high at the first stage of maturation (Kimri) and increased sharply at the second stage (Rutab), then decreased significantly at the final dried stage of maturation (Tamr). Aflatoxins were detected in 12% of the samples, although aflatoxigenic *Aspergillus* were detected in 40% of the varieties examined; all were at Kimri stage only. No aflatoxins or aflatoxigenic *Aspergillus* species were detected at the final edible stage of maturation. The absence of aflatoxins in fresh dates has also been described by Ragab *et al.* (2001). A possible explanation is the antifungal effect of date extract as

described by Shraideh *et al.* (1998). Exposure of yeast to 5% date extract showed evidence of weakening in the cell wall of the yeast, with indications of cell distortion and partial collapse in some cases. Increasing the concentration of date extract (20%, w/v) led to more drastic damage to the yeast, which resulted in cell lysis and concurrent leakage of cytoplasmic material with eventual cell death.

Natural occurrence of aflatoxins on oranges in Egypt was investigated by Ragab *et al.* (1999). Thirty-two percent of oranges collected from a local market contained either all four major aflatoxins or a combination of aflatoxin B_1 and aflatoxin G_1. By inoculation of surface-intact oranges with *A. parasiticus,* Ragab *et al.* (1999) showed that aflatoxin formation may occur in the peel of oranges in spite of their high content of oils. For *A. flavus,* it has been shown that citrus oil exhibits an antifungal action (Varma and Verma, 1987). Mycotoxin production of *A. parasiticus* on oranges is positively correlated with temperature and relative humidity (Ragab *et al.*, 1999). Aflatoxins diffused into the edible portion of the fruit but appeared to be degraded with time. A similar observation previously had been reported by Varma and Verma (1987) for orange juice. A decrease in aflatoxin production with time was observed for *A. flavus* grown on oranges and in orange juice, but the authors suggested that aflatoxins were degraded by the mycelium of *A. flavus* itself. The production of aflatoxins was maximum when the biomass reached its optimal value and rapidly declined after the mycelium started to autolyze.

Singh and Sumbali (2000) demonstrated that mature jujube fruits are a favorable substrate for infection and aflatoxin production by *A. flavus* strains. Among the mycoflora on the surface of jujube (*Ziziphus mauritiana*) *A. flavus* consistently was recorded during the entire period of fruit development. Fifty isolates of *A. flavus* isolated from the preharvest fruits caused extensive postharvest rot of mature jujube when inoculated. When all of these isolates were also screened for their aflatoxigenic potential in mature jujube, 54% of the isolates tested positive for different aflatoxins (B_1, B_2, G_1, and G_2) at levels ranging from 31 to 2874 μg/kg.

B. OCHRATOXIN A

Depending on the geographic region and climate, ochratoxin A is produced by *Penicillium* or *Aspergillus* species. In recent years, much work has focused on the identification of ochratoxin producing fungal species on grapes in different climatic regions. In the past, the ability to produce ochratoxin A was believed to be restricted to *A. ochraceus* and closely related species from the section *Circumdati* and to *P. verrucosum,* but it has become evident that species from the *Aspergillus* section *Nigri* also are able to produce

this mycotoxin. Abarca *et al.* (2001) speculate that other species may be additional sources for ochratoxin production in their natural environments.

The difference in temperature requirements for mold growth may explain the observation reported by Pietri *et al.* (2001), in which *Penicillium* species occur on grapes only at the beginning of ripening in contrast to *Aspergillus* species, which were constantly associated with grapes during ripening and harvest in Italy. *P. verrucosum* and *A. alutaceus,* formerly known as *A. ochraceus,* were supposed to be the most important producers of ochratoxin A in grapes. Battilani *et al.* (2003a) reported that aspergilli were dominant to penicillia, with the section *Nigri* predominating. *A. carbonarius* probably plays an important role because of the high percentage of positive strains and the amount of ochratoxin A produced on grapes. Aspergilli section *Nigri* were already present on grape bunches early in the season, and their frequency increased during later growth stages. A total of 48 *A. niger* strains were isolated from Argentinian grapes, of which eight could produce ochratoxin A, and 16 of 53 *A. niger* strains from Brazilian grapes produced ochratoxin A (Rosa *et al.*, 2002). A key role for *A. carbonarius* concerning ochratoxin A production in grapes and musts from France was also reported by Sage *et al.* (2002).

Similar results were reported from Magnoli *et al.* (2003), who analyzed the mycoflora of Argentinian grapes. Of 63 strains belonging to the *Aspergillus* section *Nigri* and tested for ochratoxin A production, 41.3% were producers; levels of ochratoxin A produced ranged from 2 to 24.5 ng/ml of culture medium. The presence of ochratoxigenic strains of the *Aspergillus* section *Nigri* may be an important source of ochratoxin A in grapes from tropical and subtropical zones. *P. purpurogenum* has also been isolated from grapes, but was not shown to produce ochratoxin A (Cabanes *et al.*, 2002).

C. PATULIN AND CITRININ

Patulin and citrinin are both produced by *Penicillium* species. Citrinin was first isolated from *P. citrinum,* from which it derived its name. Today, it is known that apart from *Penicillium* species like *P. citreonigrum, P. citrinum, P. expansum,* and *P. verrucosum*, citrinin is also produced by other species like *A. candidus, A. terreus,* and *Monascus purpureus* or *M. ruber* (Weidenbörner, 2001). The occurrence of citrinin is mainly associated with rice and other cereals, but citrinin has also been detected in raisins (Meister, 2003) and in apples (Martins *et al.*, 2002).

A large number of molds produce patulin. Patulin is primarily known as a toxin produced by *P. expansum* in foods but may also be formed by *P. claviforme, P. cyclopium, P. equinum, P. glandicola, P. commune, P. lapidosum, P. melinii, P. novaezeelandiae,* and *P. griseofulvum,* as well as

Aspergillus clavatus, A. giganteus, A. terreus, and *Byssochlamys nivea* (Davis and Diener, 1987).

Early studies report patulin occurrence on bananas, pineapple, grapes, peaches, apricots, plums, and tomatoes, but the authors do not present data on incidence and concentration of contamination (Frank *et al.*, 1976; Thurm *et al.*, 1979). Inoculation experiments showed that patulin may be produced by *Penicillium* species on a variety of foods and especially a variety of fruits, but the natural occurrence of patulin is limited to fruits and is mainly associated with apples and apple products. According to Weidenbörner (2001), two factors are responsible. Besides an inactivation of patulin by certain compounds in foods, patulin-producing molds represent only a low percentage of the total mold strains isolated from most foods ranging from 0.9% for cornmeal to 1.42% for European-style dry sausage. Furthermore, an antifungal activity of bacteria against *P. expansum* has been described. Florianowicz (2001) investigated the antifungal activity of 11 selected bacterial cultures and five microfungi in different phases of their growth in respect of their activity against *P. expansum*. Two *Bacillus* species, *B. megaterium* and *B. subtilis,* and three strains of the genus *Lactobacillus, L. casei, L. delbrueckii* subspecies *bulgaricus*, and *L. lactis* subspecies *cremoris,* were active against *P. expansum.* Inhibition of patulin production by *P. expansum* using fruit oils was observed by Hasan (2000). Complete inhibition of patulin formation by *P. expansum* grown on glucose-Czapek's-apple medium after seven days at 25°C was achieved in the presence of 0.2% lemon oil. A lower but still very strong inhibition was observed with 0.05% lemon oil or orange oil at both concentrations.

In rotten apples, *P. expansum* is the predominating mold. Strains of *P. expansum* cause blue mold rot of apples after infection following preharvest insect or storm damage of the surface tissue or damage caused by rough gathering, prolonged storage, or improper postharvest handling (Logrieco *et al.*, 2003). Available literature on the occurrence of patulin has been reviewed by Drusch and Ragab (2003). Up to 130 mg of patulin/kg have been detected in the lesions of apples, with no correlation between the size of the lesion and the patulin concentration (Beretta *et al.*, 2000).

Martins *et al.* (2002) investigated the natural co-occurrence of patulin and citrinin on 351 samples of seven apple varieties. The percentage of samples contaminated with patulin only was 68.6%, whereas contamination with citrinin only was 3.9%. Co-occurrence of both mycotoxins occurred in 19.6% of the samples. The maximum mean patulin concentration was 80.5 mg/kg for Richared variety, and the maximum mean citrinin concentration was 0.92 mg/kg for Rome beauty variety. Because the ratio of weight of the rotten area to the total weight was about one-third, a direct risk for mycotoxin ingestion for consumers seems unlikely. Apples with such a high

proportion of rotten tissue are usually not consumed or processed. Demirici *et al.* (2003) detected patulin in cherries, mulberry, raspberry, and strawberry. In 31 of 41 samples of fruits collected from the Turkish market, these authors found up to 746 μg of patulin/kg in raspberries, up to 572 μg of patulin/kg in strawberries, and up to 426 μg/kg in mulberries. Cherries were contaminated most frequently with patulin, 9 of 10 samples contained patulin, with a mean concentration of 37 μg/kg.

D. *ALTERNARIA* TOXINS

Scott (2001) summarized the available literature on the natural occurrence of *Alternaria* toxins in fruits. The most frequently detected toxins were alternariol, alternariol methyl ether, and tenuazonic acid in apples, mandarin, melon, and alternariol and its methyl ether in red currant, raspberry, strawberry, gooseberry, and blackberry. Stinson *et al.* (1980) were able to detect *Alternaria* toxins after the authors isolated *Alternaria* strains from blueberries, broke the skin, and inoculated the berries after steam disinfection. In contrast, Tournas and Stack (2001) did not detect *Alternaria* toxins after infection of blueberries with *A. alternata.* As discussed earlier in this chapter, fruits become more susceptible to mold invasion during ripening, and this may be a crucial point for inoculation experiments.

Depending on the substrate and the strain of *A. alternata,* the mycotoxin profile produced may vary. Black and gray strains of *A. alternata* produced tenuazonic acid, alternariol, and alternariol methyl ether, when grown on rice substrate or whole mandarin fruits. Gray strains of *A. alternata* also produced altertoxin I, and when cultivated on autoclaved rice, both strains were able to produce altenuene (Logrieco *et al.*, 1990). Investigations carried out on the natural occurrence of mycotoxins in infected fruits showed that samples of the two kinds of mandarin heart rot showed different mycotoxin profiles. In black-rot samples, tenuazonic acid, alternariol methyl ether, and alternariol (up to 87, 1.4, and 5.2 mg/kg) were found, whereas tenuazonic acid (up to 174 mg/kg) was the only detectable mycotoxin in gray-rot samples (Logrieco *et al.*, 1990). Traces of tenuazonic acid have been reported by Bottalico and Logrieco (2001) for samples of black-rot melons.

Tournas and Stack (2001) investigated mycotoxin formation by two *Alternaria* strains (ATCC 66868 and ATCC 56836) cultivated on strawberries, grapes, and apples at various temperatures ranging from 5 to 21°C. *A. alternata* and its toxins were not a major problem in strawberries because of the presence of fast-growing molds like *Botrytis* and *Rhizopus* that overgrew and inhibited *Alternaria*. Growth of *Alternaria* species and mycotoxin formation may also limit storage stability of fruits. Alternariol and alternariol methyl ether were detected in grapes even when stored at 5°C, whereas

in apples, a temperature of more than 10°C was required for mycotoxin formation. Alternariol methyl ether concentration in apples was up to 14 mg/kg, which may constitute a problem when apples are stored for extended periods of time during the winter and spring season under uncontrolled atmosphere (Tournas and Stack, 2001). Scott (2001) pointed out that more work is needed on the development of reliable methods for the determination of *Alternaria* toxins. However, data from the studies cited give sufficient evidence that *Alternaria* toxins frequently occur and that the occurrence may not be limited to fruits. Generally, the presence of *Alternaria* toxins in fruit products serves as an indicator of the use of poor-quality raw materials.

E. RARELY DETECTED TOXINS

A detailed mycotoxin profile of a *Penicillium vulpinum* strain isolated from apples (formerly *P. claviforme*) was described by Kozlovskii *et al.* (2000). The authors detected viridicatin, cyclopenin, and a-cyclopiazonic acid, but the strain did not produce detectable levels of patulin and citrinin. Venkatasubbaiah *et al.* (1995) detected by thin-layer chromatography, high-performance liquid chromatography (HPLC), mass spectrometry (MS), and ^{1}H nuclear magnetic resonance spectrometry, among other toxic substances, the trichothecene mycotoxins trichothecolone and trichothecolone acetate in liquid cultures of *Peltaster fructicola,* one of the molds that cause sooty blotch of apple. Feldmann *et al.* (2003), however, were unable to confirm the results for different strains of *P. fructicola* with a more sensitive HPLC/MS/MS system. The authors discussed the trichothecene identification of Venkatasubbaiah *et al.* (1995) as possibly resulting from HPLC matrix disturbances or delay phenomena.

Jiménez and Mateo (1997) screened corn and rice cultures of five species of *Fusarium* originally isolated from banana. In these cultures, they detected the type A trichothecenes deoxynivalenol (DON) and 3-acetyl DON in *F. graminearum,* the type B trichothecenes T-2 tetraol and neosolaniol in *F. acuminatum,* zearalenone in *F. graminearum* and *Fusarium equiseti,* a-zearalenol in *F. equiseti,* and fumonisin B1 in *F. moniliforme* and *F. proliferatum.* Moretti *et al.* (2000) isolated 120 *F.* strains from rotted fig fruits mainly belonging to the species, *F. ramigenum, F. solani,* and *F. subglutinans*, and at a lower frequency, *F. proliferatum*. Cultivated on maize kernel media, fusaric acid was produced by all species at very low amounts, but one strain of *F. subglutinans* produced a high level of this toxin. *Liseola* section species produced beauvericin, fumonisins, and fusaproliferin. As reviewed by Logrieco *et al.* (2003), exposure of plants to *Fusarium* toxins and their relative toxicological risk are not well understood. For example, toxigenic *Fusarium* species are important pathogens and root colonizers of various fruits

(e.g., banana, mango, and pineapple) and vegetables (e.g., potato), but no reports of the natural occurrence of *Fusarium* mycotoxins in products obtained from these plants exist.

In conclusion, the growth of mycotoxigenic mold species on fruits is not always an indication of the presence of mycotoxins, but they may also be absent due to the growth of nontoxigenic fungal strains. Temperature and humidity may significantly affect mycotoxin formation in the field or after harvest, and fungicides may be applied to prevent or inhibit mold growth. Because aflatoxins occur in the field only in tropical and subtropical regions, figs, dates, and citrus fruits should be monitored with special attention for signs of mold growth. Ochratoxin A, patulin, citrinin, and the toxin mixture of *A. alternata* may be present in fruits from nearly every fruit-growing region of the Earth, but it should be considered that several of the toxin-producing species have a relatively narrow host range.

IV. MYCOTOXINS IN FRUIT PRODUCTS AND IMPACT OF PROCESSING ON MYCOTOXIN CONCENTRATION

The effects of postharvest storage and processing of fruits on fungal growth and a possible contamination with mycotoxins after harvest is of special interest. Fruits should be harvested at optimum maturity and should be handled gently to prevent bruises and punctures that permit fungal invasion. Good sanitation should be maintained, moldy fruits and fruits with skin breaks and bruises should be culled out, and washing with hot water or fungicide treatments may also be applied (Splitstoesser, 1987). However, the application of fungicides to fruits after harvest to reduce decay has been increasingly curtailed by the development of pathogen resistance to many key fungicides, the lack of replacement fungicides, negative public perception regarding the safety of pesticides, and consequent restrictions on fungicide use. Biological control of postharvest diseases has emerged as an effective alternative. Because wound-invading necrotrophic pathogens are vulnerable to biological control, antagonists can be applied directly to the targeted area (fruit wounds), and a single application can significantly reduce fruit decays as reviewed by Janisiewicz and Korsten (2002).

In terms of product safety, diffusion of mycotoxins in infected fruits and possible health risks associated with the processing or consumption of the remainder of an infected fruit after removal of visibly rotted tissue is of interest. Laidou *et al.* (2001) investigated the diffusion of patulin in the flesh of pears inoculated with four pathogens, *P. expansum, A. flavus, Stemphylium vesicarium,* and *A. alternata*. *P. expansum* and *A. flavus* penetrated more rapidly into the flesh than *S. vesicarium* and *A. alternata* because of the

utilization of substrate or the mechanism of breaking host defense. The selected strain of *A. flavus* did not produce patulin. Patulin diffused up to 6 mm in sound tissues of pears inoculated with *P. expansum* or *A. alternata* and up to 18 mm in sound tissues of pears inoculated with *S. vesicarium,* indicating that the absence of rots does not guarantee an absence of the mycotoxin (Laidou *et al.*, 2001), but diffusion is limited to the vicinity of rotted parts of the fruit. Patulin did not diffuse more than 2 cm from the infected zone into apples inoculated with *P. expansum* as determined by a stable isotope dilution assay (Rychlik and Schieberle, 2001). In contrast, in tomatoes, diffusion of patulin occurred throughout the whole fruit. The authors predicted that patulin may easily penetrate through low-viscous foods containing high amounts of water like blueberries, grapes, or melons. Similar results have been reported for ochratoxin A by Engelhardt *et al.* (1999) in different visibly rotted fruits after removal of rotted areas.

This approach gives only qualitative information about diffusion of mycotoxins into sound tissue and only in cases in which diffusion occurs. Freedom from ochratoxin A in sound tissue may result from the absence of ochratoxin A in the rotted area or from lack of diffusion. On the other hand, a positive result for ochratoxin A in sound tissue gives proof of diffusion. Ochratoxin A was detected in the sound tissue of cherries, strawberries, and tomatoes, but not in nectarines and apricots (Engelhardt *et al.*, 1999). In peaches with cleaved moldy stones, Engelhardt *et al.* (1999) detected up to 0.21 μg of ochratoxin A/kg, and its frequency of contamination was up to 50%. In conclusion, trimming of fruits can reduce mycotoxin content to a certain degree, depending on the type of fruit. Consumers should keep in mind that visibly sound fruits after trimming may still contain mycotoxins. Nevertheless, for industrial processing of certain fruits like apples, trimming is a useful tool for reduction of possible mycotoxin contamination and, therefore, leads to an increase in product safety.

As stated by Scott (1984), published information on mycotoxins in foods processed for human consumption is limited. Information on the extent that mycotoxins persist through processing is important for risk management by food processors and regulatory authorities. Whereas past research mainly focused on cereal processing, this chapter summarizes available literature on major categories of fruit products: dried fruits, fruit juices, wine and cider as fermented fruit juices, and fruit purees and jams.

A. DRIED FRUITS

The most important dried fruits produced for human consumption are raisins, sultanas, figs, apricots, and dates. Because all these fruits are cultivated in warm climates, mycotoxins associated with these fruits are aflatoxins and

ochratoxin A. Aflatoxins in figs are mainly produced by *A. flavus* or *A. parasiticus* (Doster and Michailides, 1998). To determine the likely origin of ochratoxin A in dried vine fruits, a mycological study of 50 samples (currants, raisins, and sultanas) from the Spanish market was conducted by Abarca *et al.* (2003), who found that 96.7% of *A. carbonarius* isolates and 0.6% *A. niger* varietas *niger* isolates were ochratoxin producers. Among the black aspergilli, *A. carbonarius* showed a consistent ability to produce ochratoxin A and is, thus, considered the most probable source of ochratoxin A contamination in dried vine fruits.

Few data on mycotoxin contamination of dried fruits (raisins, figs, prunes, dates, and apricots) have been published. Table IV shows the most recent surveys on the occurrence of ochratoxin A and aflatoxins in dried fruits. The frequency of contamination varies widely from 0 to 57% for aflatoxins and from 20% to 95% for ochratoxin A, respectively. In dried vine fruits, high concentrations of ochratoxin A up to 53.6 μg/kg have been reported. Mean ochratoxin levels in dried vine fruits were generally lower and ranged from 1 to 3 μg/kg. Stefanaki *et al.* (2003) reported that sultanas were less contaminated than currants. Aflatoxin contamination of dried fruits is usually below 2.5 μg/kg. High aflatoxin levels in dried figs of up to 1342 μg/kg have been found by Waizenegger (2001); however, the fruits were sorted before analysis, and data, therefore, reflect contamination of individual figs rather than the contamination level of a whole batch. The data of Waizenegger (2001) exemplify that individual moldy fruits may be highly contaminated with mycotoxins and that contamination in whole batches of dried fruits may be at considerably lower levels.

Mycotoxin contamination of dried fruits may start with fungal contamination on the trees, increase during harvesting and sun drying, and continue to accumulate during storage because of rewetting and improper storage conditions. Factors influencing mold growth and subsequent mycotoxin formation, and possible preventive measures have been reviewed by Drusch and Ragab (2003). These authors mention that several factors, such as cultivar susceptibility to fungal invasion, environmental stress conditions like drought, insect activity, mechanical damage, nutritional deficiencies, temperature, and humidity during growth determine possible mycotoxin contamination of the final product. Plant pathogens may infect a variety of tissues in addition to the fruits, and, thus, the removal of diseased branches and other plant parts helps to reduce the incidence of spoilage. Cultural practices that lower humidity in the growing area, such as weed control and the proper spacing of plants, often have a beneficial effect because growth of fungi is favored by moist conditions. Sanitation in the orchard and vineyard is important for control of spoilage organisms that overwinter in cankers, dead branches, and fallen fruit (Splitstoesser, 1987).

TABLE IV

DATA FROM RECENT SURVEYS ON AFLATOXINS AND OCHRATOXIN A IN DRIED FRUITS

Mycotoxin	Commodity	Total no. of samples (n)	No. of positive samples n (%)	Maximum (μg/kg)	Median (μg/kg)	Mean (μg/kg)	Reference
Ochratoxin A	Raisins, sultanas, currants	60	53 (88)	53.6	—	—	MacDonald *et al.*, 1999
	Raisins, currants	106	101 (95)	21.4	0.32	—	Engel, 2000
	Raisins, currants	59	10 (17)	19.0	0.92	2.64	Möller and Nyberg, 2003
	Raisins, currants	59	12 (20)	34.6	0.2	1.17	Möller and Nyberg, 2003
	Currants, sultanas	81	60 (74)	13.8	1.2	2.6	Stefanaki *et al.*, 2003
	Dried vine fruits	300	220 (73)	21.3	0.7	2.0	Food Standards Agency, 2003a
Ochratoxin A	Figs	34	27 (79)	3.95	0.02	—	Engel, 2000
Ochratoxin A	Prunes	31	26 (84)	0.07	0.03	—	Engel, 2000
Aflatoxins B_1, B_2, G_1, G_2	Raisins, sultanas, currants	60	0 (0)	—	—	—	MacDonald *et al.*, 1999
Aflatoxin B_1	Raisins	100	2 (2)	300	260	260	Youssef *et al.*, 2000
Aflatoxins B_1, B_2, G_1, G_2	Figs	42	7 (17)	1342	215	—	Waizenegger, 2001
	Figs	21	12 (57)	<2.5	—	—	Food Standards Agency, 2002
Aflatoxins B_1, B_2, G_1, G_2	Prunes	16	5 (31)	<2.2	—	—	Food Standards Agency, 2002
Aflatoxins B_1, B_2, G_1, G_2	Dates	12	6 (50)	<2.5	—	—	Food Standards Agency, 2002
Aflatoxins B_1, B_2, G_1, G_2	Apricots	12	1 (8)	<2.2	—	—	Food Standards Agency, 2002

Other key elements of preharvest management are cultivar selection, judicious use of insecticides and fungicides, and if necessary irrigation. Different fungicides (copper oxychloride, mancozeb, benomyl, captan, thiram, chlorothalonil, and prochloraz) were successfully applied for reducing *A. flavus* and *A. parasiticus* in dried figs and subsequently aflatoxins (Tosun and Delen, 1998). Maturation stages were taken into consideration for application time and type of fungicides. Treatment types involved tree and soil applications at wintering and budding stages, respectively, to reduce possible fungal sources on the trees and treatment at fruiting, at ripening and at shriveling. The latter were applied only to the soil under the trees where dried figs dropped to eradicate the soil-borne fungi that are mostly present in the topsoil level. A last form of treatment was applied to both the soil surface of the drying place and to the storage room before the fruits were stored.

Splitstoesser (1987) summarized some important control practices at harvesting, grading, and packing stages as follows: (1) Harvest fruit when at optimum maturity, (2) handle the fruit gently to prevent bruises and punctures that would permit the entry of saprophytic fungi, (3) maintain good sanitation to minimize the buildup of mold on fruit-contact surfaces. Mechanical harvesters, lug boxes, and packinghouse equipment should be cleaned and sanitized regularly. Live steam, formaldehyde, and fumigation with chlorine gas were some of the treatments used to destroy fungal spores. (4) Moldy fruit and those showing skin breaks and bruises should be culled out during the sorting and grading operations.

Drying of the fruits is usually carried out directly in the orchard. Raisins are harvested from the grapevine and sun-dried on the ground. It is important to prevent direct contact of the grapes with the soil to hamper fungal colonization on the fruits. In contrast, figs are left on the trees until they shrivel. The figs fall to the ground and are gathered up and dried further in the sunlight on drying devices for about five days. After drying, the sound fruits are stored in farmer storehouses.

The most detailed investigation on the influence of harvesting and drying techniques on mycotoxins in dried figs has been published in 1995 by Özay *et al.* (1995). Apart from classic sun-drying as described earlier, the authors investigated the effectiveness of solar drying. For solar drying, radiation energy from the sun was used for heating air up to a temperature of 60–65°C. The air was forced through a drying chamber, in which figs were placed on a wire mesh. In solar-dried figs, moisture content and a_w value (15.3% and 0.566%, respectively) were lower than in sun-dried figs. Özay *et al.* (2005) concluded that picking fruits from the tree and application of solar drying were the most effective treatments to reduce fungal contamination in dried figs. Waizenegger (2001) investigated a possible correlation between fluorescence and aflatoxin contamination and its use for reducing

aflatoxins in dried figs. The fluorescence, bright green-yellowish under ultraviolet light, is caused by kojic acid, a metabolite of certain *Aspergillus* species. Doster and Michailides (1998) reported that bright green-yellowish fluorescence in naturally infected figs was associated with decay by only four fungal species: the aflatoxin-producing species *A. flavus* (both L and S strains) and *A. parasiticus,* and the aflatoxin non-producers *A. tamarii* and *A. alliaceus.* Figs infected with *A. flavus* or *A. parasiticus* and showing no bright green-yellowish fluorescence were occasionally contaminated with aflatoxins, whereas other figs showing bright green-yellowish fluorescence and infected with *A. flavus* or *A. tamarii* had no aflatoxins. In a different study, Steiner *et al.* (1988) found that removal of bright green-yellow fluorescent figs from a 56-kg batch effectively lowered the original contamination level from 22.6 to 0.3 μg/kg aflatoxin B_1. All 52 bright green-yellow–positive figs contained kojic acid in concentrations between 8 and 6900 mg/kg. Thirty-seven (71%) fruits were contaminated with aflatoxin B_1 in concentrations between 5 μg and 76 mg/kg, and 15 (29%) fruits were contaminated with aflatoxin G_1 at levels of 5 μg to 180 mg/kg.

However, fluorescence was sometimes only detectable after cutting of the fruits (Doster and Michailides, 1998; Waizenegger, 2001). Nevertheless, screening of figs for bright green-yellowish fluorescence is a possibility for reducing aflatoxin contamination in dried fruits for human consumption, although acceptable quality-control measures for routine analysis should be nondestructive. Doster and Michailidis (1998) pointed out that bright green-yellow fluorescence might be of use to remove aflatoxin-contaminated figs for certain specific situations in California such as the reduction of aflatoxin contamination during the manufacturing of fig paste, because the figs are cut into quarters during processing.

B. FRUIT JUICE

Apples and pears are the fruits most frequently contaminated with patulin. As a consequence, most scientific publications on mycotoxin contamination of fruit juices deal with the occurrence of patulin in apple juice. Table V shows the most important surveys on the occurrence of patulin in apple juice for the last five years. The frequency of contamination ranged from 13 to 81%. Apart from one Turkish study with a mean of 140 μg/L (Yurdun *et al.*, 2001), mean patulin concentrations were rather low and usually below 50 μg/L. The highest patulin concentration was 733 μg/L, but concentrations up to 1150 μg/kg have occasionally been reported for commercially available apple juice (Beretta *et al.*, 2000).

Beretta *et al.* (2000) also observed a statistically significant difference between apple juices produced from conventionally and organically grown

TABLE V
DATA FROM RECENT SURVEYS ON PATULIN CONTAMINATION IN APPLE JUICE

Origin of samples	Unit	Total no. of samples (n)	No. of positive samples n (%)	Maximum	Mean	Median	Reference
United Kingdom	µg/L	199	110 (55)	171	14.3	7.1	European Commission (Directorate-General Health and Consumer Protection), 2002b; Food Standards Agency, 1999
Taiwan	µg/L	71	12 (17)	39.9	–	–	Lai *et al.*, 2000
South Africa	µg/L	31	8 (26)	45	–	–	Leggott and Shephard, 2001
Turkey	µg/L	45	27 (60)	732.9	139.9	–	Yurdun *et al.*, 2001
Sweden	µg/kg	39	5 (13)	<50	–	–	Thuvander *et al.*, 2001
Austria	µg/kg	242	114 (47)	50	14.9	1.6	European Commission (Directorate-General Health and Consumer Protection), 2002b
Belgium	µg/kg	117	27 (23)	59	16	<LOQ	
France	µg/kg	67	37 (55)	130	14.3	3.0	
France	µg/kg	122	49 (40)	37	11.6	1.7	
Germany	µg/kg	1248	320 (26)	415	22.1	4.2	
Turkey	µg/L	30	12 (40)	106.9	35.1	16.8	Demirci *et al.*, 2003
Italy	µg/L	44	11 (25)	74.2	26.7	–	Riteni, 2003
Belgium and imported	µg/L	43	35 (81)	38.8	9.0	6.0	Tangni *et al.*, 2003

apples. The highest value for apple juice from conventional agriculture was 3.0 μg/kg and for apple juice from organic agriculture was 28.2 μg/kg. These authors suggest that the application of fungicides in conventional agriculture was the reason for the lower contamination. Apart from the use of fungicides, patulin content in apple juice is significantly determined by the quality of apples as influenced by harvesting and storage conditions, by the different processing steps during juice production and by other juice ingredients.

The quality of apples for juice production is determined by the proportion of rotted and decayed apples, because patulin concentration in the brown-rotten area of apples is usually very high. Sydenham *et al.* (1997) reported up to 6.3 mg of patulin/kg in the rotted areas of apples; however, Beretta *et al.* (2000) detected up to 113 mg of patulin/kg in the same areas.

Kadakal and Nas (2002b) used apples, classified by the decay proportion on the fruit surface as sound, 30, 60, or 100% decayed, in the production of apple juice, to determine the effect of apple decay proportion on the patulin content of apple juice. Patulin increased in apple juice samples as the decay proportion increased. Patulin in juice samples produced with apples that were sound, 30, 60, and 100% decayed, were 0–15.9, 47.1–500.3, 156.4–2257.5, and 54.9–2508.6 μg/kg. Similar results have been reported by Jackson *et al.* (2003). Patulin was not detected in juice pressed from fresh tree-picked apples but was found at levels of 40.2–374 μg/L in juice pressed from fresh ground-harvested (dropped) apples. Another possible source of patulin contamination may be contamination of apple juice with *P. expansum*. McCallum *et al.* (2002) observed extensive fungal growth and high patulin levels after inoculation of apple cider with different isolates of *P. expansum.* Concentrations of 538–1822 μg/ml in apple ciders were associated with incubation at room temperature (25°C), and potentially toxic patulin levels of 75–396 μg/ml also were found in refrigerated ciders (4°C) inoculated with *P. expansum*.

Removal of rotten fruits by sorting is an effective technique to reduce possible patulin contamination in apple juice. Jackson *et al.* (2003) did not detect patulin in juice pressed from tree-picked apples stored for 4–6 weeks at 0–2°C after sorting but found it at levels of 0.97–64.0 μg/L in juice pressed from unculled fruits stored under the same conditions. Cider from controlled-atmosphere–stored apples that were culled before pressing contained 0–15.1 μg/L of patulin, whereas cider made from unculled fruit contained 59.9–120.5 μg/L of patulin. The importance of removing contaminated apples from the initial processing line during apple juice production was studied during three consecutive seasons by Leggott *et al.* (2000). Patulin concentration of 440 μg/kg was significantly reduced to 200 μg/kg after removal of rotten and damaged apples. Sydenham *et al.* (1997) investigated the effect of storage in the open on patulin content in apples and

apple juice. Storage of apples in the open is unavoidable in countries in which only limited storage facilities for cold or modified atmosphere storage are available. Over a period of 33 days, mean patulin levels in unprocessed fruits increased from 90 to 2445 μg/kg but decreased to between 75 and 695 μg/kg following a water wash step. Subsequent removal of rotten fruit decreased patulin levels further to between 55 and 405 μg/kg.

After washing, sorting, and pressing, raw apple juice usually is clarified to remove suspended solids. For this purpose, juice is treated with pectinolytic enzymes and/or nonenzymatic fining agents like bentonite, gelatine, caseinate, or chitosan. Solid particles are removed by centrifugation, filtration or ultrafiltration. Bissessur *et al.* (2001) evaluated the effectiveness of several clarification processes, namely clarification with bentonite, enzymatic (pectinase) treatment, paper filtration, and centrifugation for the reduction of patulin. Pressing followed by centrifugation resulted in an average toxin reduction of 89%. Total toxin reduction using filtration, enzymatic treatment, and fining were 70, 73, and 77%, respectively. Patulin reduction was due to the binding of the toxin to solid substrates that were verified by analyzing the clarified juice as well as the filter cake, pellet, and sediment.

Table VI gives an overview on the effect of single processing steps and treatments on patulin content in apple juice. In agreement with the observation of Bissessur *et al.* (2001), washing and pressing are the most effective steps for reducing patulin concentration in apple juice. Washing of ground-harvested apples before pressing reduced patulin levels in juice by 10–100%, depending on the initial patulin levels and the type of wash solution used (Jackson *et al.*, 2003). Hence, patulin is a good indicator of the quality of the apples used to manufacture juice. It is recommended that avoidance of ground-harvested apples and the careful washing and sorting of apples before pressing are good methods for reducing patulin levels in juice. As a consequence, the Codex Committee on Food Additives and Contaminants (CCFAC) presented a draft for a "Code of practice for the prevention of patulin contamination in apple juice and apple juice ingredients in other beverages" (Codex Alimentarius Commission, 2002). According to the CCFAC, postharvest management systems based on HACCP for the reduction of patulin in apple juice should be considered.

Table VI also shows that the use of activated charcoal seems to be another effective step in reducing patulin contamination in apple juice. Leggott *et al.* (2000) observed a reduction of patulin from 110 to 75 μg/L by a combination of depectinization, charcoal treatment, and ultrafiltration, which was probably because of the adsorption of patulin on the activated charcoal. No further removal of patulin occurred during the remainder of the juicing process. Leggott *et al.* (2001) reported that the type of charcoal

TABLE VI
REDUCTION OF PATULIN CONTAMINATION BY INDIVIDUAL PROCESSING STEPS DURING APPLE JUICE PRODUCTION

Treatment	Initial concentration	Reduction (%)	Reference
Washing	90/345/2445 μg/kg	17/75/72	Sydenham *et al.*, 1997
	47–339 μg/kg	21–31	Acar *et al.*, 1998
	2010 μg/kg	78	Leggott *et al.*, 2000
	<60–374 μg/L	10–100[a]	Jackson *et al.*, 2003
Pressing	2000 μg/L	52.5	Bissessur *et al.*, 2001
Centrifugation	2000 μg/L	36.5	Bissessur *et al.*, 2001
Clarification	47–339 μg/kg	15–49	Acar *et al.*, 1998
(gelatine/bentonite)	2000 μg/L	24.5	Bissessur *et al.*, 2001
Enzymatic treatment (depectinization)	2000 μg/L	20.5	Bissessur *et al.*, 2001
Filtration	2000 μg/L	17.5	Bissessur *et al.*, 2001
Depectinization, limited clarification and ultrafiltration	163–313 μg/kg	19–29	Acar *et al.*, 1998
Activated charcoal	120 μg/L	45–80	Leggott *et al.*, 2001
	117 μg/kg	23	Kadakal *et al.*, 2002
	62.3 μg/kg	57	Kadakal and Nas, 2002a

[a]Calculated based on the differences in patulin level in the final product.

used for patulin reduction had to be carefully selected. Different steam-activated carbons exhibited similar adsorption isotherms at a dosage level of 1 g/L and achieved patulin reduction rates of 70–80%. In contrast, chemically activated carbon was less effective in removing patulin and achieved only a 45% reduction at a dose of 1 g/L. Huebner *et al.* (2000) developed an ultrafine activated carbon bonded onto granular quartz to produce a composite carbon adsorbent with a high carbonaceous surface area, good bed porosity, and increased bulk density for the reduction of patulin levels from aqueous solutions and apple juice. Fixed-bed adsorption with 1 g of composite carbon adsorbent was also effective in reducing patulin concentrations (20 μg/L) in a naturally contaminated apple juice, and breakthrough capacities increased with temperature. The composite carbon adsorbent offered a higher initial breakthrough capacity than pelleted activated carbon. However, the appearance and taste of apple juice may be affected by the treatment process.

Efficient chemical decontamination strategies for patulin-contaminated foods like apple juice and apple juice concentrate do not exist. In the presence of sulfhydryl groups or sulfite, patulin is degraded rapidly (Aytac

and Acar, 1994; Fliege and Metzler, 2000). As reviewed by Steiner *et al.* (1999) at acidic pH, a reversible binding of sulfite to patulin occurs. The resulting hydroxysulfonate still includes the conjugated lactone ring, which is the toxicologically relevant structure. Furthermore, because of its allergenic potential, the use of sulfite in apple juice, which is frequently consumed by infants and young children, is not recommended.

As reported by Drusch *et al.* (2004), the presence of oxygen and free radicals is necessary for a rapid degradation of patulin by ascorbic acid. Oxidation of ascorbic acid in the presence of oxygen and metal ions is a possible source of these radicals. Because patulin degradation leveled off after complete oxidation of ascorbic acid, the initial concentration of ascorbic acid and its rate of degradation were important factors in patulin degradation. Because of the low oxygen content in the head space of a food package, addition of ascorbic acid to products like apple juice before filling is not an effective decontamination strategy. Furthermore, the toxicological potential of the resulting patulin degradation products remains unknown. Yazici and Velioglu (2002) investigated the effect of added thiamine hydrochloride, pyridoxine hydrochloride, and Ca-d-pantothenate at various doses to reduce the patulin content of apple juice concentrate. Addition of thiamine hydrochloride (1000 mg/kg), pyridoxine hydrochloride (625 or 875 mg/kg), and Ca-d-pantothenate (1000 or 2500 mg/kg), and storage at 4°C for six months yielded 55.5–67.7% of patulin reduction compared to only 35.8% for the control. Other quality parameters like clarity, color, and turbidity were not affected by this treatment.

Apart from patulin, *P. expansum* also may produce other mycotoxins like chaetoglobosin A and C, the communesins A and B, and the expansolides A and B. Larsen *et al.* (1998) described the production of all these mycotoxins by *P. expansum* when grown on black currant and cherry juice. In particular, chaetoglobosin A was found in juice when sufficient oxygen was available. These authors pointed out that this may pose a risk during juice production, particularly when juice tanks are only partly emptied and oxygen from the head space is available. Therefore, Larsen *et al.* (1998) recommend that tank head spaces may be supplied with nitrogen to control mycotoxin production during storage of juices. Andersen *et al.* (2004) also detected chaetoglobosins and communesins in naturally infected cherry and gooseberry juice samples. Because these samples did not contain patulin, a patulin-negative sample does not necessarily mean that the sample is free from fungal metabolites. These authors suggested that chaetoglobosin A may be a better indicator of growth of *P. expansum* in fruit products than patulin, and that in order to increase safety of fruit products, a simultaneous method for determination of patulin and chaetoglobosin A should be developed.

Ochratoxin A is mainly found in grape juice (Belli *et al.*, 2002; Cerutti *et al.*, 1982; Delage *et al.*, 2003; Fritz, 1983; Majerus *et al.*, 2000; Zimmerli and Dick, 1996). The concentration in red grape juice is usually much higher than in white grape juice. Zimmerli and Dick (1996) found up to 289 ng of ochratoxin A/L in red grape juice compared to 5 ng/L in white grape juice. Majerus *et al.* (2000) analyzed 27 and 64 samples of white and red grape juice, respectively. The 50th percentile of ochratoxin A in the samples was 90 ng/L for white grape juice samples compared to 270 ng/L for red grape juice samples. With 5.3 μg/L, the maximum ochratoxin A concentration in red grape juice also was much higher than in white grape juice (1.3 μg/L). A possible explanation is that a prolonged enzymatic treatment at increased temperature is done during production of red grape juice to increase color yield. This processing step may favor fungal growth and ochratoxin A production. However, in 12% of red grape juice samples and in 22% of white grape juice samples, no ochratoxin A was detectable (Majerus *et al.*, 2000), and, therefore, possibilities for prevention of mycotoxin contamination already exist. Majerus *et al.* (2000) found that all samples of orange juice ($n = 30$) and apple juice ($n = 33$) were free from ochratoxin A.

Abdel-Sater *et al.* (2001) found aflatoxin B_1 and G_1 in apple juice–based drinks marketed in Egypt up to 20–30 μg/L, aflatoxin B_1 in guava juice (12 μg/L), but not in mango juice or grape juice– and peach juice–based drinks. Taking into account that apple juice–based drinks analyzed in the study of Abdel-Sater *et al.* (2001) contained only 20% of apple juice, the initial contamination with aflatoxins of the juice was up to 150 μg/L. Data like this can only be attributed to poor hygienic conditions of the raw material and/or the production facilities. Abdel-Sater *et al.* (2001) and Ragab (1999) also emphasized the necessity of introducing concepts of good manufacturing practices and HACCP in this context.

In the last few years, because of the development of reliable liquid chromatographic (LC) methods with diode array detection or MS detection for quantification of low levels of *Alternaria* toxins, the presence of alternariol and alternariol methyl ether in fruit juices has attracted attention. Delgado and Gómez (1998) detected alternariol and alternariol methyl ether natural contaminants in 16 of 32 samples of apple juice concentrate. Levels of alternariol ranged from 1.35 to 5.42 ng/ml, and alternariol methyl ether was present at concentrations of up to 1.71 ng/ml. Similar concentrations have been reported by Lau *et al.* (2003), who describe sensitive LC-MS and LC-MS-MS confirmatory procedures based on atmospheric pressure chemical ionization with negative ion detection for the determination of alternariol and alternariol methyl ether. The natural occurrence of alternariol in nine samples of apple juice and in single samples of some other clear fruit beverages like grape juice, cranberry nectar, raspberry juice, and prune

nectar at levels of up to 6 ng alternariol/ml were confirmed. *Alternaria* toxins were stable in fruit juice. Scott and Kanhere (2001) investigated the stability of alternariol, alternariol methyl ether, and altertoxin I in apple juice. Alternariol and alternariol methyl ether were stable after heating of juice for 20 min at 80°C or during storage at room temperature for 20 days, and no apparent loss was observed by these authors.

C. WINE AND OTHER ALCOHOLIC BEVERAGES

The most important mycotoxin in wine is ochratoxin A. Several studies on ochratoxin A concentration in wine are available (Battilani and Pietri, 2002; Burdaspal and Legarda, 1999; Delage *et al.*, 2003; Lau *et al.*, 2003; Majerus *et al.*, 2000; Markaki *et al.*, 2001; Pietri *et al.*, 2001; Soleas *et al.*, 2001; Stefanaki *et al.*, 2003; Tateo *et al.*, 2000; Visconti *et al.*, 1999; Zimmerli and Dick, 1996), which show large differences in the incidence and level of ochratoxin A contamination. Levels of ochratoxin A are higher in red wine than levels in rosé and white wine in all these studies. The most comprehensive review was published in 2002 by the European Commission (European Commission [Directorate-General Health and Consumer Protection], 2002a). The total number of samples was 1470, with 59% positive. The levels of contamination ranged from 3 ng/kg (Spain) to 15,600 ng/kg (Italy). The weighted European mean level was 357 ng/kg. The most recent data for ochratoxin A in red wine are summarized in Table VII.

Several authors observed an interrelation between ochratoxin A concentration and geographical region. Majerus *et al.* (2000) analyzed 41 samples of European white wine and 94 samples of European red wine. These authors classified results according to wine regions as defined by the European Common Market Organization for Wine following natural criteria like soil, climate, and topography and lumped the results to one northern region including Germany, northern France, and northern Italy and one southern region covering central and southern Italy, southern France, Greece, and Spain. With a 90% percentile value of 0.42 μg/L for white wine and 0.11 μg/L for red wine, samples from the southern region had a higher level of ochratoxin A than samples from the northern region with a 90% percentile value of less than 0.01 μg/L for white wine and 0.06 μg/L for red wine. As shown in Table VII, Pietri *et al.* (2001) reported a similar gradient for Italian red wine and for Italian white dessert wine from different regions of Italy. All authors emphasized that the warm climatic conditions in southern regions of Europe favor growth of *Aspergillus* species and, therefore, favor ochratoxin A formation in wines from these regions.

TABLE VII
CONCENTRATION OF OCHRATOXIN A IN RED WINE

Origin of the samples	n	Range (ng/L)	Mean (ng/L)	Median (ng/L)	Reference
Europe	91	<3–603	—	54	Burdaspal and Legarda, 1999
Southern Italy	27	<10–7630	—	895	Visconti *et al.*, 1999
Worldwide		<10–7000	—	172	Majerus *et al.*, 2000
Morocco	14	28–3240	—	650	Filali *et al.*, 2001
Mediterranean countries	31	<2–3400	—	—	Markaki *et al.*, 2001
Italy (total)	96	<1–3177	—	90	Pietri *et al.*, 2001
Northwest Italy	23	<1–79	—	2	
Northeast Italy	19	<1–227	—	90	
Central Italy	30	<1–1450	—	134	
Southern Italy	24	10–3177	—	1264	
Worldwide	580	<50–200	—	—	Soleas *et al.*, 2001
Greece	104	<50–2690	—	90	Stefanaki *et al.*, 2003
South Africa	9	180–390	246	—	Shepard *et al.*, 2003
South America	22	28–70	39	—	Rosa *et al.*, 2004

Zimmerli and Dick (1996) pointed out that apart from climatic differences, different practices in grape cultivation (e.g., use of pesticides or different cultivars, and wine making) may influence the ochratoxin A concentration in wine. The latter include time and condition of storage of harvested grapes, type of maceration, and time and temperature of fermentation.

The difference in wine making is also the reason for the differences in ochratoxin A concentration between white, rosé, and red wine. White grapes are immediately pressed after harvest, whereas red wine grapes are mashed and macerated to extract anthocyanins from the berry skins. Maceration lasts either for several days at elevated temperature or for several hours using pectolytic enzymes after heating of the must to 80°C.

All cited studies show that ochratoxin A contamination of wine is a result of mold infection in the field and subsequent processing conditions and, therefore, dispute the statement of Zimmerli and Dick (1996) that ochratoxin A in wine of southern European origin is formed only after the harvest of the grapes. Zimmerli and Dick (1996) assumed that climatic conditions in the south of Europe are less humid than in central Europe and are, therefore, less favorable for mold growth on the field. The authors suggested that ochratoxin A–producing molds growing in the barrels and processing of moldy fruits significantly caused ochratoxin A contamination in wine. They gave further evidence to their hypothesis by analyzing must and wine

samples, in which similar levels of ochratoxin A contamination were observed. In the presence of ethanol, ochratoxin A is rather stable. Zimmerli and Dick (1996) report a conversion of ochratoxin A to its ethyl ester ochratoxin C under acidic conditions in the presence of ethanol.

Fining of wine, a common winery practice, reduced ochratoxin A levels in wine. Fining involves the addition of an adsorptive compound to reduce levels of certain compounds like protein particles that would cloud the wine and phenolic compounds like tannins that could cause bitterness and astringency in wine. Dumeau and Trione (2000) assessed the ability of winemaking additives to remove ochratoxin A from red wines. Cellulose at 50 g/hL or inertized yeasts at 50 g/hL removed only small amounts of ochratoxin A (8 and 13%). Silica gel at 50 g/hL removed 30% of ochratoxin A. A 50% reduction in ochratoxin A concentration could be achieved with activated charcoal at 20 g/hL or silica gel in combination with gelatin. Castellari *et al.* (2001) showed that in particular activated carbon and potassium caseinate remove high amounts of ochratoxin A from red wine. Fining agents were used at levels of 10 and 150 g/hL. Because of low adsorption of total polyphenols, the activated carbon can be used at a dosage higher than 10 g/hL and might completely remove ochratoxin A from red wine.

Nevertheless, good manufacturing practice (GMP) is an important tool for preventing ochratoxin A contamination in wine. According to Soleas *et al.* (2001), a maximum level of 200 ng of ochratoxin A/L is easily achievable. To investigate the possible relation between quality of wine and ochratoxin A concentration, Tateo *et al.* (2000) analyzed Italian table wines in multicomponent packages. Ochratoxin A was detected in 97% of the samples, and the concentration was above 1000 ng/L in 52% of the samples. The authors stated that these levels are generally higher than those found in good quality–bottled wines.

Apart from ochratoxin A in wine, for alcoholic beverages, the occurrence of patulin in apple cider has occasionally been reported. The frequency of contamination and the detected concentrations of patulin are generally very low (Armentia *et al.*, 2000; Food Standards Agency, 2003b; Jackson *et al.*, 2003; Tangni *et al.*, 2003). Moss and Long (2002) studied the fate of patulin in the presence of *Saccharomyces cerevisiae,* a yeast commonly used for hard cider production. *S. cerevisiae* degraded patulin during active fermentative growth, but not when growing aerobically. Products of patulin degradation were more polar than patulin, and these authors identified E-ascladiol as one major metabolite of patulin. One possible explanation for the presence of patulin in cider after fermentation is an addition of patulin-contaminated apple juice to cider. The addition of apple juice to cider is a common practice for the production of sweet apple cider in European countries and South Africa (Tangni *et al.*, 2003).

D. MARMALADES AND JAM

Traditional marmalades and jams are made almost entirely from fruits and sugar. Additional ingredients may be gelling agents, starch syrup, and acids. Marmalades and jams are thickened by boiling to a final moisture content of 30% and a total sugar concentration of approximately 60%. Consequently, traditional marmalades and jams have a reduced water activity (a_w) of 0.8–0.9, which allows mold growth, but which may not be favorable for mycotoxin production. As previously described (Table III), for *Aspergillus* species the difference between minimum a_w value for mold growth (a_w 0.77–0.83) and mycotoxin formation (a_w 0.83–0.88) is rather small. In contrast, for *Penicillium* species, the minimum a_w value required for fungal growth is between 0.82 and 0.85, whereas mycotoxin production requires a minimum a_w value of 0.99. Conditions for mold growth and mycotoxin formation in marmalades and jams change, when the level of added sugar decreases, as is the case for diabetic products. The a_w value is much higher and products have to be stabilized using preservatives.

Lindroth *et al.* (1978) studied the effects of storage temperature and water activity on patulin production by *P. expansum* in black currant, blueberry, and strawberry jams over a storage period of six months. Reduction of storage temperature from 22 to 4°C decreased both hyphal growth and patulin production. When the water activity of stored jam was reduced by the addition of 20 and 44% sugar, toxin production in the jams was reduced to 0.08–10% of the maximum occurring in unsweetened jams, despite that the addition of sugar stimulated hyphal growth. Katsumata *et al.* (2002) investigated the effects of 17 spice oils to inhibit the growth of *P. expansum* on strawberry jam and found cassia, cinnamon, and clove oils to be effective. Cinnamic aldehyde and eugenol demonstrated high inhibitory effects on the growth of this mold. The combined use of two constituents revealed that the combination of cinnamic aldehyde and β-caryophyllene was highly effective for growth inhibition, although β-caryophyllene demonstrated only a weak effect when used alone. Patulin and citrinin were not detected in the jam on which *P. expansum* grew.

Ochratoxin A formation in apricot jam has been investigated by Ruhland *et al.* (1998). Apricot jam prepared conventionally was inoculated with ochratoxin A–producing *A. ochraceus* and *P. verrucosum.* Fungal growth was detected after two weeks and mycotoxin production after five weeks. Ochratoxin A contents reached up to 86.3 µg/kg for *A. ochraceus*—and 94.6 µg/kg for *P. verrucosum*–inoculated jams. Seven of twelve naturally contaminated jam samples contained 0.09–14.33 µg/kg ochratoxin A. In a market survey, Engel (2000) could not detect ochratoxin A at a concentration higher than 0.1 µg/kg in 42 commercial jam samples.

In summary, a frequent and continuous occurrence of mycotoxins in fruit products is limited to aflatoxins in figs, to ochratoxin A in dried vine fruits, grape juice, and wine, and to patulin in apple juice. Although in the fruit-producing countries due to climatic conditions, fungal growth and mycotoxin contamination are not unavoidable, the principles of good agricultural practices are key elements in the prevention of possible mycotoxin contamination as outlined in this review for aflatoxins in dried figs. Manual sorting of the fruits or sorting techniques like analysis of bright green-yellowish fluorescence help to reduce mycotoxin levels in the fruits before processing or consumption. Trimming damaged or rotted fruit before juice pressing is an effective tool for reducing mycotoxin levels in juices like apple juice. However, concerning direct consumption of fruits, it has to be kept in mind that mycotoxins may penetrate through parts of the fruit into the sound tissue or throughout the whole fruit. Analysis of patulin may not be sufficient for evaluation of the mycological status of apple juice and an additional analysis for chaetoglobosin A may be necessary. More data on the occurrence of chaetoglobosin A in apple juice should be collected to substantiate this recommendation. Removal of patulin from juices using physical adsorption techniques is not applicable, because the color and taste of the product are adversely affected.

Ochratoxin A is mainly associated with grapes and derived products. Red grape juice contains higher levels of the toxin, because a prolonged maceration and an enzymatic treatment are applied to increase color yield. Because ochratoxin A is stable in ethanol, wine also is frequently contaminated with ochratoxin A, although fining reduces ochratoxin A levels to a certain degree.

V. IMPACT ON HUMAN NUTRITION

Before an evaluation of the impact of a possible mycotoxin contamination in fruits on human health, the general procedure of risk assessment and the outcome thereof for mycotoxins by international agencies is briefly described. With the establishment of the World Trade Organization in 1995, standards and recommendations elaborated by the Codex Alimentarius Commission (CAC) reflect the international consensus for health and safety requirements (Moy, 1998). The CAC is an intergovernmental institution, which was founded in 1963 by the World Health Organization (WHO) and the Food and Agriculture Organization of the United Nations (FAO).

Risk assessment for mycotoxins is a multistep process that involves the CAC and the Joint FAO/WHO Expert Committee on Food Additives (JECFA). The CCFAC is responsible for the subsequent step, risk

management. Another important institution is the International Agency for Research on Cancer (IARC) of the WHO, which provides scientific backgrounds and evaluations concerning the carcinogenicity of chemical substances. Table VIII gives an overview on risk assessment for aflatoxins, ochratoxin A, and patulin.

Apart from hazard identification and characterization, a vital step in risk assessment is the exposure assessment for a population and the contribution of different food groups to the total intake. For ochratoxin A, a reevaluation has been performed by the JECFA (2001a). As stated by the JECFA, 85% of newly available data on ochratoxin A originated from Europe. This is probably because the European Union has established maximum levels for ochratoxin A for different commodities and research was focused on ochratoxin A in those products for years. Assessments of international ochratoxin A intake have been made by the JECFA on the basis of data on mean consumption combined with the weighted mean level of contamination. Because ochratoxin A occurs mainly in the diet of European countries, data on food consumption in Europe obtained from the Global Environmental Monitoring System/Food Contamination Monitoring (GEMS/Food) were considered the most relevant for risk assessment in the evaluation of JECFA (2001a). The submitted data on levels of contamination were aggregated according to the recommendations of a FAO/WHO workshop to obtain a weighted mean. Using this approach, the mean total intake of ochratoxin A was estimated as 45 ng/kg of body weight/wk assuming a body weight of 60 kg. Wine contributed about 10 ng/kg of body weight/wk to the mean intake (22%), whereas grape juice contributed 2–3 ng/kg of body weight/wk (6.7%). Dried fruits contributed less than 1 ng/kg of body weight/wk (JECFA, 2001a).

The situation concerning the contribution of fruits and derived products to the total ochratoxin A intake may change if either the basis for food consumption data or ochratoxin A concentration is changed. This becomes evident from data of a nationwide ochratoxin A survey performed in Germany (Cholmakov-Bodechtel *et al.*, 2000). Intake estimation of Cholmakov-Bodechtel *et al.* (2000) was based on the analysis of 7000 food samples from the German market and a questionnaire on nutritional and consumption habits of 2500 persons. The mean total daily ochratoxin A intake of adults was 39.9 ng. Wine including champagne contributed 1.4% and foods from the category juices, table water, and other nonalcoholic beverages 6.6%. Based on data for the highest plausible portion size from the questionnaire and the 90th percentile of ochratoxin A concentration, calculated total daily ochratoxin A intake increased to 247.9 ng. Furthermore, the contribution of wine including champagne rose to 11.3%, and the contribution of foods from the category juices, table water, and other nonalcoholic beverages was 12.5%.

TABLE VIII

SUMMARY OF THE OUTCOME OF THE RISK ASSESSMENT BY THE INTERNATIONAL AGENCY FOR RESEARCH ON CANCER AND THE CODEX ALIMENTARIUS COMMISSION FOR AFLATOXINS, OCHRATOXIN A, AND PATULIN IN FOODS FOR HUMAN CONSUMPTION[a]

	International Agency for Research on Cancer degree of evidence of carcinogenicity			
	Humans	Animals	Overall for humans	Codex Alimentarius Commission
Aflatoxins	Strong	Strong	Class 1 (carcinogenic to humans)	No tolerable intake defined; presence of aflatoxins in food should be reduced to irreducible levels[b]
Aflatoxin B_1	Strong	Strong		
Aflatoxin B_2		Limited		
Aflatoxin G_1		Strong		
Aflatoxin G_2		Inadequate		
Ochratoxin A	Inadequate	Strong	Class 2B (possibly carcinogenic to humans)	Provisional tolerable weekly intake: 0.1 μg/kg of body weight
Patulin	Inadequate; no data available	Inadequate	Class 3 (not classifiable as to its carcinogenicity to humans)	Provisional tolerable daily intake: 0.4 μg/kg body weight

[a]Codex Alimentarius Commission, 2002; International Agency for Research on Cancer, 1993; Joint Expert Committee on Food Additives (JECFA), 1998; Joint Expert Committee on Food Additives, 2001b.

[b]According to the JECFA, the irreducible level is defined as the concentration of a substance that cannot be eliminated from a food without involving the discarding of that food altogether, severely compromising the ultimate availability of major food supplies. Also referred to as the *ALARA principle* ("as low as reasonably achievable").

Another crucial point is that the contribution of fruits and derived products contaminated with ochratoxin A still may contribute significantly to the total ochratoxin A intake of individual groups within a population, which may possess a dietary pattern different from the average adult consumer. Within a study on the Europe-wide assessment of ochratoxin A intake (European Commission [Directorate-General Health and Consumer Protection], 2002a), the United Kingdom provided data for ochratoxin A intake by children aged 1.5–4.5 years. Total dietary ochratoxin A intake of the children was 3.55 ng/kg body weight/day. With 2.2 ng/kg body weight/day, dried fruits contributed 62% to total dietary ochratoxin A intake.

The situation is similar for patulin. Based on available risk analysis studies, dietary intake of patulin by the general population is not supposed to be a health risk, but again, infants and young children as heavy consumers of possibly contaminated apple products may be exposed to higher levels of patulin. The U.S. Food and Drug Administration (FDA) published in 2001 a background paper on patulin in apple juice, apple juice concentrates, and apple juice products, in which dietary intake of patulin was assessed (FDA, 2001). In this study, the FDA considered the estimated exposure to patulin for drinkers of "all ages" and for small children in two age categories, children younger than 1 year and children 1–2 years old using a probabilistic modeling, the so-called "Monte Carlo analysis." Two scenarios were calculated, one on the basis of no control measures of producers in which all available data on patulin in apple products were taken into account. In the second scenario, on the basis of a maximum patulin level of 50 μg/kg, producers do not place products with patulin levels higher than 50 μg/L on the market. The results of the assessment indicated that if no controls for patulin levels are carried out by producers, the estimated 90% percentile patulin exposure for apple juice drinkers of all ages was approximately 0.26 μg/kg of body weight/day. The exposure for children younger than 1 year was 0.4 μg/kg of body weight/day and exposure for children 1–2 years was 1.7 μg/kg of body weight/day, four times the provisional tolerable daily intake. Furthermore, the results showed that if processors implement controls for patulin at the 50-μg/kg level, the estimated 90% percentile decreased to 0.1 μg/kg of body weight/day for drinkers of all ages, to 0.27 μg/kg of body weight/day for the younger than 1-year-old age-group and to 0.67 μg/kg of body weight/day for 1- to 2-year-old children. Based on these data, the FDA recommended a 50-μg/kg action level for patulin in apple juice, apple juice concentrates, and apple juice products (FDA, 2001).

An assessment of the dietary exposure to patulin in Europe showed that as far as the comparison with the provisional tolerable daily intake of 0.4 μg/kg of body weight/day is concerned, from the data reported, the exposure seems to be quite below that value (European Commission

[Directorate-General Health and Consumer Protection], 2002b). Apple juice and apple nectar represented the main source of intake in Austria, Belgium, France, Germany, Portugal, and the United Kingdom for all groups of population taken into consideration, particularly for young children. Cider, including drinks based on cider, provided a considerable contribution to the total intake of consumers in France; for male adults in France it was the main contribution.

Concerning the contribution of fruits, dried fruits, and fruit-containing products to the total dietary intake of aflatoxins, no data are available. The latest risk analysis on aflatoxins was performed by the JECFA in 1998. The JECFA stated that although a wide range of foods may be contaminated with aflatoxins, they have been most commonly associated with groundnuts, dried fruits, tree nuts, spices, figs, crude vegetable oils, cocoa beans, maize, rice, cottonseed, and copra. Because only few data on dried fruits were available, the JECFA intake estimation for aflatoxin B_1, B_2, G_1, and G_2 is almost entirely based on aflatoxin contamination data and intake data for cereals and nuts. However, heavily aflatoxin-contaminated dried fruits, especially figs, may pose a health risk for consumers and maximum levels as set in the European Union, and effective control measures are unavoidable. In 2002, special conditions on the import of figs, hazelnuts, pistachios, and certain products derived thereof originating in a specific third country had to be established by the European Union. Maximum limits had been considerably exceeded several times, and after carrying out a mission to the country, the European Commission's Food and Veterinary Office (FVO) stated "that the control procedures in place for dried fig consignments intended for export into the European Community do not ensure that the consignments comply with the maximum levels established in EC legislation. Insufficient training of responsible officials, insufficient sampling and testing procedures and insufficient evidence that the export certificates correlate to the concerned consignment have been observed" (European Commission, 2002).

As discussed, aflatoxins are genotoxic and carcinogenic, and for substances of this type, there is no threshold below which no harmful effect is observed. Subsequently, no tolerable daily intake has been set by the JECFA or any other international or national authority. Limits for foods are set as low as reasonably achievable. The current legislation in the European Union includes a maximum level of 2 μg/kg for aflatoxin B_1 and 4 μg/kg aflatoxin B_1, B_2, G_1, and G_2 for dried fruits and products processed thereof, intended for direct human consumption or as an ingredient in foodstuffs.

In conclusion, the risk of an acute toxicosis or long-term chronic health effects of a mycotoxin contamination through consumption of mycotoxin-contaminated fruits and fruit products is relatively low compared to other food groups such as cereals. The occurrence of an endemic poisoning by

consumption of mycotoxin-contaminated fruits has so far not been reported in contrast to mycotoxin poisonings by consumption of cereals. Nevertheless, regional problems may arise in tropical or subtropical areas, where climatic conditions favor mold growth and mycotoxin formation, where food consumption pattern may be unvaried, and where hygienic conditions during storage and processing may be poor.

Certain groups of a population may be at risk for elevated exposure to mycotoxins if certain fruit products significantly contribute or even dominate the daily diet. This has especially been shown for infants and young children for patulin in apple juice and ochratoxin A in dried vine fruits. Furthermore, wine and cider may significantly contribute to ochratoxin A intake of adults. As a consequence, maximum mycotoxin concentrations for certain fruit products have been established in several countries.

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HUMAN PROTOZOAN PARASITES IN MOLLUSCAN SHELLFISH

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I. SEAFOOD IN THE AMERICAN DIET

The popularity of seafood in the American diet is high (Munoz, 1999; Wallace *et al.*, 1999) and steadily increasing as seafood items tend to represent a healthy contribution of low-fat proteins in a balanced diet (Munoz, 1999; Rippey, 1994; Wallace *et al.*, 1999). However, some concerns have been raised worldwide regarding health risks, particularly from molluscan shellfish contaminated with human pathogens (Feldhusen, 1990; Todd *et al.*, 1992; citations in Table I).

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TABLE I

REPORTS OF ***CRYPTOSPORIDIUM*** IN MOLLUSCAN SHELLFISH INTENDED FOR HUMAN CONSUMPTION (CHRONOLOGICAL ORDER)

Reference; Shellfish species	Geographic location	Identification level; detection techniques	Comments
Fayer *et al.*, 1998; *Crassostrea virginica*	Choptank, Severn, Miles, Wye, Potomac, and Wicomico Rivers; Chesapeake Bay, USA	*Cryptosporidium parvum*; IFA, mouse bioassay	Infectious oocysts in hemolymph and gills; most infected oysters at a site near a large cattle farm
Fayer *et al.*, 1999; *C. virginica*	Fishing Bay, Tangier Sound, and Wicomico, Nanticoke, Potomac, and Patuxent Rivers; Chesapeake Bay, USA	*C. parvum* Genotype 1 and 2; IFA, mouse bioassay, PCR, PCR-RFLP	Oocysts detected in oysters and water; infectious oocysts in hemolymph and gills
Freire-Santos *et al.*, 2000; *Dosinia exoleta, Venerupis pullastra, V. rhomboideus, Venus verrucosa, Mytilus galloprovincialis, Ostrea edulis, Ruditapes philippinarum*	Galicia, northwest Spain, bounded to the Atlantic Ocean; Italy; England	*Cryptosporidium* species; malachite green, safranine, methylene blue, carbol-fuchsin, auramine-rhodamine, IFA	Depuration ineffective for *Cryptosporidium*, positive relationships between fecal coliforms and *Cryptosporidium* in shellfish
Gomez-Bautista *et al.*, 2000; *M. galloprovincialis*, *Cerastoderma edule*	Galicia, northwest Spain, bounded to the Atlantic Ocean	*C. parvum* genotype 2, IFA, mouse bioassay, PCR, RFLP	Oocysts infectious, $>10^3$ oocysts/mollusc, most contaminated shellfish near river banks with grazing cattle

Lowery *et al*., 2001; *Mytilus edulis*	Ireland, shores of Belfast Lough	*C. parvum* Genotype 1; IMS-IFA, PCR, PCR-RFLP	Anthropogenic source(s) of contamination
Fayer *et al*., 2002; *C. virginica*	Chesapeake Bay, Maryland	*Cryptosporidium* species; IFA	Oyster contamination coincided with rainfalls and increased stream flow
Gomez-Couso *et al*., 2003a; *Dosinia exoleta, Venerupis pullastra, V. rhomboideus, Venus verrucosa, Mytilus galloprovincialis, Ostrea edulis, Ruditapes philippinarum*	Galicia, northwest Spain, bounded to the Atlantic Ocean	*Cryptosporidium* species; IFA	Viable *Cryptosporidium* oocysts detected in 34% of sampled bivalves; depuration was found to be ineffective in oocyst removal
Negm, 2003; *Caelatura Iaronia pruneri*, *Donax trunculus limiacus*	Alexandria, Egypt	*Cryptosporidium* species, *Cyclospora* species, microsporidia; conventional stains, mouse bioassay	*Cryptosporidium* species oocysts and microsporidian spores were infectious; *Cyclospora* species oocysts were noninfectious
Fayer *et al*., 2003; *C. virginica*	Atlantic Coast from Maine to Florida	*C. parvum*, *C. hominis*; *C. meleagridis*, IFA, PCR, genotyping, mouse bioassay	65% commercial harvesting sites contaminated with *Cryptosporidium*
Gomez-Couso *et al*., 2004; *C. virginica*	United Kingdom	*C. parvum*, *C. hominis*; multiplexed nested PCR	*C. parvum* and *C. hominis* detected in 11% of sampled bivalves.

Since the late 1800s when shellfish-related illnesses were first reported in the United States, there have been more than 400 epidemics of food-borne diseases and more than 14,000 gastroenteritis cases related to consumption of contaminated molluscan shellfish (Rippey, 1994). New York and Florida accounted for more than 50% of these epidemics (Rippey, 1994). In New York alone from 1980 to 1994, molluscan shellfish accounted for 64% (n = 204) of all food-borne outbreaks in which the etiologic agent was identified and 41% of outbreaks caused by an unknown etiologic agent (Wallace *et al.*, 1999).

Consumed oysters are more likely than other seafood items to contain infective microorganisms because they recover and contain pathogens from surrounding waters and are very often eaten raw (Rippey, 1994; Wallace *et al.*, 1999). In the United States, 8% of approximately 33 million food-borne illnesses annually have been linked to the consumption of raw oysters (Altekruse *et al.*, 1999). Clams, mussels, cockles, and scallops are less of a public health concern because they are usually consumed cooked or steamed, which significantly alters the infectivity of potential pathogens (Rippey, 1994).

A. GASTROENTERITIS RELATED TO WASTEWATER AND SEWAGE DISPOSAL

Food-borne infections due to consumption of molluscan shellfish have been classified into three categories based on the origin of the etiologic agent (Rippey, 1994): (1) allochthonous agents (i.e., pathogens associated with sewage disposal, wastewater effluents, agricultural and urban runoff, and overboard disposal of toilet contents), (2) autochthonous agents (i.e., pathogens or marine biotoxins indigenous to coastal waters, and (3) pathogens derived from postharvest contamination due to inappropriate storage or processing. This chapter focuses specifically on allochthonous agents, that is, waterborne protozoan parasites originating from anthroponotic and zoonotic sources and found in a variety of commercial and feral molluscan shellfish species.

From 1898 to 1990, more than 75% of gastroenteritis outbreaks and more than 79% of gastroenteritis cases due to consumption of shellfish contaminated by sewage or wastewater-derived pathogens were due to an unknown etiologic agent (Rippey, 1994). In general, outbreaks and cases of gastroenteritis of unknown etiology occur more frequently in late spring and late fall, roughly coinciding with periods of the most intense feeding (i.e., filtering) and, thus, pathogen bioaccumulation (Rippey, 1994). It is believed that in more than 93% of molluscan shellfish–associated outbreaks, the shellfish were probably contaminated at the sites from which they were harvested as opposed to postharvest contamination (Wallace *et al.*, 1999).

II. BIAS IN REPORTING OF MOLLUSCAN SHELLFISH–VECTORED ILLNESSES

The data reported in medical literature most likely represent a small portion of actual gastroenteritis cases, because the true incidence of shellfish-vectored illnesses is believed to be underestimated as much as 20-fold or more (Hauschild and Bryan, 1980; Mead *et al*., 1999). There are several reasons for such underreporting. First, there is a lack of mandatory federal requirements for reporting of gastroenteritis of an unspecified nature (gastroenteritis is not a reportable illness), and physicians and state health departments are not obligated to forward case reports to federal or state authorities (Rippey, 1994; Wallace *et al*., 1999). Second, many cases of gastroenteritis are mild and self-limiting, which do not require treatment by a physician (Rippey, 1994; Wallace *et al*., 1999). Third, not all outbreaks are recognized or reported, and sporadic cases of food-borne illnesses are not detected by the existing food-borne disease surveillance system (Wallace *et al*., 1999). Fourth, it is difficult to epidemiologically ascribe a diarrhetic disease outbreak to a specific food item, particularly when small numbers of people are showing symptoms (Archer and Kvenberg, 1995; Rippey, 1994; Wallace *et al*., 1999). Fifth, for some infectious agents, symptoms may not become apparent immediately, but long after the implicated food items have been consumed or discarded (Rippey, 1994; Wallace *et al*., 1999). Finally, the accuracy of the tagging system is not perfect (Rippey, 1994).

III. ASSOCIATION OF HUMAN WATERBORNE PARASITES AND MOLLUSCAN SHELLFISH

Cryptosporidium parvum, Giardia lamblia, Cyclospora cayetanensis, and *Toxoplasma gondii* are human protozoan enteropathogens in which transmission is associated with water (Graczyk *et al*., 1997d; Lindsay, 2001; Lindsay *et al*., 2001; Ortega *et al*., 1993; Wolfe, 1992). *C. parvum*, *G. lamblia*, *C. cayetanensis* infections cause gastroenteritis, which is predominantly manifested by diarrhea (Graczyk *et al*., 1997d; Ortega *et al*., 1993; Wolfe, 1992). *T. gondii* causes serious congenital complications in fetuses born to mothers infected for the first time during pregnancy (Lindsay, 2001). Medically, the most important is *Cryptosporidium* because it significantly contributes to the mortality of people with impaired immune systems due to the lack of effective therapy (Blagburn and Soave, 1997). Although *G. lamblia* (synonyms: *Giardia intestinalis* and *Giardia duodenalis*) and *C. cayetanensis* cause serious prolonged diarrheal illness in adults and children worldwide, the infections usually respond well to pharmacological

treatment (Ortega *et al.*, 1993; Wolfe, 1992). *C. parvum, G. lamblia,* and *T. gondii* are anthropozoonotic pathogens (Graczyk *et al.*, 1997d; Lindsay, 2001; Lindsay *et al.*, 2001; Wolfe, 1992). All of these parasites produce a long-lasting and environmentally resistant infectious stage (e.g., *Cryptosporidium, Cyclospora*, and *Toxoplasma* oocysts and *Giardia* cysts, which can be transmitted via water). *Cryptosporidium* oocysts pollute coastal waters via point sources of contamination such as wastewater discharges, leaky septic tanks, urban runoff, recreational activities, and agricultural runoff predominantly from livestock operations, such as cattle farms (Graczyk *et al.*, 2000a, c). Clinical infections are mainly confined to calves, which can shed up to 10^6 oocysts/g of their feces and exceed 10^9 oocysts in daily output (Anderson, 1981). As many as 10^6 oocysts/ml can be found in human diarrhetic feces (Rose, 1997). The infectious dose of *C. parvum* for immunosuppressed people has not been established, but it is believed that the disease can be caused by a single oocyst (Rose, 1997). Mortality rates due to *C. parvum* among these individuals vary from 52 to 68% (Rose, 1997). In addition to *Cryptosporidium, Giardia, Toxoplasma,* and *Cyclospora,* human infectious microsporidia such as *Encephalitozoon intestinalis, Encephalitozoon hellem,* and *Enterocytozoon bieneusi* are emerging anthropozoonotic pathogens that inflict considerable morbidity on healthy people and can be associated with mortality in immunosuppressed populations (Bryan and Schwartz, 1999). The transmissive stages of all these parasites (i.e., oocysts, cysts, and spores) are resistant to environmental stressors and, therefore, relatively ubiquitous in the environment (Kucerova-Pospisilova *et al.*, 1999; Rose *et al.*, 1997; Wolfe, 1992). *Cryptosporidium* and *Giardia* are very frequently transmitted via water (Rose *et al.*, 1997; Wolfe, 1992). Considerable evidence indicates involvement of water in the epidemiology of microsporidia as well (Cotte *et al.*, 1999; Dowd *et al.*, 1998; Fournier *et al.*, 2000; Sparfel *et al.*, 1997).

Molluscan shellfish are suspension- or sediment-feeding organisms that filter unicellular algae, bacteria, other microorganisms, and detrital particles in the approximately 1–30 μm size range (Kennedy *et al.*, 1996; McMahon, 1991). Bivalves have an important role in aquatic habitats; by filtering suspended particles, they clarify the water and generally improve water quality (McMahon, 1991). The diameter of *Cryptosporidium, Cyclospora,* and *Toxoplasma* oocysts does not exceed 6, 8, and 10 μm, respectively, and *Giardia* cysts are oval and no longer than 15 μm (Graczyk *et al.*, 1997d; Lindsay, 2001; Lindsay *et al.*, 2001; Ortega *et al.*, 1993, Wolfe, 1992). Microsporidian spores range from 1.5 to 4 μm (Graczyk *et al.*, 2004). Thus, cystic stages of these parasites fall within the range of particles filtered by bivalve molluscs. Multiple *in vitro* and *in vivo* experimental studies demonstrated that cysts can be efficiently recovered from water and then retained and concentrated in shellfish tissue (citations in Table III).

Historically, *C. parvum* oocysts of waterborne origin were first identified in the tissue of blue mussels in Ireland (Chalmers *et al*., 1997), initiating worldwide investigation of this pathogen in molluscan shellfish (Graczyk, 2003a,b). Since then, multiple studies demonstrated that these filter-feeding organisms can harbor environmentally derived protozoan parasites as a result of concentrating the recovered particles (Graczyk, 2003b). Because of the vast amount of publications related to human protozoan parasites in molluscan shellfish, this chapter classifies available reports into three categories presented in Table I, Table II, and Table III.

An interesting epidemiological discovery is the identification, for the first time, of human infectious microsporidia spores, *E. intestinalis* and *E. bieneusi,* in molluscan shellfish; zebra mussels (*Dreissena polymorpha*) (Graczyk *et al*., 2004). Microsporidia infects a variety of vertebrate and invertebrate hosts, and approximately 14 species have been reported to infect people (Kotler and Orenstein, 1999). Of these, *E. intestinalis* and *E. bieneusi* have been reported to be zoonotic and to infect domestic animals and livestock (Bornay-Llinares *et al*., 1998; Breitenmoser *et al*., 1999; Buckholt *et al*., 2002; Deplazes *et al*., 1996; Graczyk *et al*., 2002; Rinder *et al*., 2000). In humans, they cause serious gastroenteritis, as well as urinary and sometimes ocular infections (Graczyk *et al*., 2004). Although the actual transmission route of this specific spore species is not known, it is quite possible that infectious spores of human or animal origin passed to the aquatic environments via feces or urine (Bryan and Schwartz, 1999). Spores of microsporidia have been detected in a variety of surface waters (Avery and Undeen, 1987), and water as a source of human infections has been concluded from epidemiological data (Cotte *et al*., 1999). Spores of *E. intestinalis* and *E. bieneusi* have been detected in surface waters (Dowd *et al*., 1998; Sparfel *et al*., 1997).

Cryptosporidium oocysts have also been identified in feral bivalves, supporting the concept that estuarine shellfish can be used in the sanitary assessment of water quality as biological indicators for contamination of water and sediment. Publications listed in Table II postulated the values of filter feeders as an alternative monitoring system in aquatic habitats for waterborne contamination. For example, in North America, bivalves such as zebra mussels serve as an excellent biological indicator of chemical, viral, and bacterial pollutants (e.g., in the Great Lakes and the St. Lawrence River), because they can bioaccumulate such pollutants in their tissue (Brieger and Hunter, 1993, de Lafontaine *et al*., 1999, Horgan and Mills, 1999). Zebra mussels and *Corbicula* clams very efficiently concentrate *C. parvum* and *G. lamblia* in relation to low ambient concentrations (Graczyk, 2003b). In addition, zebra mussels are also able to recover spores of human-infective species of microsporidia such as *E. intestinalis* and *E. bieneusi* (Graczyk *et al*., 2004). Bivalves such as zebra mussels or

TABLE II

REPORTS OF HUMAN WATERBORNE INTESTINAL PARASITES IN FERAL MOLLUSCAN SHELLFISH (CHRONOLOGICAL ORDER)

Shellfish species	Geographic location	Reference	Infectious agent, comments
Mytilus edulis	Ireland, Sligo area	Chalmers *et al*., 1997	*Cryptosporidium*, oocysts detected in water (22% prevalence) and mussels (8% prevalence)
Crassostrea virginica	Choptank, Severn, Miles, Wye, Potomac, and Wicomico River; Chesapeake Bay, USA	Fayer *et al*., 1998	*C. parvum*, infectious for mice, oocysts in hemolymph and gills, most infected oysters at a site near a large cattle farm
Macoma balthica, M. mitchelli	Rhode River; Chesapeake Bay, USA	Graczyk *et al*., 1999c	*Giardia duodenalis* assemblage A, on average 36 cysts/clam, contaminated clams in wildlife refuge
Ischadium recurvum	Severn and Wicomico River; Chesapeake Bay, USA	Graczyk *et al*., 1999b	*Cryptosporidium*, on average 55 oocysts/g of flesh and hemolymph homogenate
I. recurvum, C. virginica, M. balthica	Chesapeake Bay, USA	Graczyk *et al*., 2000b	*Cryptosporidium* species, *G. duodenalis* genotype (assemblage) A
Dreissena polymorpha	St. Lawrence River near Sainte-Foy; Quebec, Canada	Graczyk *et al*., 2001	*C. parvum* genotype 1, anthropogenic source, oocysts in hemolymph and flesh, 4.4×10^2 oocysts/mussel
D. polymorpha	Shannon River, Ireland	Graczyk *et al*., 2004	*C. parvum, G. lamblia, Encephalitozoon intestinalis, Enterocytozoon bieneusi*. Viable parasites identified by fluorescence *in situ* hybridization (FISH) method.

Corbicula clams are convenient for such purposes because they form dense populations and clusters, which facilitate the collection of large samples, do not have economic value, have a relatively small size, and are easily collected throughout the year (McMahon, 1991).

A. QUANTITATIVE ESTIMATION OF REMOVAL OF HUMAN WATERBORNE PARASITES

Zebra mussels collected from the St. Lawrence River, Canada, near a wastewater discharge site contained on average approximately 440 *C. parvum* oocysts/mussel (Graczyk *et al.*, 2001). Knowing the *C. parvum* retention rate as 4.9×10^2 oocysts/mussel/24 hr (Frischer *et al.*, 1999) and *D. polymorpha* densities of approximately 3.0×10^4 specimens/m^2 for adult (>1 year old) mussels (McMahon, 1991), it has been calculated that during 24 hours, approximately 1.3×10^7 waterborne *C. parvum* oocysts can be removed by each square meter of mussel bed in the St. Lawrence River (Graczyk *et al.*, 2001).

The concentration of *C. parvum* observed in zebra mussels from the Shannon River, Ireland (Graczyk *et al.*, 2004), was much lower than that reported from the St. Lawrence River (Graczyk *et al.*, 2001). However, in the St. Lawrence River, mussels originated from sites affected by wastewater discharge, and in the Shannon River, no apparent sources of water contamination have been identified near any of the sites. Considering the natural densities of zebra mussels (McMahon, 1991), and the fact that on average approximately eight parasites per mussel have been identified in the Shannon River study (Graczyk *et al.*, 2004), at least 2.4×10^5 pathogens/24 hr can be potentially removed per square meter of zebra mussel bed in the Shannon River.

B. CAN HUMAN PARASITES INFECT MOLLUSCAN SHELLFISH?

A single report suggests indigenous infection of *Cryptosporidium* in bivalves (Azevedo, 1989). Organisms supposedly resembling *Cryptosporidium* species merozoites were found in the gill epithelium of Portuguese clams (*Ruditapes decussatus*); however, as no other stages were observed, this finding was considered by the author as inconclusive (Azevedo, 1989).

C. A PUBLIC HEALTH THREAT FROM MOLLUSCAN SHELLFISH CONTAMINATED WITH *CRYPTOSPORIDIUM*

Before 1992, the association between contamination derived from animal fecal wastes and the occurrence of shellfish-vectored illnesses was inconclusive (Stelma and McCabe, 1992). In 1994, enterohemorrhagic *Escherichia*

TABLE III

EXPERIMENTAL EXPOSURE OF MOLLUSCAN SHELLFISH TO TRANSMISSIBLE STAGES OF HUMAN INTESTINAL WATERBORNE PARASITES (CHRONOLOGICAL ORDER)

Shellfish species	Reference	Parasite species	Comments
Crassostrea virginica	Fayer *et al.*, 1997	*Cryptosporidium parvum*	*In vivo* retention, infectious oocysts in hemocytes, gills, and gut, oocyst after 1 wk in oysters
Corbicula fluminea	Graczyk *et al.*, 1997c	*C. parvum*	*In vitro* phagocytosis, 82% of 1.1×10^5 oocysts phagocytosed after 2 hr
C. fluminea	Graczyk *et al.*, 1997a	*Giardia duodenalis*	*In vitro* phagocytosis, 86% of hemocytes showed phagocytosis after 2 hr exposure
C. virginica	Graczyk *et al.*, 1997b	*C. parvum*	*In vitro* phagocytosis, 95% of hemocytes showed phagocytosis after 2 hr exposure
C. fluminea	Graczyk *et al.*, 1998a	*Cyclospora cayetanensis*	Retention rate: 4.6×10^2 oocysts/clam/24 hr
C. fluminea	Graczyk *et al.*, 1998b	*C. parvum*	Retention rate: 1.9×10^5 oocysts/clam/24 hr
C. virginica	Graczyk *et al.*, 1998c	*C. parvum*, *G. duodenalis*	Specific detection of *C. parvum* and *G. duodenalis* in tissue of oysters carrying oyster diseases
C. fluminea	Graczyk *et al.*, 1999a	*G. duodenalis*	Retention rate: 1.3×10^3 cysts/clam/24 hr

Mytilus edulis	Graczyk *et al.*, 1999b	*C. parvum*	Detection threshold: 19 oocysts/g spiked-to-recovered oocysts: 51%
M. galloprovincialis	Tamburrini and Pozio, 1999	*C. parvum*	Retention rate: 1×10^7 oocysts/mussel/24 hr, infectious oocysts in hemocytes, gills, and gut, oocyst after 2 wks in oysters
Ruditapes philippinarum	Freire-Santos *et al.*, 2001	*C. parvum*	*In vivo* retention, oocysts in gills and gut, oocyst infectious after 48 hr in clams
C. virginica	Lindsay *et al.*, 2001	*Toxoplasma gondii*	*T. gondii* oocysts can be removed from water by oyster and retain their infectivity
Ostrea edulis, Tapes decussatus	Freire-Santos *et al.*, 2002	*C. parvum*	Infectivity of *C. parvum* oocysts demonstrated at 31 days after contamination
Mytilus galloprovincialis	Arkush *et al.*, 2003	*T. gondii*	*T. gondii* oocysts retain infectivity for at least 3 days in bivalve tissue
Dreissena polymorpha, C. fluminea	Graczyk *et al.*, 2003	*C. parvum, Giardia lamblia*	*D. polymorpha* and *C. fluminea* can accumulate human waterborne parasites in proportion to ambient concentrations
C. virginica	Lee and Lee, 2003	*Eimeria acervulina*	Oocysts retained in oyster tissue no longer than 96 hr after contamination
Corbicula japonica	Izumi *et al.*, 2004	*C. parvum*	Single exposure to pathogen results in a rapid intake and secretion of viable oocysts

coli O157 became of major concern (Rippey, 1994). This bacterium has not been associated with shellfish; however, its frequent occurrence in cattle indicated potential public health problems with shellfish harvested from waters affected by runoff from cattle farms (Rippey, 1994).

Beginning in 1998, multiple studies worldwide indicated that molluscan shellfish intended for human consumption can be contaminated with *Cryptosporidium* (citations in Table I). So far there has been no reported outbreak (or case) of food-borne cryptosporidiosis linked to consumption of raw oysters in the United States. However, more than 40% of all food-borne infections linked to oyster consumption are in the category of an unknown etiologic agent (Anonymous, 1996). In addition, 20% of the general U.S. population are vulnerable to *C. parvum* infection (Gerba *et al.*, 1996), the epidemiology of enteric infections (i.e., cryptosporidiosis) indicates an association with consumption of raw shellfish (Fang *et al.*, 1991), and it is believed that in the United States and Canada, the true incidence of shellfish-vectored gastroenteritis is considerably underestimated (Hauschild and Bryan, 1980). As stated earlier in this chapter, there is no mandatory federal requirement for reporting of gastroenteritis of an unspecified nature and physicians, and state health departments are forwarding case reports to federal authorities (Rippey, 1994; Wallace *et al.*, 1999).

D. GLOBAL PROBLEM

Protozoan parasite contamination of molluscan shellfish destined for human consumption is not limited to North America, and it appears as a global problem. Such contamination has been reported from Spain (Freire-Santos *et al.*, 2000; Gomez-Bautista *et al.*, 2000; Gomez-Couso *et al.*, 2003a), United Kingdom (Freire-Santos *et al.*, 2000; Gomez-Couso *et al.*, 2004; Lowery *et al.*, 2001), Italy (Freire-Santos *et al.*, 2000), and Egypt (Negm, 2003). More importantly, in some intensive seafood production regions such as northwest Gallicia, Spain, where molluscan shellfish production is the most important industry, cases of self-limiting diarrhea associated with consumption of raw oysters and clams are often reported (Freire-Santos *et al.*, 2001). Also, molluscan shellfish consumed raw are seriously considered a food vehicle for *Cryptosporidium* transmission in Switzerland (Baumgartner *et al.*, 2000).

E. POTENTIAL METHODS OF SANITIZATION

Molluscan shellfish destined for human consumption can be subjected to processing such as depuration, irradiation, ozonation, and high-pressure processing in order to remove or inactivate potential microbiological

contaminants. These methods have been applied predominantly to purge or inactivate bacterial and viral agents, and the published information on their efficiency for protozoan parasites is limited. Gomez-Couso *et al*. (2003a) demonstrated that depuration was ineffective in removing *C. parvum* oocysts from mussels, oysters, clams, and cockles harvested from contaminated waters. This laboratory also demonstrated that molluscan shellfish contaminated with *C. parvum* oocysts can spread this contamination within the commercial depuration plants to other aquatic organisms processed in such facilities (Gomez-Couso *et al*., 2003b).

IV. METHODS USED FOR IDENTIFICATION OF HUMAN PROTOZOAN PARASITES IN MOLLUSCAN SHELLFISH

Methods for identification of human protozoan parasites in the tissue of molluscan shellfish include (1) immunofluorescent antibodies (IFA) alone or in combination with immunomagnetic separation (IMS), (2) polymerase chain reaction (PCR) alone or combined with restricted fragment length polymorphism (RFLP) for genotyping, (3) multiplexed nested PCR, and (4) fluorescence *in situ* hybridization (FISH). Infectivity of the parasites recovered from the shellfish are usually assessed by mouse bioassays.

Because *Cryptosporidium, Giardia,* and microsporidia can infect a variety of nonhuman hosts (Graczyk *et al*., 1997; Kotler and Orenstein, 1999; Wolfe, 1992), identification of human infectious species is a challenge. Another challenge is determination of the viability of these environmentally recovered pathogens, because they may be nonviable and, thus, not of epidemiological importance. Although molecular methods are very sensitive and specific, they do not assess infectivity of the pathogens recovered from shellfish. Both challenges are met by the FISH technique. FISH employs fluorescently labeled oligonucleotide probes targeted to species-specific sequences of 18S ribosomal RNA (rRNA), and therefore, identification of pathogens is species specific (Graczyk *et al*., 2002, 2004; Hester *et al*., 2000). Also, because rRNA has a short half-life and is present only in numerous copies in viable organisms, FISH allows for differentiation between viable and nonviable pathogens (Dorsch and Veal, 2001; Graczyk *et al*., 2002; Hester *et al*., 2000; Jenkins *et al*., 2003; Vesey *et al*., 1998). FISH has been combined with direct IFA against the wall antigens of *Cryptosporidium* and *Giardia,* and this approach has been successful for detection of *C. parvum* and *G. lamblia* in shellfish samples (Graczyk *et al*., 2004). For identification of viable pathogens such as *C. parvum, G. lamblia,* or human-infective microsporidia, FISH is advantageous over other techniques including PCR because it allows simultaneous species-specific identification, visualization,

and viability assessment of single pathogen cells. Such resolution is not available or is extremely impractical with any other technique. For example, using highly sensitive RT-PCR, the lowest number of *C. parvum* oocysts that can be assessed for viability is 10^3 (Jenkins *et al.*, 2000).

V. WHY ARE THE SHELLFISH CONSUMPTION–CAUSED ILLNESSES NOT ANTICIPATED TO DECLINE?

There are several reasons that shellfish-vectored outbreaks and cases of gastroenteritis are not projected to decline and *Cryptosporidium* is being identified in bivalve molluscs intended for human consumption.

1. The fecal coliform count, which is the main standard indicator for waterborne fecal contamination, is not reliable in determining the quality of water at shellfish-harvesting sites (Anonymous, 1996; Rippey, 1994; Wilson and Moore 1996). The transmissive stages of human protozoan parasites can persist in aquatic environments for a longer time as compared to enteric and indicator bacteria (Graczyk and Schwab, 2000; Power and Collins, 1989; Richards, 1988). Although *C. parvum* oocysts are excreted with feces, waterborne *Cryptosporidium* pollution does not correlate with fecal coliform counts (Graczyk *et al.*, 2000c; Rose, 1997). Thus, waters considered to be "safe" based on the fecal coliform standards may, in fact, be contaminated by human enteric parasites (Anonymous, 1996; Graczyk and Schwab, 2000; Rippey, 1994; Wallace *et al.*, 1999).
2. Animal operations such as individual farms or huge industrial animal production facilities (e.g., beef and dairy cattle) located near shores can generate enormous surface runoff, particularly under adverse weather conditions, and can cause water pollution (Fayer *et al.*, 1998, 1999; Freire-Santos *et al.*, 2000; Gomez-Bautista *et al.*, 2000).
3. Deficiencies at the sewage treatment plants such as volume limitations related to designed capacity of a plant under adverse weather conditions such as heavy rainfall allow the discharge of large amounts of unprocessed waste waters. In addition, the periodic breakdown in particle removal or inadequate disinfection can deliver human enteropathogens into shellfish-harvested waters (Rippey, 1994).
4. Transmissive stages of human enteric parasites are resistant to environmental degradation (heat, sunlight, temperature fluctuations, etc.) and may even remain infectious after exposure to chemical water treatment processes such as chlorination (Graczyk and Schwab, 2000; McDonnell *et al.*, 1997). These pathogens can still be infectious even after the oyster meat has been processed (Graczyk and Schwab, 2000; McDonnell *et al.*, 1997) and are very poorly (i.e., slowly) depurated (removed) from molluscan

shellfish tissue (Power and Collins, 1989; Richards, 1988; Schwab *et al.*, 1998).

5. Increased fecal pollution determined on the fecal coliform counts has decreased the total area of coastal habitats approved for harvesting of molluscan shellfish, particularly oysters, for human consumption (Rippey, 1994). Thus, large and very productive areas have been closed, resulting in illegal harvesting of oysters from unapproved or closed but profitable waters (Rippey, 1994). Such criminal activity unavoidably affects public health when contaminated shellfish enter the market (Rippey, 1994).

6. Improper postharvest handling and transportation of molluscan shellfish (i.e., temperature abuse) affect oysters directed for consumption in a raw form (Rippey, 1994). Holding of oysters at temperatures higher than 4°C in transit or in the market place can contribute to multiplication of bacterial enteropathogens (Rippey, 1994).

7. Many shellfish-related outbreaks have more than one contributing factor (Wallace *et al.*, 1999). For example, contaminated ingredients added to raw or lightly cooked molluscs have been also reported as contributing factors for food-borne infections (Wallace *et al.*, 1999).

8. Development of new molecular techniques that can be applied to a wide variety of food items has dramatically increased the sensitivity and specificity of detection of human enteric parasites in the tissue of molluscan shellfish (Schwab *et al.*, 1998, 2000; citations in Table I).

VI. CONCLUSIONS

1. Food-borne illnesses following consumption of molluscan shellfish continue to occur despite that (1) testing of waters for fecal coliforms from which oysters are harvested for human consumption demonstrate that the water quality met the criteria of the National Shellfish Sanitation Program (NSSP), (2) oysters harvested from the NSSP-approved waters are considered "safe" with regard to fecal pollution, (3) sanitation procedures at oyster-harvesting facilities met standards set by the state authorities, and (4) in most instances, neither confirmed evidences of improper handling or processing of outbreak-implicated oysters nor the environmental sources of pollution were detected. These facts indicate that the monitoring of water for fecal coliform at molluscan shellfish-harvesting sites may not be sufficient indicating the presence of human waterborne parasites such as *Cryptosporidium, Cyclospora, Giardia, Toxoplasma,* and human infectious microsporidia (Fayer *et al.*, 1998; Graczyk and Schwab, 2000; Rippey, 1994; Wallace *et al.*, 1999).

2. Oocysts of *C. parvum* can retain their infectivity in molluscan shellfish (Fayer *et al.*, 1997; Freire-Santos *et al.*, 2001; Tamburrini and Pozio, 1999), and standard depuration processes applied to commercially harvested bivalve molluscs are not effective for *Cryptosporidium* (Freire-Santos *et al.*, 2000, 2001; Gomez-Bautista *et al.*, 2000).

3. Reducing the number of outbreaks of food-borne diseases due to bivalve molluscs will require the coordinated efforts of different agencies involved in water quality assessment, shellfish harvesting and processing, disease surveillance, and consumer education (Anonymous, 1996; Rippey, 1994; Wallace *et al.*, 1999). It may be useful to reduce or eliminate economic incentives for illegal harvesting of shellfish from unapproved or prohibited waters that results in contaminated shellfish reaching the market place (Rippey, 1994).

4. Estuarine molluscan shellfish, both commercial and feral, can be used for the sanitary assessment of water quality as biological indicators for water and sediment contamination with *Cryptosporidium, Giardia, Toxoplasma,* and *Cyclospora* (Xiao *et al.*, 1998, 2001; citations in Tables I and II).

5. Prevention of food-borne gastroenteritis due to consumption of molluscan shellfish relies on consumer education and thorough cooking of shellfish (Fayer *et al.*, 1998, 1999; Freire-Santos *et al.*, 2001; Rippey, 1994; Wallace *et al.*, 1999). Education should be particularly focused on populations that are predisposed to serious illness after consumption of contaminated shellfish (e.g., people with suppressed immune systems) (Fayer *et al.*, 1999; Rippey, 1994; Wallace *et al.*, 1999). Several factors may impede consumer education campaigns about the risk of raw shellfish consumption (Altekruse *et al.*, 1999).

6. It may be useful to institute guidelines for processing steps to reduce pathogen counts in molluscan shellfish (e.g., cold shock, heat shock, pasteurization, or irradiation) (Altekruse *et al.*, 1999). Because oocysts of *C. parvum* can be rendered noninfectious by heating to temperatures higher than 72°C, it has been recommended that shellfish be cooked before eating, particularly for persons with any type of immune deficiency (Fayer *et al.*, 1998, 1999; Freire-Santos *et al.*, 2001).

7. Continued surveillance for outbreaks and cases of gastroenteritis associated with consumption of raw shellfish are needed to assess the efficacy of the NSSP in preventing human illnesses (Graczyk and Schwab, 2000; Rippey, 1994; Wallace *et al.*, 1999). Public health officials should consider consumption of raw shellfish as a possible source of infection during the evaluation of a gastroenteritis outbreak (Graczyk and Schwab, 2000; Rippey, 1994; Wallace *et al.*, 1999).

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REGULATION OF HUMAN IMMUNE AND INFLAMMATORY RESPONSES BY DIETARY FATTY ACIDS

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I. INTRODUCTION

The development, maintenance, and optimal functioning of the immune system are dependent on balanced and adequate nutrition. However, either a deficiency or an excess of a number of nutrients can have adverse effects. The nutrients with the most pronounced effects in humans include amount and type of dietary fatty acids (FAs), protein energy malnutrition, vitamins A, B6, B12, C, and E, and minerals including zinc, copper, selenium, and iron. Multiple rather than single nutrient deficiencies

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are often the cause for a compromised immune system. A number of excellent review articles and books deal with the nutritional regulation of immune functions (Amati *et al.*, 2003; Calder and Kew, 2002; Field *et al.*, 2002).

Dietary fat can have diverse effects on human health based on the amounts consumed and, more importantly, on the types consumed. Dietary fat may also differentially affect certain cells, tissues, and organs depending on their stage of development. The FA composition of human tissues and organs can vary depending on the types of FAs that are consumed in the diet; that composition has been used as a biomarker for correlation with immunity and risk with disease. In addition, some dietary FAs can be transformed into potent biological mediators that have been shown to initiate or alter numerous processes in the body. For example, linoleic acid (LA, 18:2, n-6), a common component of some vegetable oils, can be converted by a number of cell types into arachidonic acid (AA, 20:4, n-6), a major precursor for the potent immunomodulatory agents, prostaglandin (PG)E_2 and leukotriene (LT)B_4, which are produced from AA by the enzymes cyclooxygenase and 5-lipoxygenase, respectively. Other 20-carbon FAs, eicosapentaenoic acid (EPA, 20:5 n-3), and dihomo-gamma linolenic (DGLA, 20:3, n-6) compete with AA as substrates for these enzymes and, thus, can decrease the production of PGE_2 and LTB_4. The eicosanoids produced from EPA and DGLA consequently have only weak effects on cells of the immune system. Docosahexaenoic acid (DHA, 22:6, n-3) is not a substrate for the cyclooxygenase and lipoxygenase; however, it can inhibit the synthesis of the n-6 eicosanoids by inhibiting the release of membrane AA (Martin, 1998). It can also be retroconverted to EPA. Because PGE_2 and LTB_4 have been linked to alterations in the immune system and to specific pathological processes, dietary fat intake has the potential to alter human disease. Reduced production of inflammatory eicosanoids by DHA, EPA, and DGLA, therefore, forms the basis for their use in the management of inflammatory diseases.

In this chapter, we provide a brief introduction to immune system and FA nomenclature. The effects of dietary FAs on the number and activity of the cells of the immune system is a main focal point. We emphasize the results of human studies and discuss animal studies as a means to understand some of the proposed mechanisms by which FAs alter immune functions. The amount of published information for various topics can be quite different, and thus, the amount of detail given varies. Further details can be found in individual papers cited or other reviews regarding the effect of dietary FAs on immune functions (Calder and Grimble, 2002; De Pablo and De Cienfuegos, 2000; Harbige, 2003; Yaqoob and Calder, 2003).

II. IMMUNE SYSTEM AND ITS RESPONSE

The human immune system is very complex and involves a number of organs, cell types, and molecules that are scattered throughout the body. The primary organs associated with the immune system include bone marrow, thymus, spleen, lymph nodes, liver, and small intestine. The cells of the immune system are mainly leukocytes or white blood cells (WBCs), a component of the hematopoietic system. In adult humans, all WBCs originate from the bone marrow, with their maturation occurring in other tissues as well. Based on the shape of their nuclei, WBCs can be divided into two major groups: mononuclear or segmented nuclei, that is, polymorphonuclear. The mononuclear cells include monocytes/macrophages and lymphocytes. The macrophages differentiate from bloodborne monocytes upon migration into tissues. They play a central role in nonspecific immunity and processes such as phagocytosis, bactericidal and tumoricidal activity, inflammation, and tissue repair. These cells are a major source of inflammatory cytokines and eicosanoids and have specific roles in regulating the activity of other immune cells (T, B, and natural killer [NK]) through those mediators. Macrophages also process and present antigens to T and B cells.

Depending on the site of maturation, specific surface markers, and functions, the lymphocytes are divided into T (thymus), B (bone marrow or bursa), NK, and LAK cells. T lymphocytes mature in the thymus and are involved in DTH and in anticancer and antifungal immunity. T helper (Th) and T suppressor (Ts) cells play a central role in immune regulation. The NK cells provide spontaneous and nonspecific immunity against tumor cells, virally infected, and chemically modified cells. The LAK cells are the primary blood or spleen lymphocytes that acquire the ability to kill certain tumors *in vitro,* when cultured with interleukin (IL)-2. They have been reported to cause regression of solid tumors when administered to experimental animals or patients with cancer and suppress the formation of metastases.

The PMN cells include neutrophils, eosinophils, and basophils. Neutrophils make up 60–75% of circulating WBCs and provide the first line of defense against microbes that penetrate the normal barriers of skin. They are extremely efficient phagocytes and are a source of inflammatory cytokines and lipid mediators such as PGs, LTs, and platelet-activating factor. Eosinophils are involved in allergy and provide protection against parasites. Basophils contain vasoactive amines such as histamine, serotonin, and heparin, as well as precursors for PGs and LTs. Release of these pharmacological materials by the basophils is responsible for the anaphylactic reaction. These factors also serve as chemoattractants for neutrophils and eosinophils to sites of inflammation.

Immunity is a state of resistance or protection from pathogenic microorganisms. There are two major types of immunity: innate and adaptive. Innate immunity is present at all times in most individuals and is thus fully functional before infectious pathogens enter the body. It does not distinguish between pathogens of different species, and the response time does not change with repeated exposures. Examples of innate immunity include mechanical barriers such as skin and the epithelial linings of lungs and gut; secreted products, such as saliva and tears; and cells including macrophages, neutrophils, and the NK cells. The adaptive immunity or specific immune response is the reaction to challenge by a specific immunogen. It is activated after the pathogen has evaded the innate response and has entered the body. It is specific for the attacking microbes, and the response time is reduced during repeated exposures. Adaptive immunity has two major classes: humoral immunity mediated by B lymphocytes and cell-mediated immunity mediated by T lymphocytes and activated macrophages. The cells of the immune system synthesize and recognize various molecules, including antibodies, complement proteins, cytokines and their receptors, adhesion molecules, growth factors, and many others. Many of these molecules are also synthesized by a diverse array of cells not belonging to the immune system. These molecules, which can act in an autocrine or paracrine manner, have pleiotropic and synergistic effects. Thus, the overall protection against the pathogen is provided by an interaction between the various cells and molecules of the immune system.

III. ALLERGY AND INFLAMMATION

Allergy or hypersensitivity is an immunologically mediated reaction to a foreign antigen (allergen), causing tissue inflammation and organ dysfunction. It generally represents the undesirable aspects of an immune reaction, whereas immunity implies the desirable effects. The immune response may be a local reaction at sites such as skin, respiratory, vasculature, gastrointestinal tract, or other visceral organs or a systemic reaction such as anaphylaxis or serum sickness. Immunologically induced inflammation may involve the reaction of the antigen with T cells and with the products of B cells (antibodies). Only antibodies of immunoglobulin (Ig) E, IgG, and IgM types are known to be involved in allergic reactions. Types I, II, and III hypersensitivity reactions are immunoglobulin mediated, whereas type IV is T-cell mediated. Type I hypersensitivity involves IgE-mediated release of vasoactive amines, AA metabolites, and cytokines from mast cells. Examples include anaphylaxis, rhinitis, asthma, allergic gastroenteropathy, and atopic dermatitis. Type II reactions involve the binding of either IgG or IgM antibodies specific for tissue or cell

surface antigens, which activates complement cascade and destroys the cells to which the antigen is bound. Examples include immune hemolytic anemia, Rh hemolytic disease, and autoimmune hyperthyroidism. In type III or immune complex–mediated hypersensitivity, antibodies of the IgG or IgM isotype form circulating immune complexes with the allergen and thereby activate complement to generate mediators of inflammation. Examples include the Arthus reaction and serum sickness. Type IV hypersensitivity is caused by T-cell activation and direct target cell lysis by cytotoxic T cells; examples include DTH and tuberculin reactions.

Inflammation is a local protective response to infection or injury whereby cells and proteins in the blood enter to remove the pathogens and repair the damaged tissue. Edema, redness, pain, and heat are the four cardinal symptoms of inflammation. Extent of reactions is determined by inflammatory mechanisms mediated by serum protein or cellular systems. Serum protein systems include complement, coagulation, fibrinolysis, and kinin; cellular systems include PMN cells, mast cells, platelets, eosinophils, lymphocytes, macrophages, and reticuloendothelial system. Insufficient responses result in immunodeficiency leading to cancer and infections; excessive responses are the cause of a number of chronic diseases like diabetes, cardiovascular disease, rheumatoid arthritis, multiple sclerosis, and Alzheimer's disease (Tracey, 2002).

The early phase mediators of inflammation are histamine and serotonin released from basophils, and the late-phase mediators include AA metabolites, neutrophil lysosomal enzymes, lymphokines, and monokines. PMN cells are involved in acute inflammation, whereas mononuclear cells are involved in chronic inflammation. Cytokines produced by the Th1 cells such as IL-2 and interferon-γ (IFN-γ) favor the development of inflammatory reactions and suppress the allergic responses, whereas cytokines produced by the Th2 cells such as IL-4, IL-5, IL-6, and IL-10 lead to IgE production and activation or production of eosinophils and mast cells (Kidd, 2003). Under normal conditions, a balance is maintained between the activity of the Th1 and Th2 cells; however, an imbalance can lead to inflammatory or allergic diseases. In addition to the cytokines produced by the T cells, those produced by other cells also regulate the immune and inflammatory responses. IL-1, IL-6, IL-8, IL-18, tumor necrosis factor-α (TNF-α), and IFN-γ and IFN-β are considered proinflammatory, whereas IL-4, IL-10, IL-1 receptor antagonist (IL-Ra), and transforming growth factor-β (TGF-β) are considered antiinflammatory. Thus, cytokines play an important role in the pathogenesis of allergic and inflammatory diseases. Commonly used markers to evaluate the degree of chronic inflammation include determination of serum inflammatory proteins (C-reactive protein [CRP], serum amyloid protein [SAA], fibrinogen), inflammatory cytokines, and eicosanoids discussed earlier, as well as adhesion molecules (intercellular

adhesion molecule-1 [ICAM-1], vascular cell adhesion molecule-1 [VCAM-1], E-selectin).

IV. DIETARY LIPIDS

Dietary lipids are composed mainly of triglycerides with only small amounts of phospholipids, cholesterol, and other sterols. Chemically, triglycerides are the triacylglycerols or a glycerol molecule esterified with three FAs. Saturated FAs have no double bonds in their carbon chain; examples include palmitic (16:0) and stearic acid (18:0). Unsaturated FAs have one (monounsaturated) or more (polyunsaturated) double bonds in the carbon chain. Depending on the position of the first double bond from the methyl end, these FAs are divided into n-9, n-6, or n-3 (also called ω-9, ω-6, or ω-3) series, with the first double bonds being between carbon 9 and 10, carbon 6 and 7, and carbon 3 and 4, respectively. Common sources of different dietary FAs are shown in Table I. Animal fats are a rich source of saturated

TABLE I
MAJOR DIETARY SOURCES OF COMMON FATTY ACIDS

Fatty acid	Source
Saturated	
Lauric (12:0)	Coconut and palm oils
Myristic (14:0)	Coconut and palm kernel oils
Palmitic (16:0)	Palm oil, milk fat, beef tallow, lard, chicken fat, cottonseed oil, chocolate
Stearic (18:0)	Beef tallow, lard, milk fat, cashew nut butter, chocolate
Omega-9 or n-9	
Oleic (OA,18:1n-9)	Olive, peanut, rapeseed, palm oils, chicken, turkey, lard, tallow
Omega-6 or n-6	
Linoleic (LA, 18:2n-6)	Sunflower, safflower, corn, soybean, cottonseed, walnut oils
Conjugated linoleic (CLA)	Hydrogenated oils, margarines, milk fat, ruminant meats
Gamma linolenic (GLA, 18:3n-6)	Borage, evening primrose, black currant oils
Arachidonic (AA, 20:4n-6)	Organ meats, eggs, fish
Omega-3 or n-3	
α-linolenic (ALA, 18:3n-3)	Flaxseed, perilla, rapeseed, walnut oils
Eicosapentaenoic (EPA, 20:5n-3)	Fish and fish oils (salmon, menhaden, mackerel, tuna)
Docosahexaenoic (DHA, 22:6n-3)	Fish and fish oils (salmon, menhaden, mackerel, tuna)

medium-chain FAs and oleic acid (18:1n-9). Plant seed oils are good sources of LA and contain only trace amounts of n-6 FAs with more than 18 carbons; animal tissues contain significant amounts of AA. Conjugated LA (CLA) and gamma LA (GLA) (18:3n-6) are other important n-6 FAs that are found in relatively small amounts in the diets but have significant physiological effects. *CLA* is a collective term for isomers of LA that have conjugated double bonds. Depending on the position and geometry of the double bonds, more than a dozen isomers of CLA have been reported; two of those isomers, cis 9, trans 11-CLA (*c*9, *t*11-CLA) and trans 10, cis 12-CLA (*t*10, *c*12-CLA), have been studied regarding their health effects. The major dietary sources of *c*9, *t*11-CLA are dairy products and ruminant meat, whereas that of *t*10, *c*12-CLA are partially hydrogenated vegetable oils from margarines and shortenings. Primrose, borage, and black currant seed oils contain considerable amounts of GLA. Dietary FAs of the n-3 series include ALA (18:3n-3), EPA, and DHA (22:6n-3). These FAs are found only in plant oils, because the animals lack the enzyme 15-delta desaturase required for inserting the double bond at n-3 carbon of LA. Flax and perilla seed oils are very rich sources of ALA, whereas deep ocean fish and the fish oils are good sources of EPA and DHA. Another good source of DHA–triglyceride is the oil from genetically engineered algae (Martek Corporation) in which DHA represents approximately 50% of the total FAs.

The three classes of unsaturated FAs are not interconvertable; however, they are metabolized by a common series of elongases and desaturases (Figure 1). A competitive interaction between FAs exists so that FAs of the 18:3n-3 family suppress the metabolism of the 18:2n-6 family and those of the 18:2n-6 family suppress the metabolism of the 18:3n-3 family, though less strongly. Both 18:2n-6 and 18:3n-3 FAs suppress the metabolism of the 18:1n-9 FAs. FA composition of the diet plays a critical role in determining the tissue FA composition; the ratios between different classes of FAs, rather than their absolute amounts, are more important.

Humans cannot insert the first double bond at C3 or C6 but can elongate and desaturate LA and ALA. Therefore, FAs with 18, 20, or 22 carbons, with two to six double bonds in *cis* configuration, and the first double bond between C3 and C4, or C6 and C7, are considered essential FAs (EFAs). In addition to their many physiological roles in processes such as growth, reproduction, and brain and eye functions, EFAs are structural components of cell membranes, involved in signal transduction, as well as ligands for nuclear receptors, required for the growth and maintenance of immune cells. In addition, they are produced and secreted during immune cell activation.

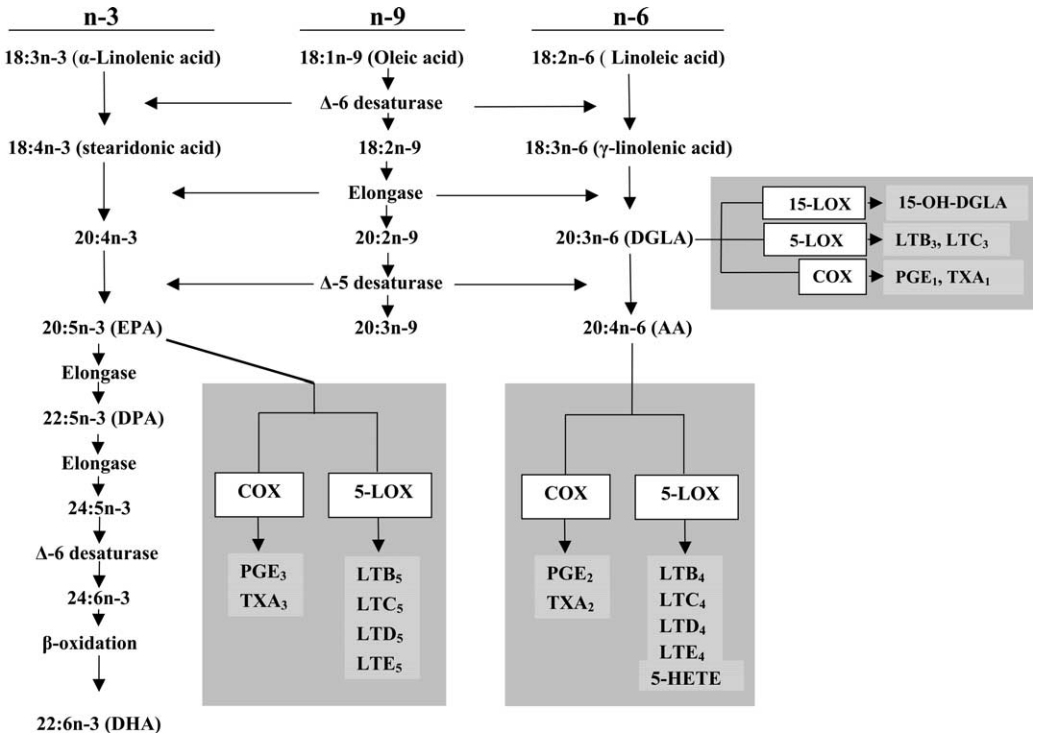

FIG. 1 Metabolism of n-3, n-6, and n-9 fatty acids and their conversion to various eicosanoids. AA, arachidonic acid; COX, cyclooxygenase; DGLA, dihomo-gamma-linolenic acid; DHA, docosahexaenoic acid; DPA, docosapentaenoic acid; EPA, eicosapentaenoic acid; HETE, hydroxyeicosatetraenoic acid; LOX, lipoxygenase; LT, leukotriene; TX, thromboxane.

V. EFFECTS OF DIETARY FATTY ACIDS ON IMMUNE AND INFLAMMATORY RESPONSES

Both the total fat intake and the ratios between FAs of different classes influence the activity of immune cells. Such information was initially obtained through epidemiological human studies, and studies conducted with cultured cells and animal models. These studies showed that EFAs are required for the growth and maintenance of the immune cells, and free FAs are produced and secreted during the activation of these cells. A number of intervention studies regarding the effects of the amount and composition of dietary fat on human immune response have been conducted, results of which are discussed in the following sections.

A. AMOUNT OF DIETARY FAT AND IMMUNE RESPONSE

Eight published studies have examined the effects of reduction in energy from fat intake on different indices of immune response (Table II). Age of the study participants and the amount and duration of change in dietary fat are the most critical factors in determining modulation of immune functions by dietary fat; this information is included in Tables II–VII. In the studies that focused on the amount of dietary fat and its effect on immune responses, total calories, and calories from proteins were held constant, whereas the amount of calories from fat was adjusted with calories from carbohydrates. Reduction in fat intake ranged from 8 to 16 energy % (en%) for a duration of 4–11 weeks. Seven of the eight studies reported an increase in some aspect of immune response when the percentage of energy from fat was decreased. Four studies reported an increase in peripheral blood mononuclear cell (PBMC) proliferation (Han *et al*., 2003; Kelley *et al*., 1989, 1992; Meydani *et al*., 1993), two reported an increase in NK cell activity (Barone *et al*., 1989) or numbers (Rasmussen *et al*., 1994), two reported an increase in the DTH response (Han *et al*., 2003; Meydani *et al*., 1993), and one reported an increase in cytokine production by monocytes (Meydani *et al*., 1993). One study (Rasmussen *et al*., 1994) reported a 20% increase in the number of circulating NK cells but no change in their activity, whereas another (Barone *et al*., 1989) found a 50% increase in NK cell activity. This discrepancy may be due to use of elderly subjects, and the increase in the polyunsaturated FA (PUFA) content of the diet; P:S ratios were 0.70 for low-fat diets and 0.37 for high-fat diets (Rasmussen *et al*., 1994). Only one report found no change in immune functions with a reduction in fat intake (Venkatraman *et al*., 1997). In that study, increasing the fat content of the diet from 32 to 41 en% for endurance athletes who ran more than 40 miles/wk did not alter lymphocyte proliferation, IL-2 secretion, or the number of circulating mononuclear

TABLE II

EFFECTS OF TOTAL FAT INTAKE ON HUMAN IMMUNE RESPONSE

Immune response and magnitude	Subjects age and sex (n)	Change in energy % from fat	Duration (wk)	Reference
PBMC P, 50–60% increase	29–40 M (8)	41 to 25	11	Kelley *et al.*, 1989
PBMC P, 80–100 % increase	30–65 W (7)	41 to 31 and 41 to 26	9	Kelley *et al.*, 1992
NK activity, 50% increase	Mean 31 M (14)	30 to 22	8	Barone *et al.*, 1989
NK number, increase 20%, NC in activity	65–81 M (13)	40 to 29	5	Rasmussen *et al.*, 1994
PBMC, P, and DTH, increase 28%	>50 M and W (17)	38 to 28	4.5	Han *et al.*, 2003
DTH, 50% increase	>40 M and W (10)	35 to 15	6	Santos *et al.*, 2003
PBMC, P, DTH, IL-1, TNF 30–60% increase	>40 M and W (10)	36 to 27	6	Meydani *et al.*, 1993
PBMC, P, and cytokine production NC	Mean 35 M and W (6–8)	41 to 32	4	Venkatraman *et al.*, 1997

Note: DTH, delayed hypersensitivity skin response; NC, no change; NK, natural killer cell; PBMC, peripheral blood mononuclear cells; P, proliferation; M, men; W, women; *mean* pertains to mean age.

cells when preexercise blood samples were tested. However, the postexercise number of total circulating mononuclear cells increased by approximately 50% in men with increase in fat intake but did not change in women; the number of circulating NK cells following exercise increased with increases in fat intake by 100% in women but did not change in men. Authors concluded that a high percentage of calories from fat did not have an adverse effect on the immune system of well-trained athletes. This may be a valid interpretation. The inconsistency in the results between men and women regarding the number of circulating mononuclear and NK cells may be due to gender differences or the small number of subjects in the study (n = 6–8).

Overall, results from all these studies suggest that the long-term reduction in fat consumption improved both *in vivo* and *ex vivo* measures of immune functions. Increased response in these immune functions will generally indicate increased protection that may be of clinical significance. Only one study has compared the effects of lipid infusion on *ex vivo* measures of immune functions and the *in vivo* markers for diseases (van der Poll *et al.*, 1995). In this study, intralipid infusion caused up to a 70% reduction in the *in vitro* secretion of IL-1β, IL-6, and TNF-α; however, it did not influence inflammatory responses to endotoxin (fever, leukocytosis, release of TNF and its receptor) and even potentiated plasma endotoxin responses (release of IL-6 and IL-8 and neutrophil degranulation). The authors concluded that hypertriglyceridemia does not inhibit the *in vivo* responses to endotoxin.

B. EFFECTS OF DIETARY N-3 FATTY ACIDS ON IMMUNE AND INFLAMMATORY RESPONSES

The source of n-3 PUFAs used in human feeding trials has been either flaxseed or linseed oil as a source of ALA, fish and fish oils as sources of EPA and DHA, purified esters of EPA and DHA, or DHA triglycerides from genetically engineered algae. There are only a few human studies in which flaxseed or fish was fed, whereas there are several dozen studies with fish oils only. Results from these studies are summarized in the following section.

1. α-linolenic acid

Results from seven studies that examined the effects of dietary ALA on indices of human immune response have been published (Table III). The amount of ALA in these studies ranged from 2 to 18 g/day for a duration of 4–24 weeks. Three of these studies (Caughey *et al.*, 1996; Kelley *et al.*, 1991; Rallidis *et al.*, 2003) reported a 20–50% reduction in various indices of immune response such as levels of serum CRP, serum amyloid protein A, and IL-6; *in vitro* secretion of IL-1β and TNF-α; or DTH response. The

TABLE III

EFFECTS OF α-LINOLENIC ACID ON HUMAN IMMUNE FUNCTIONS

Response and magnitude	Subjects age and sex (n)	ALA g/day	Duration (wk)	Reference
PBMC, P, and DTH, 20–50% decrease	21–37, M (9–10)	18	8	Kelley *et al.*, 1991
IL-1 and TNF, 30% decrease	26–36, M (13)	14	4	Caughey *et al.*, 1996
CRP, SAA, IL-6, 25–30% decrease	Mean 51, M (50)	8.0	12	Rallidis *et al.*, 2003
PBMC P, lymphocyte and monocyte cytokines, DTH, NC	25–72 M and W (29–32)	4.5 and 9.0	24	Kew *et al.*, 2003
PMN chemotaxis and superoxide production, NC	>40, M (8)	4.0	12	Healy *et al.*, 2000
PBMC P, cytokines, DTH NC	18–39, M (8)	3.5	12	Wallace *et al.*, 2003
PBMC P, NK activity, cytokines, NC	55–75 M and W (8)	2.0	12	Thies *et al.*, 2001a,b,c

Note: DTH, delayed hypersensitivity skin response; NC, no change; NK, natural killer cell; PBMC, peripheral blood mononuclear cells; P, proliferation; M, men; SAA, serum amyloid protein A; W, women; *mean* pertains to mean age.

amount of ALA in those studies ranged from 8 to 18 g/day for a period of 4–12 weeks; the LA:ALA ratios ranged from 0.6 to 1.3. Even at the highest amount of ALA (18 g/day for 8 weeks; Kelley *et al*., 1991), a number of other indices of immune status did not change, including the number of circulating WBCs, granulocytes, monocytes, lymphocytes, and their subsets; serum concentrations of IgG, IgA, C3, or C4; *ex vivo* secretion of IL-2 and IL-2R; and mitogen-induced B-cell proliferation. Thus, dietary ALA can differentially alter parameters of immune status.

The four other studies (Kew *et al*., 2003; Thies *et al*., 2001a,b,c; Wallace *et al*., 2003) reported no effect of dietary ALA on several indices of immune response tested, such as DTH, PBMC proliferation, cytokines produced by lymphocytes and monocytes, PMN chemotaxis, and superoxide production. The amount of ALA used in these studies varied from 2 to 9 g/day for 12–24 weeks. No significant differences in these studies were most likely due to the small amounts of ALA in the diets; this may explain the results from the studies by three groups (Healy *et al*., 2000; Thies *et al*., 2001a,b,c; Wallace *et al*., 2003), but not those of others (Kew *et al*., 2003), who fed ALA at 9 g/day for 24 weeks. In this study, the ratios of LA:ALA in the low and high ALA diets were 8.0 and 3.0, respectively. The amount and duration of ALA in the diets do not seem to be the sole factors that determine whether ALA will inhibit indices of immune status, because one group of investigators (Rallidis *et al*., 2003) reported inhibition with ALA at 8.0 g/day for 12 weeks and another group (Kew *et al*., 2003) did not detect inhibition with a greater amount and longer duration (ALA 9 g/day for 24 weeks). The LA:ALA ratios were 1.3 (Rallidis *et al*., 2003) and 3.0 (Thies *et al*., 2001a,b), which may explain the lack of an effect of ALA in one study (Thies *et al*., 2001a,b,c). Collectively, it appears that ALA does inhibit lymphocyte and monocyte functions, the magnitude of which depends on the amount and duration of ALA supplementation and the ratio between LA:ALA. Another factor that is important and is discussed in the following section is the concentration of antioxidants. Feeding ALA increased both the ALA (0.2–0.6%) and the EPA (0.2–0.4%) concentration of PBMC lipids but did not alter their DHA concentration (Kelley *et al*., 1993). It also increased the serum concentration of ALA from 0.5 to 3.2%. It is, therefore, difficult to determine whether the effects of dietary ALA observed in this study were caused by ALA itself or by one of its elongation products.

2. Fish

There are only two published studies in which the effects of fish consumption on indices of immune response were tested. In one study, 500 g/day of salmon containing 2.3 g of EPA and 3.6 g/day of DHA was fed to nine healthy young

men for 40 days (Kelley *et al.*, 1992). The study had a crossover design and was conducted at a metabolic unit where the diet and activity levels were strictly controlled. The salmon diet contained 3.6% less calories from fat, which were replaced by an equivalent amount of calories from carbohydrates. Feeding the salmon diet did not have any effect on a number of indices of immune response, including DTH, PBMC proliferation, and serum Ig concentration. The lack of an effect on immune status in this study was most likely due to short duration, reduction in energy from fat in the salmon diet, and supplementation of both diets with additional vitamin E. Another study was conducted with 22 men and women older than 40 years in a parallel design for 24 weeks (Meydani *et al.*, 1993). Eleven subjects consumed a high fish National Cholesterol Education Program Step 2 diet (120–188 g/day fish containing 1.2 g EPA plus DHA), and the remaining subjects received the same diet with only one-fourth the amount of fish. Lymphocyte proliferation, *ex vivo* production of IL-1β, IL-6, and TNF-α , and DTH were significantly reduced in the group fed the high fish diets. The duration of fish consumption, age of the subjects, and the lack of additional vitamin E supplementation in this study may be some of the reasons the results differ from those of other investigators (Kelley *et al.*, 1992). Additional studies are needed to establish whether there are any risks associated with increased fish consumption. From the available data, it appears that two to three servings of fish per week should be safe even without vitamin E supplementation, but that amount may not substantially alter immune response.

3. *Fish oils, eicosapentaenoic, and docosahexaenoic acids*

Most of the studies with EPA and DHA have used fish oils that contain both of these FAs; however, a few other studies have examined the effects of these FAs individually. Several studies have examined the effects of fish oil supplementation on *ex vivo* neutrophil and monocyte chemotaxis, superoxide production, phagocytosis, lymphocyte and monocyte cytokine production, lymphocyte proliferation, and *in vivo* indices of immune response. The amount of fish oil supplemented ranged from 2 to 30 g/day containing 0.55–8.0 g/day EPA plus DHA for 4–52 weeks. Many of these studies were longitudinal in which the fish oils were added to the usual diets and did not include parallel control groups. This study design increased not only the intake of EPA and DHA, but also that of total fat. Most newly published studies included placebo control groups, and the total fat intake between the experimental and placebo groups was comparable. Some studies supplemented variable amounts of vitamin E, which itself affects many indices of immune response, and others did not. Results of fish oil supplementation on immune response have varied extensively.

a. Effects of EPA and DHA on number of circulating white blood cells. Four studies have examined the effects of EPA and/or DHA on the number of circulating WBCs (Kelley *et al.*, 1998b; Meydani *et al.*, 1991b; Thies *et al.*, 2001b; Yaqoob *et al.*, 2000). The amount of EPA and/or DHA ranged from 0.7 to 6.0 g/day for 12–13 weeks; one study supplemented these FAs with a large dose of 200 mg/day α-tocopherol (Yaqoob *et al.*, 2000), and one study (Kelley *et al.*, 1998b) supplemented the diets with 6 g/day DHA only for 13 weeks. Only one of these studies found alterations in the number of circulating WBCs or their specific subsets (Kelley *et al.*, 1998b). Kelley *et al.* (1998b) found a 10% reduction in the number of circulating WBCs at the end of 13 weeks of DHA supplementation, and there was no change in the control group. This decrease in WBCs was primarily due to a reduction (21%) in the number of granulocytes and was detectable as early as 7 weeks after DHA supplementation. One reason for the lack of this effect in other studies may be the low amount of DHA in the supplements. High levels of vitamin E in one study (Yaqoob *et al.*, 2000) may have also contributed to those results. A reduction in the number of circulating granulocytes by DHA (Kelley *et al.*, 1998b) was similar to that found in rats (Atkinson *et al.*, 1997; Ohhashi *et al.*, 1998) and human patients with adult respiratory distress syndrome after feeding fish oils (Mayer *et al.*, 2002).Whether this reduction in circulating granulocytes associated with dietary n-3 PUFA was due to altered margination, proliferation, differentiation, or apoptosis needs to be investigated.

b. Respiratory burst, phagocytosis, and chemotaxis. Fifteen studies have reported the effects of EPA and DHA on neutrophil and/or monocytes chemotaxis, phagocytosis, and superoxide production (Table IV). Amount of EPA and DHA in these studies ranged from 0.6 to 14.4 g/day for 3–24 weeks. Seven of these studies included placebo controls where the total fat intake matched that of the experimental groups, and in the remaining eight studies fish oils were added to the usual diets. Nine of these studies reported a 25–70% reduction in neutrophil and/or monocyte functions with the consumption of EPA and DHA. The lowest amount of EPA and DHA that showed inhibition of neutrophil superoxide production was 2.2 g/day for 4 weeks (Thompson *et al.*, 1991), and the highest amount of EPA or DHA that failed to inhibit neutrophil and monocyte phagocytosis was 4.7 g/day for 12 weeks (Kew *et al.*, 2004). All other studies that have used EPA and DHA at more than 4 g/day, except two (Halvorsen *et al.*, 1997; Kew *et al.*, 2004), demonstrate a 30–70% reduction in phagocytosis and chemotaxis. In one of the studies (Halvorsen *et al.*, 1997), cells were stored frozen before phagocytosis assays, a possible reason for the lack of effect. However, another study (Kew *et al.*, 2004) used freshly isolated cells for a flow cytometric method to determine phagocytosis; the reasons for the

TABLE IV

EFFECT OF EPA AND DHA ON CHEMOTAXIS, PHAGOCYTOSIS, AND SUPEROXIDE PRODUCTION BY HUMAN PMN AND MONOCYTES

Function and magnitude of change	Subjects age and sex (n)	EPA–DHA (g/day)	Duration (wk)	Reference and study type
PMN chemotaxis, 25–60% decrease	25–59, M and W (8)	14	3	Sperling *et al.*, 1993
PMN chemotaxis, decreased with increase in EPA–DHA	28–66, M and W (8)	1.3, 4, 9	6	Schmidt *et al.*, 1991
PMN chemotaxis, 30–50% decrease	41–60, M and W (8)	8.6	3	Luostarinen *et al.*, 1991
Monocytes chemotaxis, superoxide production, 45–60% decrease	29–54, M and W (9)	6	6	Fisher *et al.*, 1990
PMN chemotaxis, 70% decrease	22–53, M (7)	5.4	6	Lee *et al.*, 1985
PMN chemotaxis, 30% decrease	29–49, M (12)	5.3	6	Schmidt *et al.*, 1989
PMN phagocytosis, NC	23–65, M and W (10)	4.7	12	Kew *et al.*, 2004
PMN chemotaxis, 70% decrease	M and W (4–5)	4.0	8	Payan *et al.*, 1986
Monocytes respiratory burst and phagocytosis, NC	36–56, M and W (19)	4.0 EPA, DHA	7	Halvorsen *et al.*, 1997, PC
PMN chemiluminescence, superoxide production, 27–64% decrease	M and W (6)	3.6	6	Fisher *et al.*, 1986, PC
PMN chemotaxis and superoxide production, NC	>40, M (8)	2.2	12	Healy *et al.*, 2000, PC
PMN superoxide production, 50% decrease	20–30, M and W (6)	2.2	4	Thompson *et al.*, 1991, PC
PMN/monocytes phagocytosis and respiratory burst, NC	55–75, M and W (8)	1.0	12	Thies *et al.*, 2001a, PC
PMN/monocytes phagocytosis, NC	25–72, M and W (29–32)	0.8, 1.7	24	Kew *et al.*, 2003, PC
Monocyte chemotaxis, NC	24–52, M and W (16)	0.6	12	Schmidt *et al.*, 1996, PC

Note: Studies marked *PC* (placebo control) included a control group, and others did not; PMN, polymorphonuclear leukocytes; NC, no change; M, men; W, women.

discrepancy between results from this and many other studies are not clear. The shortest intervention to demonstrate inhibition of PMN chemotaxis was 3 weeks, in a study that supplemented the usual diets with fish oils and provided 8.6 g/day of EPA and DHA (Luostarinen *et al*., 1991). Normally, high intakes of vitamin E protect from the inhibitory effects of fish oils, but in this study, PMN chemotaxis was equally inhibited in subjects provided with 9 or 45 IU of vitamin E per day. That indicates that 45 IU/day was not adequate to protect from the inhibition caused by the high intake of fish oil. After a short period of feeding EPA and DHA concentrations of less than 1.7 g/day, no inhibition of neutrophil and monocyte phagocytosis, chemotaxis, or superoxide production was observed; the amounts needed to inhibit these responses were generally similar (Table IV). Genetic variation of the subjects studied also seemed important in determining the response to EPA and DHA as one study reported a reduction in monocyte chemotaxis in type IIa but not type IV hyperlipidemic subjects (Schmidt *et al*., 1991). Long-term studies are needed to determine the effects of genetic polymorphisms, antioxidants, and the amounts and types of dietary PUFAs on monocyte and neutrophil functions discussed here.

c. Cytokine production by monocytes. Nineteen studies have investigated the effects of dietary EPA and DHA on production of inflammatory cytokines by PBMCs isolated from human volunteers fed diets containing EPA and DHA, and then stimulated with mitogens *in vitro*; two additional studies examined the effects of these FAs on serum/plasma cytokines (Table V). The amount of EPA and DHA intake varied from 0.3 to 6.0 g/day for 4–52 weeks. EPA+DHA supplementation caused a 20–90% reduction in the *ex vivo* secretion of inflammatory cytokines such as IL-1, IL-6, or TNF-α in 8 of 19 studies (Abbate *et al*., 1996; Caughey *et al*., 1996; Endres *et al*., 1989; Gallai *et al*., 1995; Grimble *et al*., 2002; Kelley *et al*., 1999; Meydani *et al*., 1991b, 1993); these FAs also caused a 10–20% reduction in the serum concentrations of CRP and IL-6 in one of two studies (Ciubotaru *et al*., 2003). It seems the reduction in cytokine production was dependent on the dose of fish oil, as 7 of the 11 studies that failed to detect a decrease used supplements with less than 2 g/day EPA plus DHA. Studies that failed to detect a reduction in cytokine production with EPA and DHA consumption of greater than 2 g/day supplemented the FAs either for a short 4-week duration (Kew *et al*., 2004) or co-supplemented with a high dose of 205 mg/day vitamin E (Yaqoob *et al*., 2000). Similarly, no reduction in plasma CRP or cytokines was detected when the diets of type II diabetic subjects were supplemented with 4 g/day DHA for only 6 weeks (Mori *et al*., 2003). The lack of an effect in that study may be due to a combination of short duration and elevated serum lipids. The importance of the duration of supplementation was

TABLE V

INHIBITION OF HUMAN MONOCYTE CYTOKINE PRODUCTION BY EPA AND DHA

Function	Subjects age and sex (n)	EPA–DHA (g/day)	Duration (wk)	Reference
IL-1β, TNFα 30–45% decrease	Mean 33, M (7)	6 (DHA)	13	Kelley *et al.*, 1999, PC
IL-1β, TNFα 50–80% decrease	20–50, M and W (15)	5.2	24	Gallai *et al.*, 1995, PC
IL-1β, IL-6, IL-8, TNF-α NC	23–65, M and W (14)	4.7	12	Kew *et al.*, 2004, PC
IL-1β, TNF-α 20–60% decrease	21–39, M and W (9)	4.6	6	Endres *et al.*, 1989
Plasma CRP, IL-6, TNF-α NC	40–75, M and W (16)	4.0	6	Mori *et al.*, 2003, PC
IL-6, 50% decrease	M and W (9)	4.0	18	Abbate *et al.*, 1996
IL-1β, TNF-α, NC	Mean 25, M and W (9)	4.0	7	Molvig *et al.*, 1991
IL-1β, TNF-α, NC	Mean 45, M and W (8)	3.2	12	Yaqoob *et al.*, 2000, PC
IL-1β, IL-1Rα, TNF-α NC	21–81 M (58)	1.1–3.1	13–52	Blok *et al.*, 1997
IL-1β, IL-6, 85–90% decrease	18–36, M and W (6–8)	2.8	8	Cooper *et al.*, 1993, PC
IL-1β, TNF-α 70–80% decrease	26–36, M (13)	2.7	4	Caughey *et al.*, 1996, PC
IL-1β, IL-6, TNF-α 50–80% decrease	23–33, W (5) 51–68 W (6)	2.4	12	Meydani *et al.*, 1991b
Serum CRP, IL-6, 10–20% decrease	Mean 60, W (10)	1.3, 2.6	5	Ciubotaru *et al.*, 2003, PC
IL-6, TNF-α >50% decrease	M (15)	0.3, 1, 2	4 each	Trebble *et al.*, 2003a
IL-1β, NC	18–39, M and W (8)	0.4–1.9	12	Wallace *et al.*, 2003, PC
TNF-α decrease or increase based on phenotype	Mean 37, M (37)	1.8	12	Grimble *et al.*, 2002
IL-1β, IL-6, TNF-α, NC	25–72, M and W (30)	0.8 or 1.7	24	Kew *et al.*, 2003
IL-1β, IL-6, TNF-α, NC	>40, W (10)	1.2	24	Meydani *et al.*, 1993, PC
IL-1β, IL-6, TNF-α, NC	55–75, M and W (8)	1.0	12	Thies *et al.*, 2001a
IL-1β, IL-6, TNF-α, NC	W (27)	0.4 or 0.7	4	Hawkes *et al.*, 2002, PC
IL-1β, IL-6, TNF-α, NC	24–52, M and W (16)	0.6	12	Schmidt *et al.*, 1996, PC

Note: Studies marked *PC* (placebo control) included a control group, and others did not; CRP, C reactive protein; IL, interleukin; TNF-α, tumor necrosis factor-α; NC, no change.

demonstrated in two studies. Although supplementation of DHA did not significantly alter immune response at 55 days, after 91 days, secreted cytokine levels were reduced by 30–45% (Kelley *et al*., 1999). Similarly, other investigators (Endres *et al*., 1989) reported a 20–45% reduction in cytokine secretion 6 weeks after the start of fish oil supplementation, whereas it was reduced by 61% at 10 weeks after discontinuing the supplement. In that study, IL-1β and TNF-α secretion was restored to the presupplement levels 20 weeks after discontinuing the supplement. One study did not detect a reduction in cytokine secretion even with a EPA–DHA supplement of 3.1 g/day for 52 weeks. However, cytokine secretion was not assessed before the start of the supplements, and the first assessment was made 13 weeks after the start of supplements (Blok *et al*., 1997).

In addition to the amount and duration of the n-3 PUFA supplementation, the amount of antioxidant nutrients and the cytokine genes polymorphism are also important factors that determine the cytokine response to n-3 PUFA. Thus, vitamin E supplements of 200 mg/day or more prevented the inhibition of cytokine secretion by monocytes (Yaqoob *et al*., 2000) and proliferation of lymphocytes cultured with Con A (Kramer *et al*., 1991); however, 30 mg/day of vitamin E supplements did not prevent the inhibition of cytokine secretion by fish oil supplements (Trebble *et al*., 2003a). Likewise, 45 IU/day failed to prevent the inhibition of PMN chemotaxis by dietary fish oil (Luostarinen *et al*., 1991). The amount of vitamin E needed to prevent the inhibition of monocyte responses by n-3 PUFA appears to depend on the amount of the n-3 PUFA, and it is several-fold greater than the usual intake of vitamin E. Furthermore, the reduction in TNF secretion in response to n-3 PUFA consumption was dependent on the polymorphism in the TNF gene (Grimble *et al*., 2002). Although further studies are needed to resolve some of the inconsistencies regarding the effects of n-3 PUFA on the secretion of inflammatory cytokines, results from the studies discussed here indicate that amounts greater than 3 g/day, particularly in the absence of high intakes of vitamin E, will inhibit monocyte and PMN functions.

d. Lymphocyte functions. Currently, 14 studies have investigated the effects of EPA–DHA supplementation on lymphocyte functions, including NK cell activity, lymphocyte proliferation, and cytokine production (Table VI). NK cell activity was examined in three studies after supplementing with EPA or DHA (Kelley *et al*., 1999; Thies *et al*., 2001a; Yaqoob *et al*., 2000) or infusion with EPA–triglyceride (Yamashita *et al*., 1991). One study used 6 g/day of DHA and found no change in NK cell activity at 8 weeks after the start of DHA supplementation, although it was significantly reduced at 13 weeks (Kelley *et al*., 1999). NK cell activity in the placebo group remained unchanged at 8 and 13 weeks. A 48% reduction in NK cell

TABLE VI
EFFECTS OF DHA AND EPA ON HUMAN LYMPHOCYTE FUNCTIONS

Lymphocyte function	Subjects age and sex (n)	EPA–DHA (g/day)	Duration (wk)	Reference & study type
P, 80% decrease	24–57, M (35)	7	10	Kramer *et al.*, 1991, PC
P, NC, NK activity 20% decrease	Mean 33, M (7)	6 (DHA)	13	Kelley *et al.*, 1998b, PC
IL-2 and IFN-γ, 25–30% decrease	20–50, M and W (15)	5.2	24	Gallai *et al.*, 1995, PC
CD25 expression, 13% decrease	M & W Psorotic patients (10)	5.1	16	Soyland *et al.*, 1994, PC
IL-2, 4, 5, and IFN-γ, NC; DHA not EPA, decreased T-cell activation	23–65, M and W (14)	4.7	4	Kew *et al.*, 2004, PC
P and IL-2, NC; 65% decrease 10 wk postsupplementation	Mean 28, M (9)	4.6	6	Endres *et al.*, 1993
P 15–33% decrease	Mean 25, M and W (9)	2.0, 4.0	7	Molvig *et al.*, 1991, PC
P, IL-2, and IFN-γ, NK activity, NC	Mean 45, M and W (8)	3.2	12	Yaqoob *et al.*, 2000, PC
P and IL-2, 30–36% decrease	23–33 and 51–68, W (5 and 6)	2.4	12	Meydani *et al.*, 1991a
P, 24 % decrease	>40, W (10)	1.2	24	Meydani *et al.*, 1993
P, IL-2, 4, 10, and IFN-γ, NC	25–72 M and W (30)	0.8, 1.7	24	Kew *et al.*, 2003, PC
IL-2, 4, 10, and IFN-γ, NC	55–75 M (8)	0.4, 0.9, 1.9	12	Wallace *et al.*, 2003, PC
P, IFN-γ, increased twofold to threefold	M (15)	0.3, 1, 2	4	Trebble *et al.*, 2003a, PC
P and NK activity 48–65% decrease; IL-2 and IFN-γ, NC	55–75 M and W (8)	1	12	Thies *et al.*, 2001b, PC

Note: Studies marked *PC* (placebo control) included a control group, and others did not. CD, cluster designation; IL, interleukin; IFN, interferon; M, men; NC, no change; NK, natural killer; P, proliferation; W, women.

activity after 12 weeks of supplementation with fish oil (EPA 720 mg + DHA 280 mg/day), but not in the group that was supplemented with 700 mg/day of DHA alone, was observed (Thies *et al*., 2001b). Results of that study suggest that EPA may be more potent in inhibiting NK cell activity than DHA. In contrast to the inhibition of NK cell activity reported with 1-g/day fish oil supplement (Thies *et al*., 2001b), another study from the same laboratory failed to detect inhibition of NK cell activity even when the fish oil supplement provided 3.2 g/day of EPA–DHA for 12 weeks (Yaqoob *et al*., 2000). The lack of inhibition of NK cell activity by fish oil in that study was most likely due to concomitant intakes of 205 mg/day of vitamin E. Infusing 30 ml of EPA–triglyceride 24 hours before the isolation of PBMCs to determine NK cell activity reduced NK cell activity by more than 50% (Yamashita *et al*., 1991). Together, these studies suggest that both EPA and DHA inhibit NK cell activity; however, results have been variable because of variance in experimental designs.

Ten studies examined the effects of EPA–DHA supplementation on the *ex vivo* proliferation of lymphocytes cultured with mitogens (Table VI). The amount of n-3 PUFA supplemented ranged from 0.3 to 7 g/day for 4–24 weeks. Six of these studies found a 24–80% reduction in lymphocyte proliferation after EPA–DHA supplementation, three found no change (Kelley *et al*., 1998b; Kew *et al*., 2003; Yaqoob *et al*., 2000), and one reported a twofold increase (Trebble *et al*., 2003b). The lack of lymphocyte proliferation in one study (Kelley *et al*., 1998b) was most likely due to the lack of EPA in the supplement. Another study (Yaqoob *et al*., 2000) included a vitamin E supplement of 205 mg/day, which may be the reason for the lack of inhibition of lymphocyte proliferation by n-3 PUFA. This interpretation was supported by another study (Kramer *et al*., 1991), which indicated that concurrent supplementation with 200 mg/day of vitamin E prevented the inhibition of lymphocyte proliferation by an EPA–DHA supplement of 7 g/day; in the absence of extra vitamin E, inhibition of proliferation by fish oil supplementation ranged from 0 to 80% depending on the concentration of Con A. The lack of an effect on lymphocyte proliferation in another study (Kew *et al*., 2003) may simply be due to a relatively low amount of 1.7 g/day of n-3 PUFA and a modest supplementation with 15 mg/day of vitamin E. These results suggest that n-3 PUFA intakes of up to 1.7 g/day may be consumed without inhibiting lymphocyte proliferation as long as there is modest supplementation of vitamin E. The increase in lymphocyte proliferation after fish oil supplementation was found in only one study (Kew *et al*., 2003). The experimental design involved concurrent supplementation with a cocktail of antioxidant nutrients including vitamins A, C, and E, selenium, and magnesium. The increase in lymphocyte proliferation in this case was most likely the result of antioxidants and not fish oils.

Eight published studies examined the effects of EPA–DHA supplementation on the *ex vivo* secretion of cytokines by lymphocytes cultured with mitogens (Table VI). Fish oil supplementation caused a 25–35% reduction in the secretion of IL-2 and IFN-γ in only two of these studies (Gallai *et al*., 1995; Meydani *et al*., 1991b). One study found no change in secreted IL-2 with an EPA–DHA supplementation of 4.6 g/day for 6 weeks; however, 10 weeks after supplementation with fish oil, IL-2 secretion was reduced by 65% (Endres *et al*., 1993). The remaining five studies that did not find a reduction in lymphocyte cytokine secretion after fish oil supplementation supplemented either with a modest amount (<2 g/day) of n-3 PUFA (Kew *et al*., 2003; Thies *et al*., 2001a; Wallace *et al*., 2003) or with the fish oil for only 4 weeks (Kew *et al*., 2004) or concurrently supplemented with high amounts of vitamin E (Yaqoob *et al*., 2000). One study that concurrently supplemented with a cocktail of antioxidants involving vitamins A, C, and E, selenium, and magnesium actually reported a threefold increase in IFN-γ secretion after fish oil supplementation (Trebble *et al*., 2003a). As stated earlier, this increase was most likely due to the antioxidants rather than the n-3 PUFA. In addition to the cytokines secreted, one study examined the effect of fish oil supplementation on lymphocyte surface expression of the receptor for IL-2 (CD25) and reported a 13% reduction after supplementing the diets with EPA–DHA at 5.1 g/day for 16 weeks (Soyland *et al*., 1994). The lowest concentration of EPA–DHA that was associated with inhibition of lymphocyte function was 1.2 g/day fed for 24 weeks (Meydani *et al*., 1993), and the highest concentration that did not inhibit was 3.2 g/day for 12 weeks (Yaqoob *et al*., 2000). Overall, the results from these studies generally demonstrate that fish oils inhibit lymphocyte functions, and the responses vary with the amount, duration, and overall composition of the diet, as well as the function tested. Long-term studies are needed to determine the concentrations of fish oils that can be consumed without compromising the immune response.

e. Reasons for inconsistencies regarding the effects of n-3 PUFA on immune functions. Most of the results presented in this chapter indicate that n-3 FAs can inhibit the immune and inflammatory responses; however, there are several exceptions. The extensive variation in the study protocols and methods used has probably contributed to the discrepancies in results from different studies. Potentially important factors related to the study protocol include antioxidant nutrient content of the diets, total fat and FA composition, ratio between n-6 and n-3 PUFAs, amount and duration of supplementation, the daily amount of EPA and DHA, age, sex, and health of the subjects, and inclusion/exclusion of a control or placebo group. Methods and cells used for assessments have varied greatly and add to the discrepancies. For example, isolated PBMCs or whole blood cells cultured in

autologous sera or fetal calf serum have been used with a wide variety of physiological and nonphysiological agents to stimulate cells and monitor their responses. Some of the assays used may have little relationship to human immunity *in vivo*. Most critical among these factors seem to be the ratio between the amounts of n-3 PUFA and vitamin E, as the latter blocks the inhibition by n-3 PUFA, as well as the amount and duration of supplementation. Interaction between n-3 PUFA and vitamin E may involve protein kinase C alpha, as EPA and DHA stimulate this enzyme, whereas α-tocopherol inhibits its activation (Madani *et al*., 2001). Responses also vary with the immune function being tested; for example, mitogen-induced lymphocyte proliferation in contrast to cytokine secretion appears to be more sensitive to n-3 PUFA. Despite these differences, n-3 FAs have been successfully used in the management and prevention of several inflammatory and allergic diseases.

C. EFFECTS OF DIETARY N-6 FATTY ACIDS ON IMMUNE AND INFLAMMATORY RESPONSES

Most of the human studies dealing with the effects of n-6 FAs on immune and inflammatory responses were conducted by altering the intake of oils rich in LA. However, a few studies have also used oils rich in GLA or supplemented diets with purified CLA or AA. Effects of these FAs are discussed individually in the following sections.

1. Linoleic acid and immune response

In two studies, men and women consumed diets in which total fat and LA intake were altered (Kelley *et al*., 1989, 1991). Reduction in total fat was correlated with increased lymphocyte proliferation and DTH response (discussed in Section V.A). Changing the consumption of LA from 3 to 9 en% or 3 to 13 en% in these studies did not alter lymphocyte proliferation, DTH response, serum concentrations of immunoglobulins, and the number of circulating WBCs (Kelley *et al*., 1989, 1991), even when the excretion of urinary PGE_2 in the high LA group was significantly increased (Blair *et al*., 1993). Another study reported that adding 9 g/day of sunflower oil for 12 weeks to the diets of healthy subjects did not alter lymphocyte proliferation, NK cell activity, or the production of cytokines by lymphocytes and monocytes (Yaqoob *et al*., 2000). Similarly, NK cell activity did not differ between two groups of healthy men when their diets were supplemented with 15 g/day of either an oil rich in saturated FAs such as coconut oil or LA (safflower oil) for 2 months (Hebert *et al*., 1990). As discussed earlier, NK cell activity in this study was increased with a reduction in total fat intake. In contrast,

results from a study with elderly Danish men with a mean age of 71 years showed that NK cell activity was inversely related to the serum LA concentration (Rasmussen *et al.*, 1994). Poor health status of the elderly and a wide range in the P:S ratio of the diets may have made it possible to detect the negative effect of LA on NK cell activity.

FA composition of the adipose tissue reflects the long-term FA composition of the diet. In a group of 94 healthy American men, the P:S ratio for adipose tissue FAs ranged between 0.54 and 1.01. Within this range, the P:S ratio had no effect on a number of indices of immune response tested (Berry *et al.*, 1987). Results of the animal studies dealing with LA feeding have been reviewed (Crevel and Saul, 1992) and are generally consistent with what has been reported in humans, but in some studies, unrealistically high levels of LA were found to inhibit lymphocyte proliferation and DTH response. Taken together, these studies do not suggest a suppression of human immune response by modest increases in the consumption of LA, even if these changes were adequate to increase the levels of urinary PGE_2. An increase in the production of proinflammatory eicosanoids with increased consumption of LA may be a concern in the exacerbation of inflammatory diseases.

2. *CLA and human immune response*

There are only four published reports in which the effects of CLA supplementation on immune cell functions in humans have been examined. The first was a metabolic unit study that was conducted with 17 healthy women (Kelley *et al.*, 2000, 2001). For the first 30 days of the study, all subjects consumed a basal diet supplemented with 6 g/day of sunflower oil (standardization diet). The supplement for 10 women was replaced with 6 g/day of Tonalin, which provided 3.9 g of a mixture of CLA isomers for the ensuing 63 days; 7 women received a placebo supplement, which served as a control throughout the study. Immune cell functions were evaluated with fasting blood samples. Delayed hypersensitivity skin response to a battery of seven recall antigens was determined at the start and end of the intervention. All subjects were immunized with influenza vaccine on study day 65, and the serum antibody titers were determined using the blood samples drawn before (preimmunization) and 92 (postimmunization). The total CLA concentration in circulating WBCs at the end of the study increased eightfold when compared to the concentration on study day 30; however, it did not significantly alter the concentration of other FAs. CLA supplementation did not modify the number of circulating total WBCs, lymphocytes, granulocytes, or monocytes. It had no effect on proliferation of lymphocytes cultured with PHA, Con A, or influenza vaccine, *in vitro* production of IL-2, IFN-γ, IL-1, or TNF-α, as well as the production of inflammatory eicosanoids, PGE_2,

and LTB_4 when cells were cultured with mitogens in medium containing 10% autologous sera. Serum influenza antibody titers and DTH responses were also not affected by CLA supplementation. Overall, results of this study suggest that short-term supplementation with a modest level of CLA to healthy adult women was well tolerated but had no beneficial effect on the measured parameters of human immune response. This is consistent with the observed CLA effects on body composition and energy metabolism (Zambell, 2000). Results from another study indicate no change in serum concentrations of TNF-α and IL-6 when overweight/obese volunteers supplemented their diets with a 3.4-g/day isomer mixture of CLA or purified t10, c12-CLA for 12 weeks or an equivalent amount of olive oil (Riserus *et al.*, 2002). On the other hand, concentrations of serum CRP and urinary PGF_2-α were doubled at the end of t10, c12-CLA supplementation compared to the other two groups. These results suggest that t10, c12-CLA may exacerbate oxidative stress and inflammatory response. In a study with experienced resistance-trained men, supplementing their diet with a mixture of 6 g/day of CLA isomers for 4 weeks caused a 25% decrease in the ratio of circulating neutrophils to lymphocytes, whereas the ratio did not change in an olive oil–supplemented group (Kreider *et al.*, 2002). From the available data, it is not possible to determine whether the reduction in this ratio was due to a decrease in neutrophil numbers or an increase in lymphocyte numbers. In this study, CLA supplementation also did not significantly affect gains in bench- or leg-press weight. Whether higher amounts or longer duration of CLA would affect human immune cell functions or increase health risk remains to be determined.

In a preliminary human study, the effects of two mixtures of c9, t11 and t10, c12 CLA isomers (80:20, or a 50:50 mixture) on antibody response to hepatitis B vaccination, delayed hypersensitivity skin response, and other indices of immune cell functions in healthy men were examined (Albers *et al.*, 2003). The amount of CLA supplemented was 1.7 g/day for 84 days. CLA supplementation did not alter delayed hypersensitivity skin response or many other indices of immune cell functions tested. Mean antibody titers against hepatitis B did not differ significantly between the three groups; however, the number of subjects attaining antibody titers higher than 10 IU/L was significantly greater in the 50:50 group compared to the 80:20 or placebo groups. The validity of such arbitrary titers as seroprotective may be questionable, particularly when they are only twofold to threefold higher than the preimmunization titers. Because the 50:50 mixture contained more of the t10, c12-CLA isomer than the 80:20 mixture, the authors concluded that t10, c12-CLA enhanced the antibody response to hepatitis B, whereas the c9, t11-CLA did not alter that response. Those results are not consistent with the observed effects of CLA on influenza antibody titers (Kelley *et al.*,

2000). Whether that was antigen specific, sex specific, or the result of differences in CLA isomers used needs to be addressed in studies. Even if t10, c12-CLA enhances antibody response to specific antigens, this benefit will have to be balanced against the risks of increased lipid peroxidation and inflammation caused by this isomer (Kelley and Erickson, 2003).

3. GLA and human immune response

Results from eight studies have been published regarding the effects of GLA supplementation on human immune functions (Table VII); only three of those studies had a placebo control (Thies *et al.*, 2001c; Wu *et al.*, 1999; Yaqoob *et al.*, 2000). In seven of these studies, GLA was provided orally, and in the eighth study, GLA was infused (DeLuca *et al.*, 1999). Sources of GLA in these studies included borage, black currant, evening primrose seed oils, or a synthetic triacylglycerol; amounts of GLA ranged from 0.7 to 3.3 g/day for a maximum period of 24 weeks.

PBMC proliferation was examined in five studies; it was reduced by 65–80% in two studies (Rossetti *et al.*, 1997; Thies *et al.*, 2001c) and did not change in two studies (Fisher and Harbige, 1997; Yaqoob *et al.*, 2000). In the fifth study, lymphocyte proliferation in response to PHA increased significantly (30–60%) in both the GLA and the placebo group (Wu *et al.*, 1999). The increase in response to PHA was not significantly different between the GLA and control groups. Furthermore, GLA had no effect on lymphocyte proliferation when the cells were stimulated with Con A. Thus, only two of the five studies showed inhibition of lymphocyte proliferation. In one report (Rossetti *et al.*, 1997), the study included only two men; one supplemented his diet with 10 g of borage oil (GLA 2.4 g/day). Blood samples were taken at 5, 12, and 24 weeks after the start of GLA supplementation. Lymphocyte proliferation in response to antibodies against CD3 and CD4 progressively decreased with time, with greater than 80% inhibition at 24 weeks. The other individual in the study received similar supplements for 6, 8, and 11 weeks. Lymphocyte proliferation was again decreased, remained decreased 4 weeks after discontinuation, and returned to the presupplement levels 12 weeks after discontinuation. Even if the study included only two subjects, the data are quite convincing regarding the inhibitory effect of GLA. It is possible that the increase in total fat intake of 10 g/day rather than GLA or presenting the results as stimulation indices has contributed to some of the effects observed in this study. Results from another study (Thies *et al.*, 2001c) are even more difficult to interpret. This was the only placebo-controlled study, and it reported a 65% reduction in lymphocyte proliferation with only a GLA supplement of 700 mg/day for 12 weeks. The supplement was a blend of 21:5:74 of palm oil, sunflower seed oil, and a GLA-rich triacylglycerol.

TABLE VII

EFFECTS OF γ-LINOLENIC ACID ON HUMAN IMMUNE FUNCTIONS

Function and effect	Subjects age and sex (n)	GLA (g/day)	Duration (wk)	Reference and study type
P, IFN-γ, TNF-α, NC; IL-4 and IL-10, 50% decrease wk 8 and 12; TGF 300% increase wk 12	Adult M and W (8)	3.3	4, 8, 12	Fisher and Harbige, 1997
P 80% decrease wk 11	24–40, M (2)	2.4	6, 8, 11	Rossetti *et al.*, 1997
Auto-induction of IL-1β 75% decrease wk 8; 17% after LPS stimulation	23–63, M and W (4–5)	1.8	4, 8	Furse *et al.*, 2002
P, NK activity, IL-1, 2, 10, IFN-γ, TNF-α, NC	Mean 45, M and W (8)	1.1	4, 8, 12	Yaqoob *et al.*, 2000, PC
P, 65% decrease wk 12; NK activity, IL-1, 6, 10, NC	55–75, M and W (8)	0.8	4, 8, 12	Thies *et al.*, 2001b, PC
P, 30–60% increase; IL-1β, IL-2, NC	>65, M and W(15)	0.7	8	Wu *et al.*, 1999, PC
IL-1β, IL-6, TNF-α, 20–25% decrease	Mean 20, M and W (10)	0.5	6	Watson *et al.*, 1993
IL-1β, TNF-α 40% decrease	29–46, W (4)	2.4	24 hr	DeLuca *et al.*, 1999

Note: Studies marked *PC* (placebo control) included a control group, and others did not. IL, interleukin; IFN, interferon; LPS, lipopolysaccharide; NC, not changed; P, proliferation; TNF, tumor necrosis factor; TGF, transforming growth factor.

Inhibition of lymphocyte proliferation was observed after PBMCs were stimulated with Con A in a medium supplemented with 10% autologous plasma. It is possible that GLA in this supplement was more readily available than that from natural oils, or some other component of the mixture contributed to the inhibition of lymphocyte proliferation. In another study (Fisher and Harbige, 1997), GLA was associated with a 30% decrease in PBMC proliferation, but the results did not attain statistical significance. Even if the inhibition of lymphocyte proliferation was significantly reduced, it could not definitely be attributed to GLA, because the study did not have a placebo group, although the response was tested before and after supplementation with 15 g/day borage oil. Another study that failed to detect inhibition of lymphocyte proliferation was placebo controlled and involved a supplement of 9 g/day of evening primrose oil (GLA 1.1 g/day) for 12 weeks (Yaqoob *et al.*, 2000). The most likely reason for the lack of an effect in this study was a high concomitant supplement with 205 mg/day of vitamin E. GLA supplementation did not alter NK cell activity (Thies *et al.*, 2001c; Yaqoob *et al.*, 2000) or the secretion of IFN and IL-2 (Fisher and Harbige, 1997; Thies *et al.*, 2001c; Yaqoob *et al.*, 2000), but it significantly reduced the secretion of IL-4 and IL-10 (Fisher and Harbige, 1997). GLA supplementation, however, caused a threefold increase in the secretion of TGF-β (Fisher and Harbige, 1997). Thus, the effects of GLA supplementation on lymphocyte functions have been quite variable. This may be due to the differences in experimental design, methods, amount, source, and duration of GLA supplementation, composition of the basal diets, and age and health status of the study subjects. Studies need to address these issues.

Six studies examined the effects of oral GLA supplementation on the *ex vivo* production of inflammatory cytokines by monocytes (Table VII); only two of these studies found a modest reduction in the secretion of IL-1, IL-6, or TNF (Furse *et al.*, 2001; Watson *et al.*, 1993), whereas the remaining four studies found no change in the secretion of these cytokines. One study that used both lipopolysaccharide (LPS) and IL-1α to stimulate the production of IL-1β found that GLA supplementation reduced the LPS-induced pro-IL-1β mRNA by only 17%, and it reduced the IL-1α–induced pro-IL-1β mRNA by 75% (Furse *et al.*, 2002). In this study, GLA supplementation also increased the secretion of IL-1 receptor antagonist. It is possible that some of these studies failed to detect the effect of GLA on the secretion of inflammatory cytokines simply because they did not use the appropriate stimulus. Overall, the effects of GLA on lymphocyte and monocyte functions appear much weaker compared with those of fish oils. Even if the effects of GLA are modest, it would be of great interest to determine whether GLA would enhance the antiinflammatory effects of EPA and DHA.

4. *Arachidonic acid and immune response*

There are only two published reports in which the effects of AA supplementation on human immune functions were examined. In one crossover study (Kelley *et al*., 1997), the diets of young healthy men were supplemented with 1.5 g/day of AA for 50 days. AA supplementation did not alter a number of indices of immune response tested, including DTH response, NK cell activity, mitogen-induced lymphocyte proliferation, or *in vitro* secretion of IL-1, IL-2, and TNF-α (Kelley *et al*., 1997, 1998a). However, it significantly increased (25–100%) the number of circulating neutrophils, the *ex vivo* production of PGE_2 and LTB_4 by the PBMC stimulated with LPS, or the secondary response to influenza vaccine. These results indicate that increased intake of AA may increase the inflammatory and allergic disorders. In the second study, AA was found to have no effect on NK cell activity when the diets of men and women older than 55 years were supplemented with 700 mg/day of AA for 12 weeks (Thies *et al*., 2001b). Moreover, it did not alter the number of circulating leukocytes, lymphocytes, and T or NK cells. Thus, the results from the two studies are consistent regarding the lack of an effect on NK cell activity, but the higher amounts used in one study (Kelley *et al*., 1998b) did increase the number of circulating granulocytes in young healthy men, which was not the case when one-half the amount was used in elderly subjects (Thies *et al*., 2001b). These results are generally consistent with those discussed for LA earlier, indicating that a modest increase in consumption of these FAs does not have adverse effects in human immune response, whereas higher concentrations may increase the inflammatory response.

D. EFFECTS OF MONOUNSATURATED, SATURATED, AND TRANS FATTY ACIDS ON IMMUNE AND INFLAMMATORY RESPONSES

There are only two studies in which the effects of diets rich in monounsaturated FAs (MUFAs) on human immune functions have been examined. In one study, we compared the effects of feeding diets containing safflower oils with either 75% oleic acid or LA to young healthy men for 80 days (Kelley *et al*., 1989). The daily intake of oleic acid in the low and high MUFA diets was 24 and 51 g, whereas that of LA was 36 and 15 g. Lymphocyte proliferation and serum concentrations of immunoglobulins and complement proteins did not differ between the two diets. In another study, the effects of refined olive oil on indices of immune response in healthy men were examined (Yaqoob *et al*., 1998). The MUFA content of the two diets was 18.4 and 11.3 en%, and the diets were fed for 8 weeks. Lymphocyte proliferation and NK cell activity did not differ between the subjects fed the high and low

MUFA diets. Results from these two studies indicate that moderate changes in the consumption of MUFAs do not have any adverse or beneficial effects on several indices of immune response as long as the total fat content of the diets are comparable. The effects may differ significantly if MUFA is being replaced by n-3 PUFA, but those should be attributed to the changes in n-3 PUFA and not MUFA.

There is only one published report that examined the effects of feeding diets enriched in saturated and trans FAs on human functions (Han *et al.*, 2002). Three diets containing 30 en% from different fat sources were fed to healthy adults for 32 days; two-thirds of the fat was supplied by either PUFAs from soybean oil, trans FAs from hydrogenated soybean oil, or saturated FAs from butter. Many indices including lymphocyte proliferation and secretion of IL-2 and PGE_2 did not differ between the three diets. Secretion of IL-6 and TNF-α did not differ between the saturated FA and the PUFA group, but these were increased by 36 and 58%, respectively, in the group fed the hydrogenated fat. Results of this study suggest an increased inflammatory response by trans FAs. This notion is supported by the results from an epidemiological study that examined the association between serum concentrations of trans FAs and markers for inflammation (Mozaffarian *et al.*, 2004). In this study, the concentration of soluble TNF receptors 1 and 2 were positively correlated with dietary concentrations of trans FAs. Concentrations of IL-6 and CRP were not associated with those of trans FAs if all subjects were included; however, positive associations were found in subjects with higher body mass index. Overall, these results are consistent with the hypothesis that trans FAs increase inflammatory response and the risk for inflammatory diseases. Further studies are needed to quantify the risk from trans FAs and to find ways to mitigate this risk.

E. MECHANISMS OF ACTION OF PUFA

While several years of research have led to the description of clear as well as ambiguous phenomenological observations with respect to effects of dietary fat on the human immune system, few if any studies have specifically focused on mechanisms. However, many animal model and cell culture studies have been done in an attempt to describe the possible mechanisms involved in FA effects on immune cells and the immune system. In addition, studies on other cell types can be used to infer possible mechanistic effects by dietary fat in animal models or humans. In the following sections, we discuss selected mechanisms of how FAs may alter immune cells and ultimately the immune system. The list is by no means exhaustive, but it is a focused approach.

1. *Structural membrane alterations: effects on lipid rafts*

Lipid rafts have gained considerable attention because of their importance as sites for signal transduction initiation in many cells including those of the immune system (Rao *et al*., 2004). Structurally, lipid rafts are patched domains within the lipid bilayer of the plasma membrane whose composition differs from the bulk for the usual bilayer (Pizzo and Viola, 2004). Operationally, they are described as detergent-resistant domains (DRMs) within the plasma membrane (Pizzo and Viola, 2004). The DRMs are rich in cholesterol and sphingolipids, which have longer and more saturated FA chains compared to the relatively more unsaturated FA chains of the typical membrane phospholipids (Pizzo and Viola, 2004). That means that sphingolipids and cholesterol are more attracted to each other and that changes in FA composition, such as from the diet, may alter DRM. Pizzo and Viola (2004) have discussed the involvement of lipid rafts in lymphocyte activation. At this time, it is suggested that during lymphocyte activation and chemotaxis, lipid rafts act as platforms to compartmentalize signaling and facilitate specific interactions (Pizzo and Viola, 2004). Two other studies (Diaz *et al*., 2002; Switzer *et al*., 2004) have shown that DHA can affect lymphocyte activation by alteration of lipid rafts. In one study, phospholipase D1 was excluded from lipid rafts after DHA treatment, thus, relocating it and allowing for its activation (Diaz *et al*., 2002). That phospholipase D activation might be responsible for the immunosuppressive effect of DHA because it is known to transmit antiproliferative signals in lymphoid cells (Diaz *et al*., 2002). In another study, n-3 PUFA enhanced the polarization and deletion of proinflammatory Th1 cells, possibly as a result of alterations in membrane microdomain (lipid raft) FA composition (Switzer *et al*., 2004).

2. *Alteration of lipid mediators*

The alteration of lipid mediators by PUFA has been reviewed in great detail (Stulnig, 2003) and is mentioned only briefly here. The function of cells of the immune system can be exquisitely sensitive to lipid mediators such as prostaglandins and leukotrienes. Those mediators are normally produced by the action of specific enzyme systems on the AA substrate that is released from membrane phospholipids pools. Thus, cyclooxygenase (COX-1 or COX-2) converts AA to prostaglandins, and lipoxygenase (e.g., 5-LOX) converts AA to HETEs and leukotrienes. The amount of substrate, the activity of the enzymes, and the amount and potency of the lipid mediators appear to be regulated by FA composition and, thus, the dietary intake of PUFA. Specifically, EPA is and DHA is not a substrate for COX and LOX and can competitively inhibit AA metabolism. In addition, DHA can inhibit

the release of AA and, thus, decrease the amount available for conversion to eicosanoids (Martin, 1998). There is also some evidence that EPA and DHA can alter PG and LT synthesis at the level of gene expression by regulating COX-2 and 5-LOX (Stulnig, 2003). Although these potent lipid mediators are continuously regarded as a link between intake of PUFA and subsequent immune system alterations, there is still debate over whether eicosanoid interference is a viable mechanism.

3. *Effects on gene expression*

Literature is replete with studies describing the role of peroxisome proliferator–activated receptor (PPAR) in metabolism and immune function. These are a family of nuclear receptors that can bind various lipophilic metabolites including FAs and their derivatives. After ligand binding, PPAR activity is involved in the regulation of many genes including those involved in adipogenesis and adipocyte function, as well as lymphocyte activation and the promotion of macrophage differentiation (Stulnig, 2003). Thus, FAs have a means of altering the expression of genes important for maintenance and modulation of the immune response. For example, agonists of the specific PPAR, PPARγ, can inhibit TNF-α, IL-6, and IL-1β expression in monocytes (Stulnig, 2003). Although PUFAs have been shown to alter PPAR activity or genes that are normally regulated by PPAR, very few studies can prove that it is a direct result of binding and activation of the nuclear receptor. Far more studies are required to elucidate how PUFAs effect PPAR signaling. Other nuclear receptors that PUFA may be involved in mediating include retinoid X receptors and liver X receptors.

4. *Effects on differentiation, proliferation, and apoptosis*

FA regulation of cellular differentiation and apoptosis has been reviewed (Rudolph *et al.*, 2001). In general, differentiation was promoted by PUFAs such as AA, EPA, and DHA and metabolites such as PGE_1, PGE_2, and LTD_4. The effect of PUFA on apoptosis was less clear-cut, with AA and GLA increasing apoptosis, whereas effects of EPA, DHA, and eicosanoids varied from stimulation to inhibition (Rudolph *et al.*, 2001).

With respect to proliferation, DHA may be responsible for arresting cells, making them susceptible to apoptosis. In one study, DHA induced cell cycle arrest in Jurkat leukemic cells, perhaps by inhibiting of pRb phosphorylation (Siddiqui *et al.*, 2003). In that study, DHA greatly reduced the level of cyclin A while increasing the level of p21 WAF1, a cellular inhibitor of cyclin A/cyclin-dependent kinase 2 (cdk2) activity. In another study with HT-29 tumor cells, DHA inhibited the phosphorylation of pRb and DNA-binding

activity of E2F-1 and prevented entry of cells to S phase, and the antioxidant butylated hydroxytoluene was able to reverse the inhibition of activation of cyclin A/cdk by DHA in a dose-dependent manner, suggesting that endogenous oxidative stress produced by lipid peroxidation in HT-29 cells may be involved in the control of cell cycle progression (Chen and Istfan, 2001). One or more of these proposed mechanisms or additional ones not outlined here may account for how FAs alter human immune response. Future studies need to be designed that elucidate a series of molecular events, which will define how FAs function in alteration of immunity.

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BACTERIA IMPORTANT DURING WINEMAKING

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Numerous bacterial species are present during the vinification process. The extent to which these species grow determines the types and concentrations of many substances that contribute to the aroma and flavor of a wine. Growth of bacterial species such as *Acetobacter*/*Gluconobacter, Lactobacillus*, and *Pediococcus* may cause spoilage of a wine through the production of

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off-flavors and aromas. Certain bacterial strains may also produce substances in wine such as biogenic amines and ethyl carbamate precursors that are of public health concern. Conversely, growth of *Oenococcus oeni* may be desirable because this bacterium conducts malolactic fermentation (an enzymatic conversion of malic acid to lactic acid that decreases wine acidity) and contributes desirable flavors and aromas to a wine. Additionally, many interactions occur between different bacterial species and with the wine yeast *Saccharomyces.* These interactions may be beneficial or detrimental to wine quality depending on the species involved. This chapter discusses current information regarding the bacteria important during the winemaking process including the impact these microorganisms have on wine quality, public health concerns, and the interactions that occur between these microorganisms and *Saccharomyces cerevisiae.*

I. INTRODUCTION

The fermentation of grape juice into wine represents a complex biochemical process involving many microorganisms. Because of this diversity, a complicated microbial ecology evolves that greatly affects overall wine quality. The microorganisms present in grape musts before fermentation have varying growth requirements and tolerances to inhibitory compounds and, thus, dominate at different times during the course of natural fermentation (Amerine *et al.*, 1980; Lonvaud-Funel, 1999). Microbial diversity also results in complex ecological interactions with both beneficial and antagonistic relationships being exhibited. For example, *Saccharomyces* has been reported to inhibit and stimulate growth of certain lactic acid bacteria (LAB) (Beelman *et al.*, 1982; Cannon and Pilone, 1993; Guilloux-Benatier *et al.*, 1985; King and Beelman, 1986). Conversely, LAB have been reported to inhibit *Saccharomyces* (Boulton *et al.*, 1996; Edwards *et al.*, 1999). As such, the interactions between microorganisms in wine are important because they may influence the success or failure of the alcoholic or malolactic fermentation, which in turn will influence the final quality of a wine.

In many countries, alcoholic fermentation is induced by inoculation with a yeast starter culture of *Saccharomyces* selected for its desirable winemaking qualities (Kunkee, 1984; Kunkee and Bisson, 1993; Rainieri and Pretorius, 2000; Reed and Chen, 1978; Reed and Nagodawithana, 1988). Starter cultures of *S. cerevisiae* strains are generally used because of to their increased ethanol and sulfur dioxide resistance and production of desirable aromas and flavors (Boulton *et al.*, 1996; Ebeler, 2001; Nykänen, 1986; Reed and Chen, 1978; Reed and Nagodawithana, 1988).

Besides *Saccharomyces*, different bacterial species increase or decrease wine quality, depending on the microorganisms involved. Because grape juice and wines are harsh environments for microbial growth due to low pH, minimal levels of oxygen, the presence of ethanol, and high osmotic pressure, only a few bacterial species are able to grow. As examples, LAB belonging to the genera *Lactobacillus, Oenococcus*, and *Pediococcus* are commonly found in wine because of tolerance to these factors (Amerine *et al*., 1980; Dicks *et al*., 1995; Fleet, 2003; Henick-Kling, 1993; Lonvaud-Funel, 1999). Other bacteria such as *Acetobacter* and *Gluconobacter* are present in grape musts, with the former being a concern in wines (Drysdale and Fleet, 1988; Du Toit and Lambrechts, 2002; Joyeux *et al*., 1984a).

Many LAB found in wines can improve wine quality by metabolizing malic acid to lactic acid in a process called malolactic fermentation (MLF). This fermentation is an enzyme-mediated decarboxylation of the dicarboxylic acid, L (−) malic acid, to the monocarboxylic L (+) lactic acid (Amerine *et al*., 1980; Kunkee, 1967; Lonvaud-Funel, 1999). MLF decreases wine acidity and is particularly important in wines produced from grapes grown in cool climates, which often have high acidity (Beelman and Gallander, 1979; Kunkee, 1967, 1974).

However, some LAB can be associated with spoilage problems including stuck alcoholic fermentations (Edwards *et al*., 1999; Huang *et al*., 1996), production of off-flavors or off-odors (Costello and Henschke, 2002; Drysdale and Fleet, 1989a; Sponholz, 1993), excessive volatile acidity (VA) (Drysdale and Fleet, 1989a; Huang *et al*., 1996), synthesis of polysaccharides responsible for ropiness (Manca de Nadra and Strasser de Saad, 1995), or other defects.

This chapter summarizes current information regarding the bacteria important during the winemaking process including *Acetobacter*/*Gluconobacter, Lactobacillus, Oenococcus*, and *Pediococcus*, as well as the impact these microorganisms have on wine quality and public health concerns. Furthermore, the interactions that occur between these microorganisms and *S. cerevisiae* and their influences on wine quality are discussed.

II. *ACETOBACTER* AND *GLUCONOBACTER*

A brief overview of the importance of the acetic acid bacteria (AAB) *Acetobacter* and *Gluconobacter* in winemaking is given here, but readers are directed to a comprehensive review by Du Toit and Pretorius (2002) for a more detailed discussion.

A. TAXONOMY, OCCURRENCE, GROWTH, AND METABOLISM IN WINE

AAB are Gram negative, aerobic, catalase-positive rods that belong to the family Acetobacteraceae, which is divided into the genera *Acetobacter, Acidomonas, Gluconobacter*, and *Gluconacetobacter* (Holt *et al*., 1994; Ruiz *et al*., 2000). *Acetobacter* and *Gluconobacter* have been isolated from flowers, fruits, wine, beer, and brewers yeast and are the primary microorganism involved in vinegar production (De Lay *et al*., 1984; Drysdale and Fleet, 1988; Holt *et al*., 1994). *Acetobacter* can also be found in garden soil and canal water (De Lay *et al*., 1984).

Gluconobacter oxydans, Acetobacter aceti, Acetobacter pasteurianus, Gluconacetobacter liquefaciens, and *Gluconacetobacter hansenii* are associated with grapes and wine (Drysdale and Fleet, 1988; Du Toit and Lambrechts, 2002; Joyeux *et al*., 1984a). *Gluconobacter* species are commonly isolated from grapes and musts but disappear as alcoholic fermentation begins, perhaps because of low ethanol tolerance or a lack of oxygen (Joyeux *et al*., 1984a). *Acetobacter* are more ethanol tolerant than *Gluconobacter* and so may survive through alcoholic fermentation (Drysdale and Fleet, 1984, 1988; Joyeux *et al*., 1984a). Commonly, *A. aceti* and *A. pasteurianus* are isolated from grapes and musts and may reach populations of 10^6 cells/g on damaged grapes (Drysdale and Fleet, 1984, 1989a; Joyeux *et al*., 1984b). Yeast present on damaged grapes can metabolize grape sugars, producing ethanol, which is oxidized to acetic acid by these bacteria (Joyeux *et al*., 1984b). Acetic acid concentrations as high as 3.9 g/L may be found in juices made from infected grapes (Drysdale and Fleet, 1989a).

Because of their aerobic nature, AAB are normally inhibited in the anaerobic environment associated with alcoholic fermentation (Joyeux *et al*., 1984b). However, *A. pasteurianus* and *A. aceti* are frequently isolated from wines stored in barrels or other vessels in the winery under semi-aerobic conditions (Joyeux *et al*., 1984a). The survival of AAB in wines may be due to the exposure of wine to air during pumping and transfer operations. For instance, Drysdale and Fleet (1989b) reported AAB populations of up to 10^8 cfu/ml in wine exposed to air during pumping-over operations. Besides the presence of oxygen, the growth of AAB is greatly influenced by the pH of the must. In a study by Du Toit and Lambrechts (2002), AAB populations decreased from 10^5 cfu/ml to 10^2 cfu/ml in musts with a pH less than 3.5, whereas higher cell viability was found in musts with a pH of 3.7.

AAB produce acetic acid through the oxidation of ethanol by two membrane-bound enzymes: an alcohol dehydrogenase and an aldehyde dehydrogenase (Saeki *et al*., 1997). The alcohol dehydrogenase oxidizes ethanol to

acetaldehyde, which is further oxidized to acetic acid by the aldehyde dehydrogenase (Saeki *et al*., 1997). Some strains of AAB can produce more than 50 g/L from ethanol, making them important in the production of vinegar (Lu *et al*., 1999).

During growth in wine, AAB use glucose as a carbon source, although it is a better carbon source for *Gluconobacter* than *Acetobacter* because not all strains of *Acetobacter* can use glucose effectively (De Lay *et al*., 1984). *Acetobacter* species metabolize sugars via the hexose-monophosphate pathway, as well as the Embden-Meyerhof-Parnas and Entner-Doudoroff pathways, producing acetate and lactate as byproducts. These compounds can be further oxidized by some strains to CO_2 and water via the tricarboxylic acid (TCA) pathway. Unlike *Acetobacter, Gluconobacter* species use only the pentose-phosphate pathway to generate energy. These organisms do not contain a functional TCA cycle and, therefore, cannot oxidize acetate or lactate to CO_2 and water (De Lay *et al*., 1984). Besides glucose, AAB are able to use other carbohydrates, which may be found in wine such as arabinose, fructose, galactose, mannitol, mannose, ribose, sorbitol, and xylose (De Lay *et al*., 1984).

B. WINE SPOILAGE

A major cause of wine spoilage by AAB is the production of excessive acetic acid. The legal limit for acetic acid in wine is 1.2–1.4 g/L, concentrations that also greatly reduce wine quality (Drysdale and Fleet, 1989a; Sponholz, 1993). Drysdale and Fleet (1989a) reported that as much as 50–60% of the ethanol content of a wine could be oxidized by these bacteria with the production of 1.5–3.75 g/L of acetic acid.

Another major byproduct that affects wine quality is ethyl acetate. This compound is highly undesirable and possesses a low flavor threshold of 10 mg/L (Berg *et al*., 1955). AAB have been shown to produce ethyl acetate concentrations of up to 140 mg/L in wine and 30 mg/L in must (Drysdale and Fleet, 1989a).

Other than acetic acid and ethyl acetate, AAB produce other compounds detrimental to wine quality including acetaldehyde, acetoin, and dihydroxyacetone. Drysdale and Fleet (1989a) reported increased concentrations of acetaldehyde in wine in which AAB had been grown. Acetaldehyde concentrations above the sensory threshold in wine (100–125 mg/L) are undesirable due to "green," "grassy," and "vegetative" off-aromas (Kotseridis and Baumes, 2000; Liu and Pilone, 2000). Dihydroxyacetone may be produced by *A. aceti* and *G. oxydans* through the metabolism of glycerol under aerobic conditions (Drysdale and Fleet, 1989a; Fugelsang, 1997). This compound

can affect the sensory quality of the wine with a "sweet/esterish" property and may also react with proline to produce a "crustlike" aroma (Boulton *et al.*, 1996; Drysdale and Fleet, 1988). Furthermore, acetaldehyde and dihydroxyacetone can bind with SO_2 in wine to produce compounds that are ineffective as antimicrobial agents (Fornachon, 1963; Hood, 1983; Romano and Suzzi, 1993). In addition, some *Acetobacter* species can metabolize lactate producing acetoin, a precursor of the flavor compound diacetyl (Romano and Suzzi, 1996).

III. *LACTOBACILLUS*

A. TAXONOMY, OCCURRENCE, GROWTH, AND METABOLISM IN WINE

Lactobacillus represents a highly diverse group of species that are gram-positive microaerophilic bacteria that vary from long to short rods or even coccobacilli (Kandler and Weiss, 1986). Lactobacilli are catalase and cytochrome negative, although a few strains decompose peroxide by a non–heme-containing pseudocatalase (Beyer and Fridovich, 1985; Johnston and Delwiche, 1962; Kandler and Weiss, 1986; Kono and Fridovich, 1983). *Lactobacillus* can have complex nutritional requirements for amino acids, nucleic acid derivatives, vitamins, fatty acids, and fermentable carbohydrates (Kandler and Weiss, 1986). *Lactobacillus* species are either homofermentative or heterofermentative with regards to hexose metabolism. Homofermenters convert glucose to DL-, D-(–), or L-(+) lactic acid via the Embden-Meyerhof-Parnas pathway without the production of CO_2, whereas heterofermenters metabolize hexoses via the phosphoketolase pathway producing DL-, D-(−), or L-(+) lactic acid, CO_2, and ethanol or acetate (Kandler and Weiss, 1986).

Species of *Lactobacillus* isolated from grapes and wines worldwide have included *L. brevis, L. buchneri, L. casei, L. cellobiosus, L. curvatus, L. fermentum, L. fructivorans, L. hilgardii, L. homohiochii, L. jensenii, L. leichmannii, L. plantarum, L. sake*, and *L. trichodes* (Chalfan *et al.*, 1977; Costello *et al.*, 1983; Davis *et al.*, 1986a,b; Dicks and Van Vuuren, 1988; Douglas and Cruess, 1936; Du Plessis and Van Zyl, 1963; Fornachon, 1957; Kandler and Weiss, 1986; Lafon-Lafourcade *et al.*, 1983; Maret and Sozzi, 1977, 1979; Pilone *et al.*, 1966; Sieiro *et al.*, 1990; Vaughn, 1955). *L. cellobiosus* is regarded as a biotype of *L. fermentum*, whereas *L. leichmannii* is now referred to as *L. delbrueckii* subspecies *lactis* (Kandler and Weiss, 1986). *L. trichodes*, is considered a synonym of *L. fructivorans* (Weiss *et al.*, 1983). Edwards *et al.*

(1998, 2000) isolated two novel *Lactobacillus* species from commercial grape wines undergoing sluggish/stuck alcoholic fermentations. Based on phenotypic and phylogenetic evidence, *L. kunkeei* and *L. nagelii* were proposed as new species (Edwards *et al.*, 1998, 2000).

The occurrence and survival of *Lactobacillus* species in wine is highly pH and ethanol dependent (Davis *et al.*, 1986a). In high pH wines (>3.5), *Lactobacillus* species often are predominant, whereas at lower pH values, other LAB such as *Oenococcus oeni* dominate (Davis *et al.*, 1986b; Henick-Kling, 1993). Ethanol tolerance varies among *Lactobacillus* species. For example, growth of *L. plantarum* ceases at ethanol concentrations of 5–6% v/v, whereas the more ethanol tolerant *L. casei* and *L. brevis* have been successfully used to induce MLF (Kosseva *et al.*, 1998; Wibowo *et al.*, 1985). Finally, *L. fructivorans* is extremely ethanol tolerant and has been isolated from high alcohol (>20% v/v) dessert wines (Amerine and Kunkee, 1968; Fornachon *et al.*, 1949).

B. WINE SPOILAGE

Lactobacillus is generally considered undesirable in wine because uncontrolled growth can lead to increases in VA or formation of other adverse odors or flavors. Some species produce excessive amounts of acetic acid (Davis *et al.*, 1986b; Edwards *et al.*, 1999; Huang *et al.*, 1996). As evidence, *L. kunkeei* can produce between 3 and 5 g/L of acetic acid in wines (Edwards *et al.*, 1999; Huang *et al.*, 1996).

In addition to its sensory effect on wine, *Lactobacillus* species have been implicated in causing stuck or sluggish fermentations. Some winemakers have observed rapid wine spoilage by microorganisms dubbed the "ferocious" lactobacilli (Boulton *et al.*, 1996). Boulton *et al.* (1996) characterized this spoilage as being very swift with abundant bacterial growth during the early stages of vinification. Huang *et al.* (1996) demonstrated that uncontrolled growth of certain LAB including *L. kunkeei* (Edwards *et al.*, 1998) could cause stuck or sluggish alcoholic fermentations. Some strains of *L. hilgardii* have also been implicated in this spoilage (Mills, 2001). In many cases in which "ferocious" lactobacilli had caused spoilage, the winemakers had not used any addition of SO_2 and the initial pH of the wines was above 3.5 (Huang *et al.*, 1996), conditions favorable to the growth of *Lactobacillus.* Further discussion of the inhibition of *Saccharomyces* by *Lactobacillus* can be found in Section VIII.C.

Heterofermentative lactobacilli have been associated with the "mousy" defect in wines (Costello *et al.*, 2001; Heresztyn, 1986). This type of spoilage is characterized by the development of an offensive odor that renders a wine

unpalatable (Costello and Henschke, 2002). Lactobacilli associated with this taint *L. brevis, L. hilgardii*, and *L. cellobiosus* (Costello *et al*., 2001), can synthesize *N*-heterocyclic bases such as, 2-ethyltetrahydropyridine, 2-acetyltetrahydropyridine, and 2-acetyl-1-pyrroline (Costello and Henschke, 2002; Heresztyn, 1986). Because synthesis of these compounds requires the presence of ethanol, this defect is associated with wines rather than musts (Heresztyn, 1986).

Certain *Lactobacillus* are also implicated in spoilage of fortified wines. Known as "Fresno mold," this spoilage is characterized by mycelial/fiber like growth in wines (Amerine and Kunkee, 1968; Fornachon *et al*., 1949) and is caused by *L. trichodes* (*L. fructivorans*) (Gini and Vaughn, 1962; Vaughn, 1955). The species is relatively sensitive to SO_2, so use of this antiseptic can prevent the growth of this microorganism (Fornachon *et al*., 1949).

IV. *OENOCOCCUS*

A. TAXONOMY, OCCURRENCE, GROWTH, AND METABOLISM IN WINE

Wine strains belonging to the genus *Leuconostoc* were originally classified as *Leuconostoc oenos* by Garvie (1967a). Later, phylogenetic studies revealed that *L. oenos* represented a distinct subline separate from other *Leuconostoc* species (Martinez-Murcia *et al*., 1993) and resulted in reassignment of this microorganism to a new genus *Oenococcus* (Dicks *et al*., 1995). Strains of *O. oeni* are described as Gram-positive, nonmotile, facultatively anaerobic, catalase-negative, ellipsoidal to spherical cells that usually occur in pairs or chains (Dicks *et al*., 1995; Garvie, 1967a). Similar to *Lactobacillus, O. oeni* are chemoorganotrophs requiring a rich medium containing complex growth factors and amino acids (Dicks *et al*., 1995; Garvie, 1967b). These microorganisms are heterofermentative and convert glucose to equal molar amounts of D-(−) lactic acid, CO_2, and ethanol or acetate via the phosphoketolase pathway (Cocaign-Bousquet *et al*., 1996; Cogan and Jordan, 1994; Krieger *et al*., 1993).

B. MALOLACTIC FERMENTATION

Numerous articles have been published documenting the enological importance of malolactic fermentation (Davis *et al*., 1985; Kunkee, 1967; Lonvaud-Funel, 1999; Van Vuuren and Dicks, 1993; Wibowo *et al*., 1985). During this

process, malic acid is metabolized to L-(+) lactic acid and CO_2, reducing wine acidity (Korkes *et al.*, 1950; Pilone and Kunkee, 1970, 1972; Wibowo *et al.*, 1985). Early work by Korkes *et al.* (1950) demonstrated that MLF was a decarboxylation catalyzed by a NAD^+ specific "malic" enzyme requiring Mn^{2+}. This enzyme was first purified from *L. plantarum* (Korkes *et al.*, 1950) and then from other LAB (Cox and Henick-Kling, 1990; Lonvaud-Funel, 1995; Lonvaud-Funel and Strasser de Saad, 1982; Schutz and Radler, 1973).

The biochemical benefit of MLF to the microorganism was puzzling at first as no apparent adenosine triphosphate (ATP) or other direct energy was detected (Pilone and Kunkee, 1972). During the conversion of malic to lactic acid, pyruvate was not formed as an intermediate, and this prompted researchers to report that MLF must serve a non–energy-yielding function (Kunkee, 1967; Pilone and Kunkee, 1972). Pilone and Kunkee (1972) observed that MLF accelerated the rate of growth of *O. oeni* and theorized that the decarboxylation reaction stimulated the utilization of carbon sources by LAB. To the contrary, Cox and Henick-Kling (1989) demonstrated that MLF yielded ATP and proposed that the ability of a cell to expel lactate and protons through a symport could theoretically generate a proton motive gradient (Δp), which in turn would yield ATP through a membrane-bound adenosine triphosphatase (ATPase).

However, there is a lack of evidence for a lactate/proton symport in *O. oeni* (Cox and Henick-Kling, 1995; Olsen *et al.*, 1991; Ten Brink *et al.*, 1985). Cox and Henick-Kling (1995) reported that lactate efflux did not produce ATP during MLF at low pH levels. Instead, the authors suggested that ATP produced during malate catabolism was linked to Δp formed during malate transport and lactic acid diffusion. In support, Poolman *et al.* (1991) reported that *Lactococcus lactis* produced a Δp composed of a membrane potential and pH gradient through the electrogenic uptake of malate, together with proton consumption as a result of decarboxylation of L-malate. Salema *et al.* (1994) proposed a model showing uptake of L-malate in the monoanionic form (the dominant species at low pH) via a uniport (Figure 1). This would cause a net negative charge to be moved inwards, thereby creating an electrical potential. L-Malate is then decarboxylated inside the cell to L-lactic acid and CO_2 in a reaction that requires one proton. The consumption of a proton in the cytoplasm would generate a pH gradient that, together with the change in electrical potential, would form a Δp across the cytoplasmic membrane. ATP generation would then occur via a membrane-bound ATPase. Salema *et al.* (1994) suggested that L-lactic acid and CO_2 leave the cell as neutral species rather then being actively transported. Later work by Salema *et al.* (1996) supported this model, as a Δp was generated *in vitro* by the action of an electrogenic uniport in conjunction with proton consumption by L-malate decarboxylation.

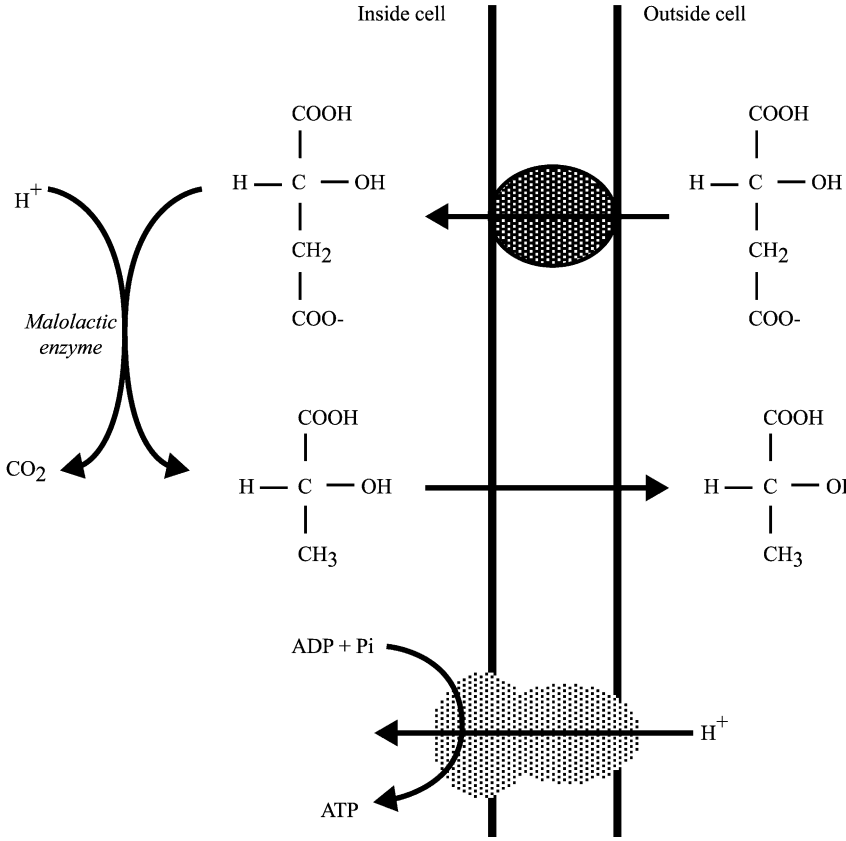

FIG. 1 Proposed model for energy generation (ATP) by *Oenococcus oeni* through conversion of malic acid to lactic acid and carbon dioxide. (Adapted from Poolman *et al*., 1991 and with permission of the Journal of Bacteriology.)

MLF most commonly occurs after alcoholic fermentation but may occur simultaneously with the primary fermentation. Because relying on natural microflora can be unpredictable and difficult to control, starter cultures of pure strains of bacteria have been developed (Henick-Kling, 1993; Krieger *et al*., 1993; Kunkee *et al*., 1964; Nielsen *et al*., 1996; Pilone, 1995). Although selected strains of *Lactobacillus* can be inoculated, *O. oeni* is the primary species to conduct MLF because of acid tolerance and the flavor profile produced (Guzzo *et al*., 1994; Krieger *et al*., 1993; Kunkee *et al*., 1964; Liu, 2002; Nielsen *et al*., 1996; Wibowo *et al*., 1985).

Exactly when to inoculate *O. oeni* during vinification is a point of contention among researchers and enologists. Some have argued that starter

cultures should be inoculated only after completion of the alcoholic fermentation, to prevent possible yeast antagonism and production of undesirable metabolites (Lafon-Lafourcade and Ribéreau-Gayon, 1984; Lafon-Lafourcade *et al*., 1983; Ribéreau-Gayon, 1985). Others contend that the inducement of simultaneous alcoholic and malolactic fermentations is feasible (Beelman, 1982; Beelman and Kunkee, 1985). Semon *et al.* (2001) concluded that the optimal time for inoculation of malolactic bacteria was dependent on the specific yeast and bacterial strains used. These authors postulated that problems associated with inoculation of bacteria before completion of alcoholic fermentation, such as excessive volatile acid and/or sluggish alcoholic fermentations, are most probably due to incompatibilities between specific strains of yeast or malolactic bacteria.

C. FLAVOR PRODUCTION

Aside from deacidification, MLF may also influence the sensory qualities of a wine by the production of many flavor and aroma compounds (Davis *et al*., 1985, 1988; Henick-Kling, 1993; Kunkee, 1967; Rankine, 1977). However, there is still debate regarding the contribution of MLF to the sensory properties of a wine. Early work by Kunkee *et al.* (1964) and Rankine (1972) indicated that MLF may not have a measurable effect on the sensory properties of a wine. On the other hand, many other studies have shown that MLF causes significant changes in wine aroma and flavor (Boido *et al*., 2002; Delaquis-Pascal *et al*., 2000; De Revel *et al*., 1999; Gambaro *et al*., 2001; Henick-Kling, 1995; Laurent *et al*., 1994; Maicas *et al*., 1999; McDaniel *et al*., 1987; Nielsen and Richelieu, 1999).

Although the exact contribution of MLF to wine flavor is still debatable, *O. oeni* is known to produce flavor and aroma compounds in wine. One of the most important is 2,3-butanedione or diacetyl (Collins, 1972; Fornachon and Lloyd, 1965; Martineau and Henick-Kling, 1995a,b; Nielsen and Richelieu, 1999; Rodriguez *et al*., 1990). Diacetyl has a distinct buttery aroma and is synthesized by wine LAB from citrate or other carbohydrates via pyruvate (Martineau and Henick-Kling, 1995a). Sensory threshold values range from 0.2 mg/L in Chardonnay to 0.9 mg/L in Pinot Noir to 2.8 mg/L in Cabernet Sauvignon wine (Martineau *et al*., 1995), with the final concentration in wine affected by many factors including bacterial strain, wine type, and redox potential (Martineau and Henick-Kling, 1995a,b; Nielsen and Richelieu, 1999). Although the presence of diacetyl at low concentrations (1–3 mg/L) is described sensorially as being "buttery" or "nutty," the compound will dominate wine aroma at higher concentrations (5–7 mg/L), resulting in spoilage (Rankine *et al*., 1969).

In addition to diacetyl, *O. oeni* produces esters, flavor compounds also important for wine flavor and aroma. Esters are primarily produced by *Saccharomyces* during alcoholic fermentation (Mason and Dufour, 2000; Nykänen, 1986; Nykänen and Nykänen, 1977; Soles *et al.*, 1982), although evidence shows that esters such as ethyl acetate, ethyl lactate, ethyl hexanoate, and ethyl octanoate can be synthesized by *O. oeni* (De Revel *et al.*, 1999; Delaquis-Pascal *et al.*, 2000; Edwards and Peterson, 1994; Maicas *et al.*, 1999; Tracey and Britz, 1989). For example, Edwards and Peterson (1994) reported that strains of *O. oeni* synthesized relatively large amounts of ethyl lactate (183–1280 μg/L) during growth in microbiological medium. In agreement, Maicas *et al.* (1999) reported that 50 mg/L of ethyl lactate was produced in wines fermented with *O. oeni*, as well as isoamyl acetate and ethyl caproate, compounds important for a pleasant fruity note in wine (Gil *et al.*, 1996; Mason and Dufour, 2000; Nykänen, 1986).

O. oeni may also influence wine flavor through the liberation of monoterpenes. These flavor compounds are often present in grapes and wine as nonvolatile flavorless glycosylated compounds (Ebeler, 2001). The liberation of monoterpenes is important for the development of certain wine aromas; however, the hydrolysis of monoglucosides requires the action of a β-glucosidase. It is known that wine yeast, in particular non-*Saccharomyces* yeast, have glycosidic activities (Charoenchai *et al.*, 1997; Delcroix *et al.*, 1994; Maicas *et al.*, 1999; McMahon *et al.*, 1999). Evidence indicates that some strains of *O. oeni* also possess β-glucosidase activity and, thus, may hydrolyze glycoconjugates and alter the sensory characteristics of wine (Boido *et al.*, 2002; Grimaldi *et al.*, 2000; Mansfield *et al.*, 2002; Ugliano *et al.*, 2003).

O. oeni may also influence the concentrations of aldehydes such as acetaldehyde. Acetaldehyde is the most abundant aldehyde found in wine and affects wine aroma, aging, and color stability (Liu and Pilone, 2000). Osborne *et al.* (2000) found that *O. oeni* can metabolize acetaldehyde, producing ethanol and acetic acid. Degradation of acetaldehyde may be desirable in some cases, because excess acetaldehyde causes an off-aroma in wine (Kotseridis and Baumes, 2000; Liu and Pilone, 2000), but undesirable in other cases because this compound plays a role in the color development of red wines (Somers and Wescombe, 1987; Timberlake and Bridle, 1976).

Aside from influencing flavor and aroma, MLF may increase the body and mouthfeel of a wine, possibly because of the production of polyols such as glycerol and erythritol (Henick-Kling *et al.*, 1994). Veiga da Cunha *et al.* (1993) demonstrated the production of glycerol and erythritol from glucose using a ^{13}C-nuclear magnetic resonance (NMR) technique, whereas

Liu *et al.* (1995) and Firme *et al.* (1994) observed the production of glycerol and erythritol by *O. oeni.*

V. *PEDIOCOCCUS*

A. TAXONOMY, OCCURRENCE, GROWTH, AND METABOLISM IN WINE

Pediococci are characterized as spherical, Gram-positive, nonmotile, catalase-negative, aerobic to microaerophilic microorganisms (Carr *et al.*, 2002; Garvie, 1986). Pediococci are the only LAB that divide in two planes, which form tetrads or large clumps of cells (Axelsson, 1998; Garvie, 1986). Currently approved species are *P. acidilacti, P. damnosus, P. dextrinicus, P. halophilus, P. inopinatus, P. parvulus, P. pentosaceus*, and *P. urinae-equi* (Garvie, 1986). The International Committee on Systematic Bacteriology has ruled that the species *P. cerevisiae* was not validly used in publications because it represented at least two species, *P. damnosus* and *P. pentosaceus* (Garvie, 1974, 1986; Raccach, 1987).

Pediococci are homofermentative, with glucose being converted to DL- or L-(+) lactate via the Embden-Meyerhof-Parnas pathway without the production of CO_2 (Garvie, 1986). *Pediococcus* are chemoorganotrophs having complex growth factor and amino acid requirements. All species require nicotinic acid, pantothenic acid and biotin, but none require thiamine, *p*-aminobenzoic acid, or cobalamin (Garvie, 1986). Growing cultures also have the ability to form L-(+) lactate from malic acid (Edwards and Jensen, 1992; Raccach, 1987).

Pediococci are commonly found associated with various plants and their products such as cabbage and sauerkraut, cucumbers and pickles, grapes and wine, and wort and grain mashes (Carr, 1970, 2002; Costello *et al.*, 1983; Edwards and Jensen, 1992; Flemming *et al.*, 1975; Mundt *et al.*, 1969; Solberg and Clausen, 1973). They may enter wine through being present on soil, grape berries, or winery equipment, but their survival is favored when pH is greater than 3.5 (Davis *et al.*, 1986a; Wibowo *et al.*, 1985). Species of *Pediococcus* isolated from wine include *P. damnosus, P. inopinatus, P. pentosaceus*, and *P. parvulus* (Davis *et al.*, 1986b; Edwards and Jensen, 1992; Garvie, 1986; Lafon-Lafourcade *et al.*, 1983).

B. WINE SPOILAGE

Growth of *Pediococcus* species in wine has been considered undesirable because of the production of off-aromas and off-flavors. Pediococci are capable of producing excessive acetoin and diacetyl, which can give undesirable aromas

and flavors at high concentrations (Sponholz, 1993). In addition, some species of *Pediococcus* are capable of degrading glycerol to acrolein, a compound that reacts with the phenolic group of anthocyanins producing a bitter taint in wine (Davis *et al.*, 1988; Du Toit and Pretorius, 2000; Sponholz, 1993).

Besides producing off-flavors, *Pediococcus* species have been implicated in the production of extracellular polysaccharides characterized as β-D-glucans (Llaubéres *et al.*, 1990). These homoglucans are produced from glucose and consist of a trisaccharide repeating unit having a (1 → 3)-linked backbone and a (1 → 2)-linked branch of one of the D-glucopyranosyl groups (Llaubéres *et al.*, 1990). Besides being visually unappealing, these polymers cause an increase in viscosity of the wine (Fugelsang, 1997; Manca de Nadra and Strasser de Saad, 1995). Pediococci associated with "ropiness" have higher tolerances to ethanol than other strains (Du Toit and Pretorius, 2000). Thus, this defect occurs in wines either during alcoholic fermentation or after bottling (Du Toit and Pretorius, 2000).

P. damnosus is the bacterium primarily implicated in the production of polysaccharides in wine (Lonvaud-Funel, 1999; Manca de Nadra and Strasser de Saad, 1995), although Manca de Nadra and Strasser de Saad (1995) isolated two strains of *P. pentosaceus* from "ropy" Argentinean wines. Strains of *P. damnosus* that produce exopolysaccharides contain an unique 4-Kb plasmid (Lonvaud-Funel, 1999; Manca de Nadra and Strasser de Saad, 1995). Taking advantage of this, Lonvaud-Funel *et al.* (1993) developed a DNA probe to detect the presence of extracellular polysaccharide-producing pediococci strains by labeling a 1.2-Kb part of this plasmid. Furthermore, Gindreau *et al.* (2001) devised a direct polymerase chain reaction (PCR) detection method to detect these strains of *P. damnosus*. The PCR detection method negated the need for the time-consuming culture and colony-isolation steps required for the DNA probe method.

Although growth of certain *Pediococcus* species in wines is undesirable, Edwards and Jensen (1992) reported that several wines from which pediococci had been isolated were not spoiled. In agreement, Edwards *et al.* (1994) reported that *P. parvulus* altered the bouquet of a Cabernet Sauvignon wine that had not undergone MLF but was not considered spoiled. Therefore, the growth of pediococci in wine may add desirable flavors and aromas under certain circumstances. Further research elucidating the impact of these microorganisms on the chemical composition, bouquet, and flavor of wines is warranted as *Pediococcus* species have been isolated from wines worldwide (Costello *et al.*, 1983; Edwards and Jensen, 1992; Fleet *et al.*, 1984; Manca de Nadra and Strasser de Saad, 1995).

VI. IDENTIFICATION OF BACTERIA IN WINE

Most bacterial species present during the vinification process have been identified by traditional microbiological techniques based on cell morphological and physiological differences. All LAB with their biochemical characteristics are described in *Bergey's Manual* (Kandler and Weiss, 1986). However, the use of physiological and biochemical criteria to identify LAB strains can yield ambiguous results because many of the bacteria have very similar nutritional requirements and grow under similar environmental conditions (Sohier and Lonvaud-Funel, 1998; Vandamme *et al.*, 1996). Furthermore, cultivation-dependent methods often exhibit biases resulting in an incomplete representation of the true bacterial diversity present (Amann *et al.*, 1995; Hugenholtz *et al.*, 1998).

As a result, much research has been undertaken to develop more specific and reliable techniques to identify bacteria present during winemaking. Method development has focused on the use of molecular biology techniques allowing direct molecular characterization of bacterial strains through DNA-DNA hybridization (Lonvaud-Funel *et al.*, 1991). Some techniques include total cell DNA hybridization (Lonvaud-Funel *et al.*, 1991; Sohier and Lonvaud-Funel, 1998), PCR methods with species-specific primers targeted at the gene encoding the malolactic enzyme (Zapparoli *et al.*, 1998) or targeted at 16S rDNA (Bartowsky and Henschke, 1999; Maria Rodas *et al.*, 2003), with separation of PCR-amplified DNA by pulsed-field gel electrophoresis (Kelly *et al.*, 1993; Zapparoli *et al.*, 2000) or denaturing gradient gel electrophoresis (Lopez *et al.*, 2003), and randomly amplified polymorphic DNA techniques (Holt and Cote, 1998; Johansson *et al.*, 1995; Reguant and Bordons, 2003; Zapparoli *et al.*, 2000). These techniques are valid tools for the study of bacterial population dynamics during MLF and for the detection of spoilage bacterial species because they allow specific identification, detection, and enumeration of bacterial species in wine.

VII. PUBLIC HEALTH CONCERNS

A. BIOGENIC AMINES

Biogenic amines are low-molecular-weight organic bases that have undesirable physiological effects on humans when absorbed at too high a concentration (Arena and Manca de Nadra, 2001; Lonvaud-Funel, 2001; Silla Santos, 1996). These compounds are formed by decarboxylation of the

corresponding amino acids by microorganisms such as LAB (Arena and Manca de Nadra, 2001; Halasz *et al*., 1994). In wine, several amino acids can be decarboxylated producing histamine, tyramine, putrescine, cadaverine, and phenylethylamine (Arena and Manca de Nadra, 2001; Lonvaud-Funel, 2001; Moreno-Arribas *et al*., 2003). The presence of high concentrations of histamine, tyramine, and phenylethylamine in wine is thought to be responsible for headaches and flushing sometimes experienced after consumption (Granerus *et al*., 1969; Ough *et al*., 1987; Rivas-Gonzalo *et al*., 1983; Sandler *et al*., 1974; Silla Santos, 1996). In addition, putrescine and cadaverine may enhance the toxicity of histamine to humans by depressing histamine oxidation (Arena and Manca de Nadra, 2001; Jarisch and Wantkle, 1996; Taylor, 1986; Taylor and Lieber, 1979; Torrea and Ancin, 2002).

Histamine is one of the most frequently found biogenic amines in wine (Lonvaud-Funel, 2001; Ough *et al*., 1987; Soufleros and Bertrand, 1988; Zee *et al*., 1983). Histamine levels ranging from undetectable to 30 mg/L have been found in wines from both European and American origins (Baucom *et al*., 1986; Ough, 1971). In general, the level of histamine in wine is usually below the toxic dose of 8 mg/L, a concentration thought to induce headaches when large amounts of wine are ingested (Ough, 1971). However, the exact toxic threshold of histamine is difficult to determine because of the presence or absence of potentiating compounds such as ethanol, aldehydes, and polyamines (such as putrescine and cadaverine), as well as the relative histamine sensitivity of different individuals (Jarisch and Wantkle, 1996; Lowenberg *et al*., 1981; Marquardt and Werringloer, 1965).

It was thought that spoilage bacteria, mainly *Pediococcus* species, were solely responsible for the production of histamine in wine (Delfini, 1989). However, some *O. oeni* strains are also able to produce histamine from histidine in wine (Lonvaud-Funel and Joyeaux, 1994). Additionally, Arena and Manca de Nadra (2001) and Moreno-Arribas *et al.* (2003) isolated strains of *L. plantarum, L. hilgardii*, and *L. brevis* capable of producing certain biogenic amines under vinification conditions. Elevated levels of biogenic amines may be found in wines that have undergone MLF due to the presence of these biogenic amine-producing LAB (Bauza *et al*., 1995; Cilliers and Van Wyk, 1985). Higher concentrations of biogenic amines have also been observed if wines remain in contact with yeast lees, probably due to the increased amount of peptides and free amino acids available for decarboxylation by LAB (Lonvaud-Funel, 2001).

The presence of biogenic amines in wines is currently not regulated worldwide (Olga *et al*., 1996). However, because of their potential health implications, wines with high concentrations may be rejected from some markets. This concern has led to the production of malolactic starter cultures that do not contain amino acid decarboxylase (Lonvaud-Funel, 2001).

B. ETHYL CARBAMATE FORMATION

Ethyl carbamate is a weak carcinogen found in many fermented foods and beverages including wine (Canas *et al*., 1989; Liu *et al*., 1994; Ough, 1976; Zimmerli and Schlatter, 1991). Ethyl carbamate is formed through the spontaneous reaction between ethanol and an ethyl carbamate precursor such as citrulline, urea, or carbamyl phosphate (Ough *et al*., 1988). Wine LAB can produce citrulline via arginine degradation, which may then react with ethanol present in the wine to form ethyl carbamate. Mira de Orduna *et al.* (2000) observed that commercially available wine LAB strains used for induction of MLF were capable of excreting citrulline from arginine degradation. In addition, Liu *et al.* (1994) demonstrated a good correlation between the excretion of citrulline and the formation of ethyl carbamate during the degradation of arginine by *O. oeni* and *L. buchneri.*

These studies demonstrate that wine LAB may contribute to ethyl carbamate formation. In the United States, there is a voluntary concentration limit of 15 ng/g for ethyl carbamate in table wines (Canas *et al*., 1994). One suggested way to achieve this goal is the development of non–arginine-degrading *O. oeni* strains for the induction of MLF (Mira de Orduna *et al*., 2001).

VIII. INTERACTIONS BETWEEN BACTERIA AND OTHER WINE MICROORGANISMS

A. ECOLOGY OF WINE MICROORGANISMS DURING WINEMAKING

Several genera of yeasts and bacteria are naturally present on grapes at the time of harvest or present on winery equipment (Fleet *et al*., 1984; Kunkee *et al*., 1965; Lafon-Lafourcade *et al*., 1983; Wibowo *et al*., 1985). During the course of alcoholic and malolactic fermentations, there is a successional growth of microorganisms (Figure 2) due to differing tolerances of inhibitory substances and varying growth requirements (Costello *et al*., 1983; Davis *et al*., 1986b; Fleet *et al*., 1984; Wibowo *et al*., 1985). Initially, non-*Saccharomyces* yeast grow during the early stages of alcoholic fermentation, but their viability rapidly decreases because of lack of oxygen and elevated ethanol concentrations, leaving *S. cerevisiae* as the dominant species to complete the fermentation (Fleet *et al*., 1984; Heard and Fleet, 1985; Holm Hansen *et al*., 2001; Nissen and Arneborg, 2003). Toward completion of alcoholic fermentation when *Saccharomyces* enter stationary/death phase, populations of *Oenococcus* can increase to conduct malolactic fermentation. Other bacteria such as *Acetobacter, Lactobacillus*, and *Pediococcus* can grow after MLF during the conservation or aging of wine.

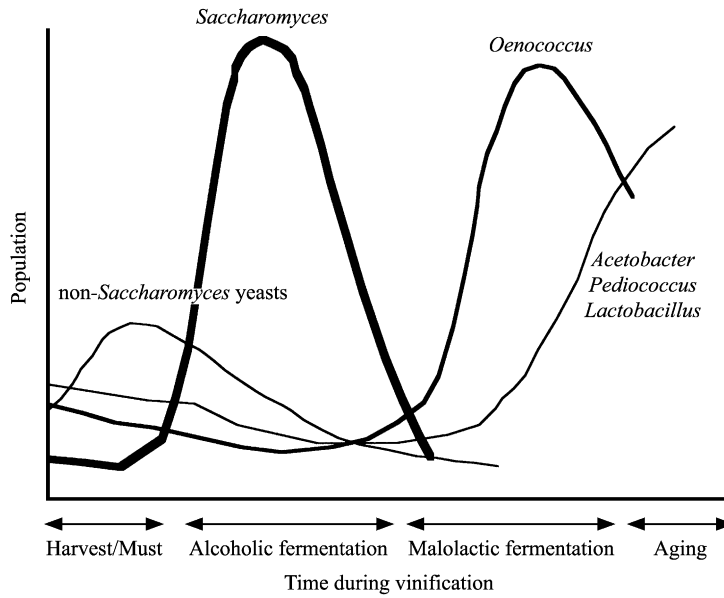

FIG. 2 General ecology of selected microorganisms during vinification.

B. INTERACTIONS BETWEEN DIFFERENT LAB SPECIES

During vinification, antagonistic interactions occur between LAB species present in wine. For example, Edwards *et al.* (1994) reported that strains of *P. parvulus* grew well in wines that had not undergone MLF, whereas the viability of all *P. parvulus* strains declined soon after inoculation into wine that had undergone MLF catalyzed by *O. oeni* (Figure 3). The authors postulated that the inhibition of *P. parvulus* may be related to the synthesis of an unidentified inhibitory substance. Inhibition of *Pediococcus* by *L. hilgardii* was noted by Rodriguez and Manca de Nadra (1995). Lonvaud-Funel and Joyeux (1993) reported that another species of *Pediococcus, P. pentosaceus*, inhibited *O. oeni* because of the accumulation of small proteolytic-sensitive thermostable compounds. Based on these results, the authors concluded that growth of certain *Pediococcus* species in wine before MLF could lead to problems inducing the secondary fermentation.

Although interactions between LAB are frequently complicated, many LAB species produce antibacterial proteinaceous substances called *bacteriocins* that have a narrow spectrum of activity against closely related species. Many excellent reviews on bacteriocins have been published (De Vuyst and

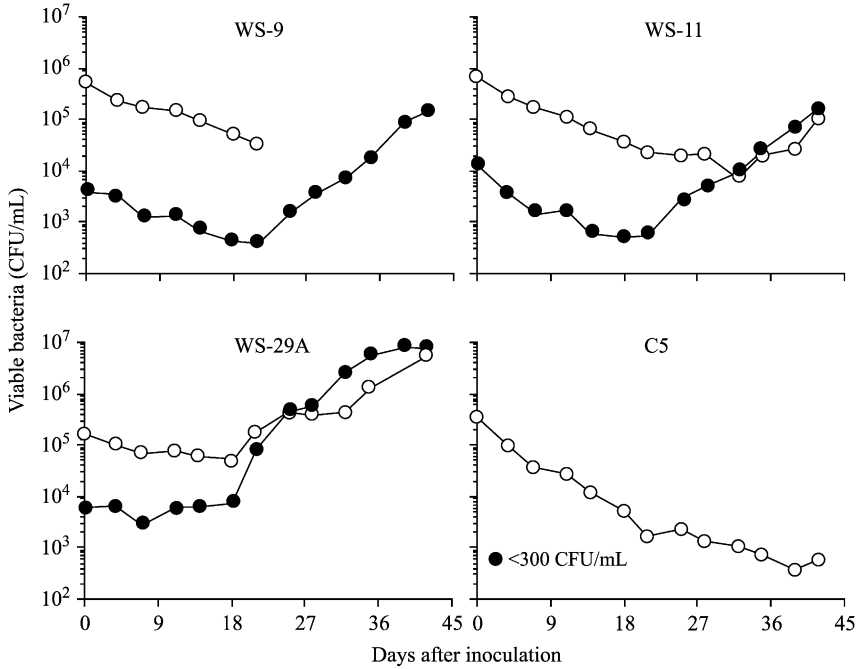

FIG. 3 Growth of *P. parvulus* WS-09, WS-11, WS-29A, and C5 inoculated into Cabernet Sauvignon MLF-negative wines (○) and MLF-positive wines (●). (Adapted from Edwards *et al.*, 1994 and with permission of the American Journal of Enology and Viticulture.)

Vandamme, 1994; Jack *et al.*, 1994; Nes *et al.*, 1996). The following discussion is restricted to bacteriocins produced by LAB present in wine.

A number of researchers have reported the production of bacteriocins by LAB species present in wine. For example, Navarro *et al.* (2000) isolated nine strains of *L. plantarum* from Rioja red wine that showed antibacterial activity, whereas Yurdugul and Bozoglu (2002) identified an isolate of *Leuconostoc mesenteroides* subspecies *cremoris* from wine that produced a bacteriocin-like inhibitory substance. Furthermore, Strasser de Saad and Manca de Nadra (1993) isolated two strains of *P. pentasaceus* that produced an inhibitory substance against strains of *Lactobacillus, Oenococcus*, and *Pediococcus.* The proteinaceous nature and narrow spectrum of activity of the inhibitory substance indicated that it was a bacteriocin.

Because of mounting consumer resistance to the excessive use of sulfur dioxide and other chemical preservatives in wine, the use of bacteriocins as preservatives has generated interest among researchers. In a study by Schoeman *et al.* (1999), bactericidal yeast strains were developed by

expressing the *pedA* pediocin gene from *P. acidilactici* in *S. cerevisiae*. The authors proposed that development of such bactericidal strains could lead to the use of *S. cerevisiae* strains capable of acting as biological control agents to inhibit the growth of spoilage bacteria. It should be noted, however, that current consumer aversion to the use of genetically modified organisms in food means the use of genetically modified wine yeast is not an option (Akada, 2002; Beringer, 2000; Boyazoglu, 2002; Dequin, 2001).

C. INTERACTIONS BETWEEN LAB AND *SACCHAROMYCES*

Winemakers have long experienced sluggish or stuck alcoholic fermentations, problems that may be attributed to insufficient nutrients to support yeast growth adequately or improper fermentation conditions (Alexandre and Charpentier, 1998; Bisson, 1999; Boulton *et al*., 1996; Groat and Ough, 1978; Ingledew and Kunkee, 1985; Kunkee, 1991; Ough, 1966; Sharf and Margalith, 1983; Tromp, 1984). In addition, the growth of unknown lactobacilli has been observed in some of these problem fermentations, leading to the speculation that uncontrolled growth of these microorganisms may also lead to sluggish fermentations (Boulton *et al*., 1996).

Evidence for the involvement of *Lactobacillus* was initially provided by Huang *et al.* (1996). Huang *et al.* (1996) observed that *Lactobacillus* spp. strain YH-15 reached a population of $>10^9$ cfu/ml two days after inoculation into a Chardonnay juice. This bacterium slowed alcoholic fermentation which eventually ceased at 5% soluble solids and was later determined to be a novel species, proposed to be *L. kunkeei* (Edwards *et al*., 1998).

Boulton *et al.* (1996) indicated that rapidly growing *Lactobacillus* species, dubbed "ferocious" lactobacilli, could produce enough acetic acid in 2–3 days to inhibit yeast metabolism. In support, *L. kunkeei* can produce between 3 and 5 g/L of acetic acid in wines undergoing stuck/sluggish fermentations (Edwards *et al*., 1999; Huang *et al*., 1996). However, Huang *et al.* (1996) noted that much lower levels of acetic acid were present in other sluggish/stuck fermentations. This indicates that acetic acid may not be the sole mechanism for the inhibition. These findings were later confirmed by Edwards *et al.* (1999), where fermentations containing *L. kunkeei* and *S. cerevisiae* were sluggish compared to the control but were not impaired with acetic acid (Figure 4). The authors concluded that acetic acid may be involved in yeast inhibition by *L. kunkeei* but that additional unidentified inhibitory mechanisms were probably involved.

The interaction between *Saccharomyces* species and *O. oeni* during the vinification process may be either stimulatory to the bacterium (Beelman *et al*., 1982; Feullat *et al*., 1985; Guilloux-Benatier *et al*., 1985; Lüthi and Vetsch, 1959) or inhibitory (Beelman *et al*., 1982; Cannon and Pilone, 1993;

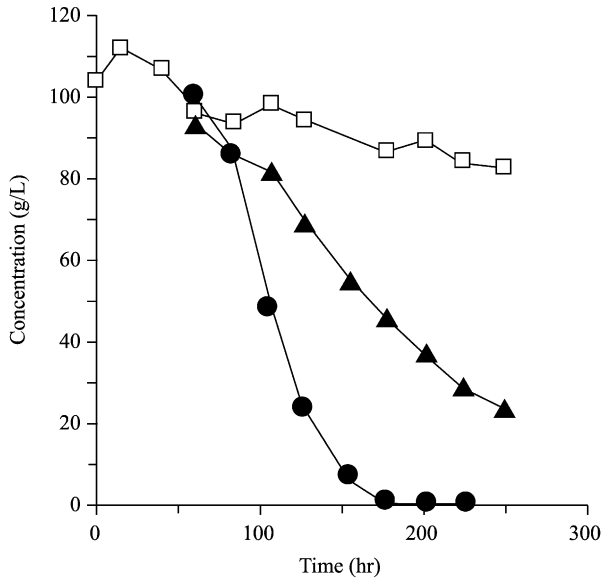

FIG. 4 Decline of glucose in a Chardonnay juice inoculated with *Saccharomyces bayanus* EC 1118 (•). *Lactobacillus kunkeei* YH-15 (□), or *S. bayarus* and *L. kunkeei* (▲). (Adapted from Edwards *et al*., 1999 and with the permission of the American Journal of Enology and Viticulture.)

Henick-Kling and Park, 1994; King and Beelman, 1986; Larsen *et al*., 2003). Inhibitory interactions have been reported in which the viability of *O. oeni* declined from 10^5 to 10^7 cfu/ml to undetectable populations soon after inoculation into wine (Beelman *et al*., 1982; Fornachon, 1968; King and Beelman, 1986; Lemaresquier, 1987; Liu and Gallander, 1983; Ribéreau-Gayon, 1985; Semon *et al*., 2001; Wibowo *et al*., 1988). This rapid decline in bacterial viability has been commonly reported, even when *Saccharomyces* and *O. oeni* are co-inoculated at similar populations (Figure 5).

Two theories have been proposed to explain this phenomenon. First, the faster growing *Saccharomyces* may remove nutrients from a grape must (Amerine and Kunkee, 1968; Beelman *et al*., 1982; Fornachon, 1968; Kunkee, 1967) because malolactic bacteria are nutritionally fastidious with complex needs (DuPlessis, 1963; Garvie, 1967b). Evidence for this was the rapid uptake of sterols, amino acids, and vitamins by yeast from grape must (Beelman, 1982; Beelman *et al*., 1982; Fornachon, 1968; King and Beelman, 1986; Lafon-Lafourcade *et al*., 1979). In support, Beelman *et al.* (1982) demonstrated that during growth in synthetic media, yeast depleted certain amino acids to concentrations that may not be sufficient for LAB growth. These authors suggest that as yeast enter stationary/death phases and lyse,

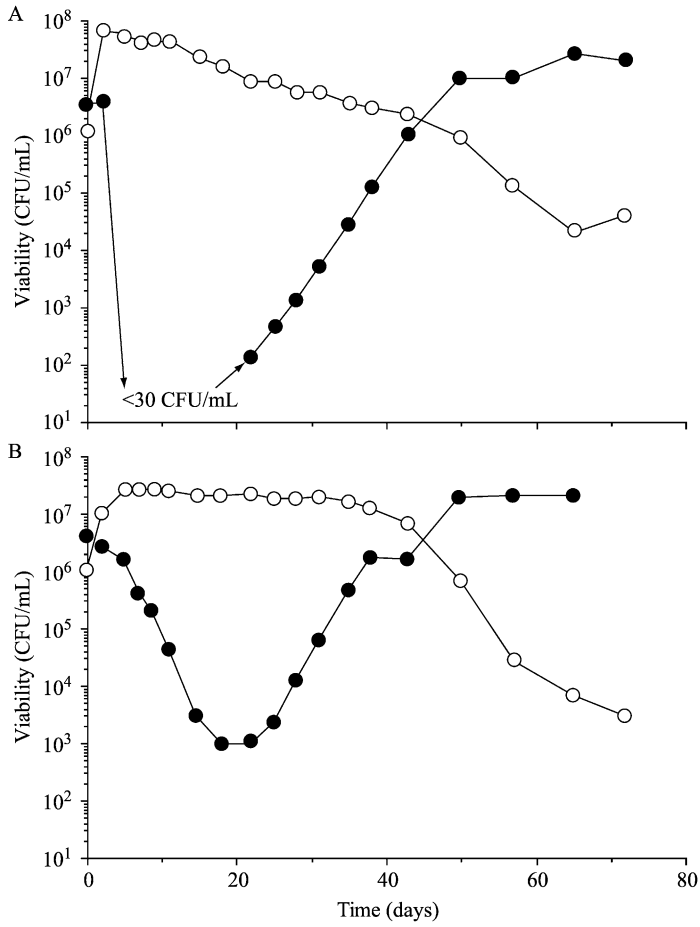

FIG. 5 Viability of *Saccharomyces cerevisiae* (○) and *Oenococcus oeni* strain EQ-54 (●) inoculated into a Chardonnay juice with the bacteria prepared using a diluted grape juice medium (A) or a lyophilized culture (B). (Adapted from Semon *et al.*, 2001 and with the permission of the Australian Journal of Grape and Wine Research.)

nutrients released into the wine would allow the recovery of *O. oeni.* Furthermore, it has been demonstrated that the autolytic activity of wine yeasts during aging on lees can affect the concentrations of amino acids, peptides, and proteins in wine (Alexandre *et al.*, 2001; Charpentier and Feuillat, 1993; Martinez-Rodriguez *et al.*, 2001).

Other studies have demonstrated that the removal of nutrients by yeast does not always explain the observed inhibition of *O. oeni*. For instance, Larsen *et al.* (2003) reported that the addition of supplemental nutrients to a

wine fermented by *S. cerevisiae* strain V1116 do not relieve the observed bacterial inhibition. Studying the impact of yeast autolysis on MLF, Patynowski *et al.* (2002) concluded that nutrient depletion by *S. cerevisiae* is not responsible for the observed bacterial inhibition. Furthermore, this research shows that the yeast produced an unidentified inhibitory factor that is progressively lost during aging. These results suggest that the second proposed theory, the production of toxic metabolites by yeast, may be responsible for the inhibition of *O. oeni*.

Saccharomyces is known to produce compounds during alcoholic fermentation that are inhibitory to *Oenococcus.* These include ethanol (Britz and Tracey, 1990; Costello *et al.*, 1983; Davis *et al.*, 1986a), SO_2 (Dott *et al.*, 1976; Eschenbruch, 1974; Eschenbruch and Bonish, 1976; Henick-Kling and Park, 1994; Romano and Suzzi, 1993; Suzzi *et al.*, 1985), medium-chain fatty acids (Capucho and San Ramao, 1994; Edwards and Beelman, 1987; Edwards *et al.*, 1990; Lonvaud-Funel *et al.*, 1988), and antibacterial proteins/peptides (Dick *et al.*, 1992). Of these compounds, SO_2 is most commonly implicated in causing bacterial inhibition (Fornachon, 1963; Henick-Kling and Park, 1994; Larsen *et al.*, 2003). SO_2 is an effective antimicrobial against wine LAB (Amerine *et al.*, 1980; Britz and Tracey, 1990; Carr *et al.*, 1976; Liu and Gallander, 1983; Ough and Crowell, 1987). When added to a must or wine, sulfur dioxide either ionizes to free SO_2 ($SO_2{\cdot}H_2O$, HSO_3^-, and/or SO_3^{2-} depending on pH level) or can become bound SO_2 by reacting with acetaldehyde, glucose, pyruvic acid, α-keto-glutaric acid, or glucose on a 1:1 molar ratio (Amerine and Ough, 1980; Burroughs and Sparks, 1973; Romano and Suzzi, 1993; Zoecklein *et al.*, 1995). Of these forms, the molecular variety of free SO_2 ($SO_2{\cdot}H_2O$) is thought to be the most antimicrobial (Edinger, 1986; King *et al.*, 1981; Macris and Markakis, 1974; Rahn and Conn, 1944; Zoecklein *et al.*, 1995).

There is conflicting information about whether some forms of bound SO_2, in particular acetaldehyde-bound SO_2, are inhibitory to wine bacteria. Early work by Fornachon (1963) reported that both *L. hilgardii* and *L. mesenteroides* were inhibited in a medium in which sulfurous acid and an excess of acetaldehyde had been added. The author determined that these bacteria could metabolize acetaldehyde-SO_2, an observation later confirmed for *O. oeni* (Osborne *et al.*, 2000). Fornachon (1963) further noted that MLF could be prevented by the presence of bound SO_2 even when the amounts of free SO_2 are negligible, possibly because of any SO_2 liberated from the metabolism of the acetaldehyde-bound SO_2. Hood (1983) provided an alternative mechanism by suggesting that any effect of bound SO_2 may be due to small amounts of free (and, therefore, molecular) SO_2 in equilibrium with the bound form. However, these results are contrary to those of Carr *et al.* (1976) who reported that acetaldehyde-bound SO_2 had no influence on the

bacterium studied (*L. plantarum*). Larsen *et al.* (2003) suggest that some forms of bound SO_2 may be more inhibitory than previously thought.

Besides being intentionally added to must/wine by winemakers, SO_2 is produced by *Saccharomyces* species during alcoholic fermentation (Dott *et al.*, 1976; Eschenbruch, 1974; Eschenbruch and Bonish, 1976; Henick-Kling and Park, 1994; Romano and Suzzi, 1993; Suzzi *et al.*, 1985). SO_2 is an intermediate produced during the assimilatory reduction of sulfate to sulfide (Amerine *et al.*, 1980; Donalies and Stahl, 2002; Eschenbruch, 1974; Rauhut, 1993; Thomas and Surdin-Kerjan, 1997). During this process, sulfate is taken up by the yeast cell via two membrane-bound permease enzymes (Rauhut, 1993) and then is reduced to sulfite via adenosine-5′-phosphosulfate and 3′-phosphadenosine-5′-phosphosulfate. Depending on needs and conditions, yeast can actively excrete sulfite via a membrane-bound sulfite pump (Avram and Bakalinsky, 1997), usually of concentrations of 10–30 mg/L, although some strains can produce amounts that exceed 100 mg/L (Eschenbruch, 1974). The presence, absence, or relative activity of genes encoding for the membrane-bound sulfite pump may explain the reported strain differences in SO_2 production by *S. cerevisiae* (Avram and Bakalinsky, 1997; Donalies and Stahl, 2002; Park and Bakalinsky, 2000).

The production of SO_2 by yeast, coupled with that added to a must/wine, has been suggested by many researchers to be the primary mechanism of bacterial inhibition during alcoholic fermentation (Fornachon, 1968; Henick-Kling, 1993; Henick-Kling and Park, 1994; Larsen *et al.*, 2003; Liu and Gallander, 1982; Lonvaud-Funel *et al.*, 1988). For instance Larsen *et al.* (2003) reported that MLF was inhibited by the high SO_2–producing yeasts *S. cerevisiae* strains V1116 and UCLM S325 (75 and 50 mg/L total SO_2, respectively), but not by a low SO_2–producing strain Saint Georges S101. Delays in entering logarithmic growth or lower peak populations of *O. oeni* were also noted in wines fermented by Zymafluor VL1, CKS 102, EC1118, and BKS 104 (Figure 6), in which total SO_2 produced ranged between 15 and 33 mg/L. However, the concentration of total SO_2 did not always correspond to the extent of bacterial growth. For example, *O. oeni* grew poorer in wines fermented by strain CKS (18 mg/L total SO_2) than it did in wines fermented by strains EC1118 and Zymafluor VL1, which contained equal or greater amounts of total SO_2. Larsen *et al.* (2003) concluded that although in some cases high SO_2–producing strains cause inhibition of MLF, other yeast strains inhibit MLF by means besides production of SO_2. In agreement, additional studies have also cast doubt over whether SO_2 produced by yeast is the sole mechanism for bacterial inhibition (Caridi and Corte, 1997; Eglinton and Henschke, 1996; King and Beelman, 1986; Lemaresquier, 1987; Wibowo *et al.*, 1988). For instance, Wibowo *et al.* (1988) found that *S. cerevisiae* inhibited the growth of *O. oeni* in wine, but that inhibition was not due to

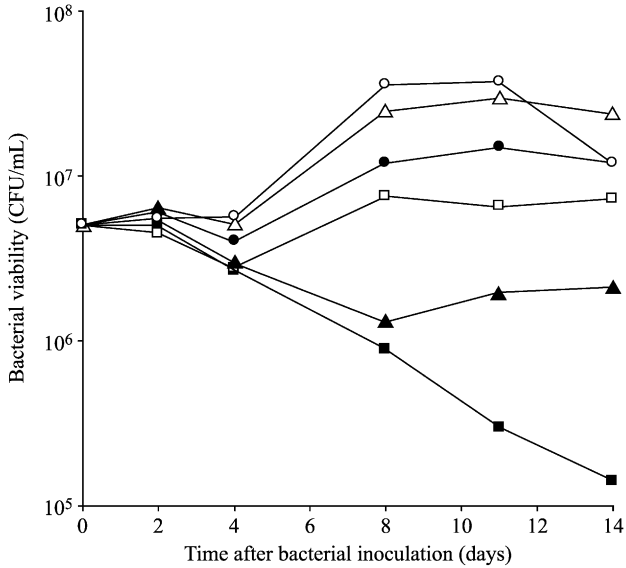

FIG. 6 Growth of *Oenococcus oeni* in Chardonnay wines prepared using yeast strains UCLM S325 (■, 50 mg/L total SO_2), Zymafluor VL1 (□, 33 mg/L total SO_2), CKS 102 (▲, 18 mg/L total SO_2), EC1118 (X[CN4], 18 mg/L total SO_2), BKS 104 (●, 15 mg/L total SO_2), or Saint Georges S101 (○, 0 mg/L total SO_2). Concentrations of total SO_2 in these wines was determined just before bacterial inoculation. (Adapted from Larsen *et al*., 2003 and with permission from the American Journal of Enology and Viticulture.)

the production of SO_2. Additionally, Eglinton and Henschke (1996) reported that production of SO_2 by S. *cerevisiae* strain AWRI 838 and related strains did not account for wines that resisted malolactic fermentation.

Wibowo *et al.* (1988) proposed that *S. cerevisiae* may inhibit *O. oeni* through the production of antibacterial proteins/peptides and not through the production of SO_2 and ethanol. In support, Dick *et al.* (1992) isolated two proteins produced by *S. cerevisiae*, which showed activity against *O. oeni.* Despite these findings, very few further studies investigating the production of antibacterial proteins/peptides by wine yeast have been undertaken.

In addition to SO_2 and antibacterial proteins/peptides, medium-chain fatty acids produced by yeast during alcoholic fermentation have also been implicated in the inhibition of malolactic bacteria (Carrete *et al*., 2002; Edwards and Beelman, 1987; Lonvaud-Funel *et al*., 1985). Inhibition of *Saccharomyces* species and some LAB by medium-chain fatty acids has been reported in grape juice and silage (Pederson *et al*., 1961; Woolford, 1975). Although this hypothesis has not been conclusively shown, Lonvaud-Funel *et al.* (1985) and

Edwards and Beelman (1987) have reported decanoic acid to be inhibitory to the growth of malolactic bacteria. Edwards and Beelman (1987) noted that decanoic acid suppressed the growth of *O. oeni* PSU-1 at a concentration of 10 mg/L, a concentration reported to be present in some wines (Houtman *et al.*, 1986). In addition, Carrete *et al.* (2002) reported that decanoic acid acted synergistically with either low pH level or ethanol to inhibit *O. oeni* ATPase, the activity of which has been linked to malolactic activity in *O. oeni* (Cox and Henick-Kling, 1995; Tourdot-Maréchal *et al.*, 1999). However, Edwards *et al.* (1990) found that MLF occurred more rapidly in wines containing 5 mg/L decanoic acid and other medium-chain fatty acids than in wines with lower levels.

IX. SUMMARY AND CONCLUSIONS

Bacteria such as *Acetobacter* and *Gluconobacter, Lactobacillus, Oenococcus*, and *Pediococcus* play important roles in determining the final quality of a wine. Several of these microorganisms can decrease wine quality. For instance, *Acetobacter* and *Gluconobacter* can cause spoilage through the production of excessive acetic acid and ethyl acetate, whereas *Lactobacillus* species may cause spoilage through increases in VA or formation of other adverse odors or flavors. *Pediococcus* species may spoil wine through the production of off-flavors such as acetoin and diacetyl and through the formation of polysaccharides. Some species of LAB can also produce biogenic amines and can contribute to ethyl carbamate formation in wine. However, not all LAB are involved in spoilage. For instance, the growth of *O. oeni* can be desirable because this species is used for MLF, a process that decreases wine acidity and contributes desirable flavors and aromas to wine.

Microbial interactions that occur in wine may be beneficial or detrimental to wine quality depending on the species involved. Examples of detrimental interactions are the inhibition of *S. cerevisiae* by *Lactobacillus* species and the inhibition of *O. oeni* by *S. cerevisiae* when MLF is desired. However, the inhibition of *O. oeni* may also be beneficial to wine quality if MLF is undesirable. Additional beneficial interactions include the stimulation of LAB growth due to yeast lysis and the inhibition of *Pediococcus* species by *O. oeni.* A better understanding of the complex interactions between LAB and *S. cerevisiae* will lead to the selection of compatible yeast and bacterial strains for the induction of alcoholic and malolactic fermentations.

In conclusion, knowledge of the bacteria involved in winemaking will allow the winemaker to minimize spoilage problems caused by AAB, lactobacilli, and pediococci and to promote or prevent MLF in a wine. Future research should include investigating the contribution of malolactic bacteria

to wine flavor, the impact of *Pediococcus* species on the sensory qualities of wine, and the mechanisms involved in the inhibition of *Saccharomyces* by *Lactobacillus* species and the inhibition of malolactic fermentation by wine yeast.

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BIOSYNTHESIS OF CONJUGATED LINOLEIC ACID IN RUMINANTS AND HUMANS

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I. INTRODUCTION

A. BACKGROUND

Nutritional quality is becoming a major issue in food choices because of growing consumer awareness of the link between diet and health. As a consequence, there is increasing consumer acceptability of the concept of "functional foods," a generic term used to describe foods or food components that have beneficial effects on human health above that expected on the basis of nutritive value (Milner, 1999). In other words, functional foods must have a relevant effect on well-being and health or cause a reduction of disease risk (Roberfroid, 1999). One of these functional food components is conjugated linoleic acid, a fatty acid found in milk fat and ruminant meat. The term *conjugated linoleic acid* (CLA) refers to a mixture of positional and geometric isomers of linoleic acid (*cis*-9, *cis*-12 octadecadienoic acid) with a conjugated double-bond system. Numerous isomers of CLA have been identified in food products, and these differ by position (e.g., 7–9, 8–10, 9–11, 10–12) or geometric orientation (cis-trans, trans-cis, cis-cis, and trans-trans) of the double-bond pair. The predominant source of CLA in human diets is ruminant-derived food products. In the United States, dairy products provide about 70% of the intake, and beef products account for another 25% (Ritzenthaler *et al.*, 2001) (Figure 1), and similar values for the contribution of different food classes are found for other countries (Parodi, 2003). The presence of CLA in ruminant milk has been known for more than 60 years. Scientists at the University of Reading, United Kingdom, first demonstrated that fatty acids obtained from summer butter differed from those obtained from winter butter by exhibiting a much stronger spectrophotometric adsorption at 230 μm (Booth *et al.*, 1933). Subsequently, Moore (1939) concluded that the adsorption at 230 μm was due to two conjugated double bonds. Parodi (1977) first identified *cis*-9, *trans*-11 octadecadienoic acid as the predominant CLA isomer in milk fat, and its structure is compared with linoleic acid in Figure 2. Although many isomers of CLA occur in ruminant fat, *cis*-9, *trans*-11 CLA accounts for about 75–90% of the total. The second most common isomer is *trans*-7, *cis*-9 CLA, representing about 10% of the total. *Trans*-7, *cis*-9 CLA co-elutes with the *cis*-9, *trans*-11 isomer on most gas liquid chromatograms, and identity of this isomer went unrecognized until it was isolated by Yurawecz *et al.* (1998) using combinations of silver nitrate high-performance liquid chromatography (HPLC), gas liquid chromatography, mass spectrometry, and Fourier transform infrared spectroscopy. The remainder of the CLA is composed of other trans-trans, trans-cis, cis-trans, or cis-cis forms, with each isomer typically representing a small portion (<1%) of the total (Bauman *et al.*, 2003; Parodi, 2003) (Table I). The

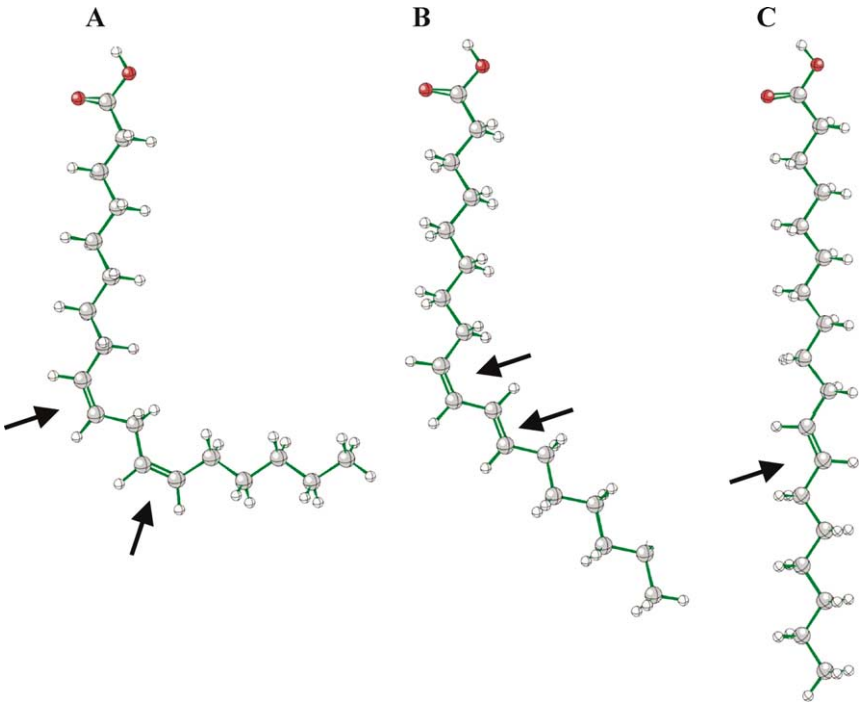

FIG. 1 Chemical structures of linoleic acid (*cis*-9, *cis*-12 18:2) (A), *cis*-9, *trans*-11 CLA (B), and *trans*-11 18:1 (C). Arrows indicate location of double bonds. (Adapted from Bauman *et al.*, 2004.)

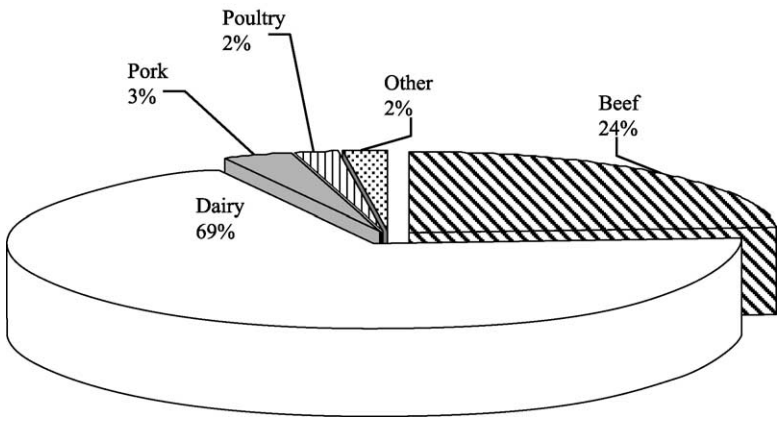

FIG. 2 Distribution of CLA sources in the U.S. diet. (Calculated from data by Ritzenthaler *et al.*, 2001.)

TABLE I
BENEFICIAL HEALTH EFFECTS OF CLA REPORTED FROM BIOMEDICAL STUDIES WITH ANIMAL MODELS

Biological effect
Anticarcinogenic (*in vivo* and *in vitro* studies)
Antiatherogenic
Altered nutrient partitioning and lipid metabolism
Antidiabetic (type II diabetes)
Immunity enhancement
Improved bone mineralization

trivial name "rumenic acid" (RA) has been proposed for *cis*-9, *trans*-11 CLA (Kramer *et al.*, 1998) and is used when discussing this isomer. A summary of all known fatty acids in milk was published by Jensen (2002).

The plethora of research related to CLA over the past 20 years was prompted by the identification of CLA as an anti-mutagen present in cooked beef (Ha *et al.*, 1987; Pariza *et al.*, 1979). Since these original findings, a number of potential health benefits of CLA have been reported (Table I). Health effects of CLA were established initially in biomedical studies of cancer, and subsequent investigations with animal models and *in vitro* cell cultures demonstrated that CLA was anticarcinogenic for many types of cancer (Belury, 2002; Parodi, 2002; Whigham *et al.*, 2000). Both RA and *trans*-10, *cis*-12 CLA are effective in reducing the formation of premalignant lesions in the rat mammary gland 6 weeks after carcinogen administration (Ip *et al.*, 2002). This is the reason there is enormous interest in the "functional food" properties of food products containing RA. Particularly noteworthy is that RA is a potent anticarcinogen when supplied in a natural form (esterified triglyceride) as a natural food component. Dietary consumption of RA-enriched butter was effective in reducing the incidence of tumors in a rat model of mammary carcinogenesis (Ip *et al.*, 1999). Though not as extensively investigated, other studies have demonstrated beneficial effects of CLA on atherosclerosis and related variables in a number of animal models (Kritchevsky, 2003). Using pure isomers, RA and *trans*-10, *cis*-12 CLA were shown to be equally effective in reducing cholesterol-induced atherogenesis in rabbits (Kritchevsky, 2003). Further, CLA has other beneficial health effects in studies with animal models. These include reducing the onset and severity of diabetes and obesity, immune modulation, and altering the rate of bone formation (Table I). Although research in these areas is limited compared with the effects of CLA on cancer, they merit consideration and further research. For in-depth reviews of these, see Pariza (1999), Whigham *et al.* (2000), Belury (2002), and Parodi (2002).

Because the presence of CLA in the human diet is reliant on ruminant products, this chapter first addresses the synthesis of CLA in ruminants. The presence of CLA in ruminant milk and meat is related to rumen fermentation and its synthesis by microorganisms through the process of biohydrogenation (BH) of dietary unsaturated fatty acids. Thus, the effect of diet and processes within the rumen is reviewed. The role of endogenous synthesis of CLA in mammalian tissues has been discovered, and this will be discussed also first as it contributes to the occurrence of CLA in ruminant products and second the significance of endogenous synthesis as a source of CLA in humans and other species.

B. THE RUMINANT DIET

Ruminant diets are extremely varied, depending on species and productive function. Diets range from all forage of variable quality (pasture, corn silage, grass or legume hay, or mixed grass and legume hay, and hay silage) to combinations of forage, cereals, and protein supplements. Many byproducts of the food industry, also highly variable in quality, may be included. A general characteristic is that ruminant diets are high in fiber (generally >30% cell wall constituents) and low in lipid content. Fatty acid content of grass and legume forages is generally lower than 5% of the dry matter and consists of a high proportion (>50%) of α-linolenic acid (all *cis* 18:3n-3). Corn silage and cereal grains contain similar quantities of fatty acid, of which linoleic acid (*cis, cis* 18:2 n-6) predominates. Diets may contain up to 5% of dry matter from supplemental fats, varying from highly saturated fats such as tallow, oilseeds, such as canola, cottonseed, and soybeans, and byproducts of vegetable oil processing. A detailed description of dietary fatty acids in ruminant diets is provided by Viviani (1970), and in more condensed form, by Palmquist (1988) and Harfoot and Hazlewood (1997); the latter also includes a modern summary of fatty acids found in ruminal organisms and their metabolic end products. Even though ruminant diets contain predominantly unsaturated fatty acids, ruminant meat and milk contain much higher levels of saturated fatty acids due to extensive BH of dietary unsaturated fatty acids in the rumen.

C. THE RUMEN MICROBES

The general consensus from numerous *in vitro* studies is that rumen bacteria are primarily responsible for BH (Harfoot and Hazlewood, 1997). Even though the rumen contains up to 10^{11} viable cells/ml and a diverse range of bacteria (Hungate, 1966), relatively few bacterial species capable of BH

have been identified, and the relative importance of individual strains to ruminal lipid metabolism *in vivo* remains largely unclear. Of those species shown to be capable of BH, *Butyrivibrio fibrisolvens* is the most extensively studied, but the metabolic activity of other species including *Ruminococcus, Eubacterium,* and *Fusocillus* strains also have been characterized (Harfoot and Hazlewood, 1997). The role of BH in ruminal metabolism has been a subject of debate, and early studies promoted the suggestion that the biological importance of BH was associated with providing fatty acid substrates for incorporation into bacterial membranes or was an essential mechanism for removing reducing equivalents (Harfoot and Hazlewood, 1997). However, most of the experiments to date are consistent with the view that the principle role of BH is to reduce the toxic effects of unsaturated fatty acids on bacterial growth (Kemp and Lander, 1984; Kemp *et al.*, 1984b). The dynamics of bacterial growth, substrate supply, and ruminal turnover were described in detail by Hungate (Chapter V, 1966) and succinctly summarized by Viviani (1970) as follows: "At an outflow of rumen contents of 6–8% hr^{-1} the mean generation time must be 12 hours if organism numbers are to be maintained. However, many are able to divide two or three times per hour; therefore, the rate of growth is far less than maximal, causing them to be in a maintenance, rather than a growing state. Thus, linolenic acid was BH to octadecanoic acid when incubated with *B. fibrisolvens* for 21 hours, whereas little BH occurred when incubation was limited to 2 hours." Significance of these observations is seen in the later work of Kim *et al.* (2000) (see Section II.C).

There is little evidence indicating that rumen protozoa are capable or involved in BH (Harfoot and Hazlewood, 1997; Williams and Coleman, 1988), and it is arguable that protozoa could satisfy their lipid requirements through the ingestion of rumen bacteria, chloroplasts, and other plant lipids.

II. RUMINAL SYNTHESIS OF CLA

A. LIPOLYSIS

Before BH of fatty acids can take place, plant lipids must become free of surrounding matrix by mastication and microbial digestive processes, followed by lipolysis of ester linkages. The lipolytic step is believed to be rate limiting for BH (Harfoot and Hazlewood, 1997). Dawson and Hemington (1974) described the digestion of grass lipids and pigments. Monogalactosyl and digalactosyl diacylglycerides are released rapidly as chloroplasts are ruptured by mastication. Deacylation was linear and more rapid for monoacylglycerides than diacylglycerides in the rumen content. Products were

fatty acids and monogalactosyl and digalactosyl glycerol; no free acylglycerol was found; conversely, monoacylglycerol and diacylglycerol were found as intermediates of lipolysis when triacylglycerol (as peanut oil) was added to the medium (Clarke and Hawke, 1970). The latter authors reported that fatty acid was released linearly in strained rumen fluid up to concentrations of 1 mg of triacylglycerol/ml. Using much higher substrate concentrations (20–100 mg/ml), Beam *et al.* (2000) also reported that rate of lipolysis *in vitro* decreased at high substrate concentrations, with lag times before initiation of lipolysis ranging from 1.3 to 2.5 hours. Using ^{14}C-labeled substrate at 0.5 mg/ml, Hawke and Silcock (1970) observed that lipolysis in strained rumen fluid conforms to first-order kinetics, with a rate constant of 0.0267 min^{-1} and a 32-minute lag. They concluded "that under normal conditions lipolysis of ingested lipids would be sufficiently rapid in the rumen to allow the full biohydrogenating capacity of the rumen microorganisms to be realized." However, differences in BH products of esterified and nonesterified linoleic acid have been shown (Moore *et al.*, 1969; Noble *et al.*, 1974). Both *in vitro* and *in vivo* presentation of free linoleic acid resulted in accumulation of vaccenic acid (VA), whereas stearic acid was the main product after infusion of corn oil (see later discussion). These observations suggest that the rate of lipolysis limits the availability of free linoleic acid in the rumen, resulting in complete BH when unsaturated fatty acids are provided in the glyceride form. However, an excessive supply of unsaturated oil in ruminant diets has negative effects on ruminal metabolism and BH (Jenkins, 1993). Several factors of the ruminal environment modify the rates of ruminal lipolysis and BH (Gerson *et al.*, 1983, 1985, 1986). These studies show that increasing dietary contents of nitrogen and fiber increased the rate of lipolysis and that lipolytic rate was decreased with increases in forage maturity. Low ruminal pH levels decrease lipolysis (Latham *et al.*, 1972); very little lipolysis occurs at pH levels of less than 6.0, whereas BH was inhibited only partially at a pH level of 5.2 (van Nevel and Demeyer, 1996).

The rate of lipolysis is related to melting point; seed oils (linseed, soya) > palm oil = tallow. Fish oils are hydrolyzed more slowly, probably caused by steric hindrance of the ester bonds (Miller and Cramer, 1969; Palmquist and Kinsey, 1994). Hydrogenated tallow (iodine number < 30) lipolysis rate was slow (Palmquist and Kinsey, 1994) or absent (Beam *et al.*, 2000), likely caused by insolubility of the substrate.

B. ISOMERIZATION

Biosynthesis of CLA by rumen bacteria arises from isomerization of polyunsaturated dietary C18 fatty acids; CLA and other ethenoic isomers occur as intermediates in the pathways of ruminal BH, whereby the unsaturated

dietary fatty acids are metabolized to stearic acid as the primary end product (Harfoot and Hazlewood, 1997). The process of BH is associated with the activity of bacteria adhering to rumen particulate matter (Gerson *et al.*, 1988; Harfoot *et al.*, 1973). Conditions required for BH to take place were described by Kepler *et al.* (1970); namely a free carboxyl group, an all-*cis* Δ-9 pentadiene system and a chain length of 18 C atoms. All methylene-interrupted *cis, cis* octadecadienoic fatty acids were examined as substrates for BH by Garcia *et al.* (1976) and by Kemp *et al.* (1984b). Although BH of all the unsaturated fatty acids occurred, conjugation of the double bonds before BH was required only for fatty acids with pentadiene structure Δ-2, 5 and Δ-9,12. Kepler and Tove (1967) purified and characterized the membrane-bound enzyme linoleate Δ-12 *cis*, Δ-11 *trans* isomerase, which catalyzes the first step in the BH of linoleic acid, from *B. fibrisolvens.* The product of this enzyme is Δ-9 *cis*, Δ-11 *trans*-octadecadienoic acid (RA), the predominant CLA isomer found in ruminant foods.

Other CLA isomers have been identified in lesser amounts in ruminant foods. Amounts and relative proportions of individual CLA isomers may be altered in response to changes in the animal's diet; for example, the *trans*-10, *cis*-12 isomer is increased by inclusion of unsaturated fatty acids in the diet and a low ruminal pH (Bauman and Griinari, 2001; Piperova *et al.*, 2002). *Trans*-10, *cis*-12 CLA is quantitatively a minor BH intermediate (Duckett *et al.*, 2002; Lock and Garnsworthy, 2002; Shingfield *et al.*, 2003) but is of major interest because of its physiological effects on fat metabolism (Baumgard *et al.*, 2000; Peterson *et al.*, 2003a). The origin of *trans*-10, *cis*-12 18:2 was unknown until a report by Kim *et al.* (2002) showed its synthesis by *Megasphaera elsdenii* strain YJ-4. *M. elsdenii* is a lactate fermenter that thrives in the rumen of animals fed high-grain diets, conditions that are known to be favorable for low milk fat syndrome in cattle (Bauman and Griinari, 2003). The characteristics of the enzyme are similar to those described for linoleate Δ-12 *cis*, Δ-11 *trans* isomerase (Kim *et al.*, 2000) in *B. fibrisolvens,* the exception being that *trans*-10, *cis*-12 CLA is the product, rather than RA. *M. elsdenii* strain YJ-4 was not found in the rumen of all animals fed high-grain diets; indeed, other strains of *M. elsdenii* often predominated, explaining the phenomenon that not all cattle are susceptible to induction of the low milk fat syndrome. In addition to the likelihood of low amounts synthesized, *trans*-10, *cis*-12 CLA is a substrate for the reductase of *B. fibrisolvens* and was BH to *trans*-10 18:1 at one-third the rate of conversion of RA to *trans*-11 18:1 (Kepler *et al.*, 1966).

Ruminal synthesis of *trans, trans* CLA isomers with double bonds in positions 9, 11 and 10, 12 also are enhanced when diets contain high amounts of concentrates (Piperova *et al.*, 2002) or are supplemented with fish oil (Shingfield *et al.*, 2003). Formation of *trans, trans* CLA is unexpected

based on established BH pathways (Harfoot and Hazlewood, 1997). The identification of *trans, trans* CLA isomers in omasal (Shingfield *et al.*, 2003) or duodenal (Piperova *et al.*, 2002) digesta of dairy cows raise some intriguing questions with regard to CLA synthesis in the rumen. Possibly linoleate Δ-12 *cis*, Δ-11 *trans* isomerase is less specific than previously thought, with ability to catalyze the formation of *trans*-9, *trans*-11 and *trans*-10, *trans*-12 CLA from linoleic acid. An alternative explanation would be that rumen bacteria express several linoleate isomerases that have not been identified and characterized.

Characterizing metabolic pathways of BH in ruminal microorganisms is extremely difficult, for several reasons. First, the organisms are strict anaerobes, which require extreme methodologies for their culture. Second, as is discussed later in this chapter, complete BH occurs in very few organisms capable of BH, thus requiring multiple species to carry out the process. Third, very few of the isolates that have been screened have BH capability; for example, Kemp *et al.* (1975) screened more than 200 isolates, 30 showed limited BH activity, and of these, only five had sufficient activity to warrant further work. Finally, many of the original well-characterized BH strains have been lost (van de Vossenburg and Joblin, 2003), thereby preventing further pursuit of their characteristics.

C. THE BIOHYDROGENATION PROCESS

Numerous *in vitro* and *in vivo* studies have elucidated the major pathways of ruminal BH (Figures 3 and 4) (Harfoot and Hazlewood, 1997). Of significance for this discussion is that the bacteria (Group A) responsible for isomerization to the conjugated diene and hydrogenation of the *cis*-Δ-9 bond are thought to be unable to complete the BH of the *trans*-11 18:1 intermediate. The reduction of *trans*- and *cis*-octadecenoic acids is thought to occur in separate organisms, collectively known as the group B bacteria (Harfoot and Hazlewood, 1997). Thus, complete BH of unsaturated fatty acids requires a balance between the two groups A and B. Reduction of *trans*-octadecenoic acids to stearic acid is believed to be the rate-limiting step, so these fatty acids can accumulate in the rumen (Keeney, 1970). However, rates of BH are higher for *trans*-octadecenoic acids with double bonds in positions Δ8–Δ10 than for those with bonds at Δ5–Δ7 or Δ11–Δ13 (Kemp *et al.*, 1984a).

The reduction of RA to VA is catalyzed by *cis*-9, *trans*-11 octadecadienoic acid reductase. Isolation of this membrane-bound enzyme from *B. fibrisolvens* indicates that it has an absolute requirement for iron and expresses maximal activity at a pH between 7.2 and 8.2 (Hughes *et al.*, 1982). Pure culture studies with *B. fibrisolvens* also have shown that the reductase is not

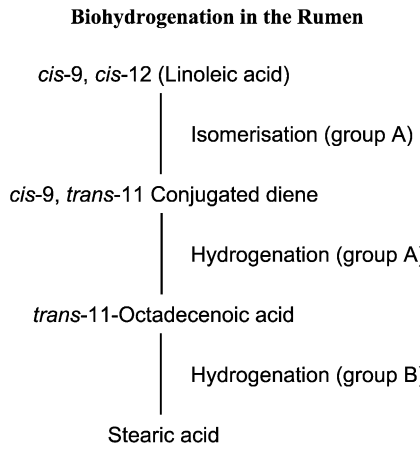

FIG. 3 Scheme for the biohydrogenation of linoleic acid; group A and group B refer to the two classes of biohydrogenating bacteria. (Used, with permission, from Harfoot and Hazlewood, 1997.)

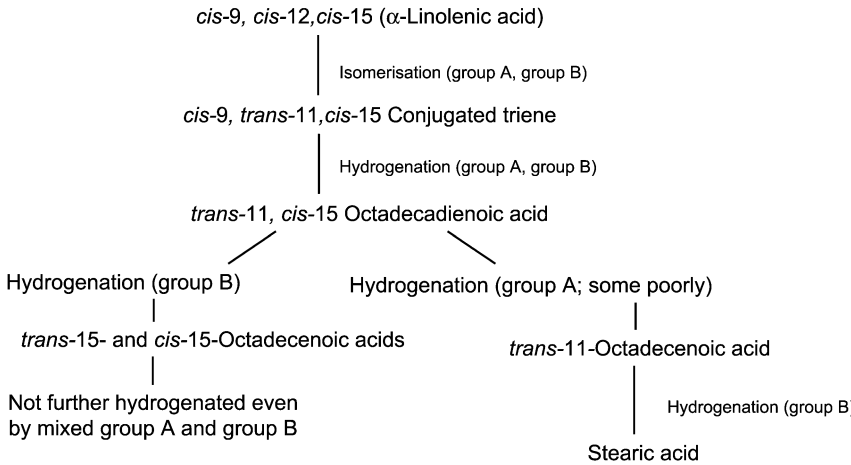

FIG. 4 Scheme for the biohydrogenation of α-linolenic acid; group A and group B refer to the two classes of biohydrogenation bacteria. (Used, with permission, from Harfoot and Hazlewood, 1997.)

highly specific and can convert *trans*-10, *cis*-12 CLA to *trans*-10 18:1 (Kepler *et al.*, 1966).

It has long been known (Noble *et al.*, 1969, 1974; Polan *et al.*, 1964) that an excess amount of free linoleic acid inhibits the final BH of VA to stearic

acid. Thus, excess free linoleic acid in the rumen environment leads to accumulation of VA. Research has shown that the highly unsaturated long-chain fatty acids of fish oil are even more effective in causing the accumulation of VA and other trans monoenoic acids (Shingfield *et al.*, 2003). These studies confirm the report of Kemp and Lander (1984), showing that stearic acid was the predominant end-product when cultures of groups A and B in late exponential growth phase were combined, but VA accumulated when small amounts of inocula were used; it was presumed that group A organisms overgrew those of group B. Presumably, group B bacteria are more sensitive to the toxic effects of long-chain polyunsaturated fatty acids (PUFAs) than group A bacteria, and therefore, VA and other trans monoenes accumulate when PUFA concentrations are increased.

Kim *et al.* (2000) brought new insight to the effects of linoleic acid concentration on RA synthesis by *B. fibrisolvens.* Growing cultures tolerated only low concentrations of linoleic acid; these cultures did not produce significant amounts of CLA until linoleic acid concentration was high enough to inhibit BH (reduction of RA to VA), upon which growth was inhibited and cell lysis commenced. Isomerization of linoleic acid was very rapid, but the isomerase did not recycle as do most enzymes in order to catalyze more substrate; CLA accumulation was proportional to cell concentration. The isomerase and reductase enzymes were linked and free CLA was not released as an intermediate. Because CLA was found to be as toxic as linoleic acid, there was no survival advantage for the organisms to release CLA. The authors concluded that CLA found in the medium (ruminal or intestinal contents) may be due to linoleic acid–dependent bacterial inactivation, cell death, or lysis. Under conditions of less than toxic amounts of linoleic acid (or other inhibitory PUFAs), the product of BH is released as VA. The organism was found to tolerate higher concentrations of linoleic acid after adaption; this could explain the common observation of high initial concentrations of CLA in milk when unsaturated fats are fed, decreasing as time on diet increases (Bauman *et al.*, 2000).

The initial step in BH of α-linolenic acid is similar to that for linoleic acid, resulting in formation of a conjugated diene, with an additional isolated double bond (Figure 4). However, some isolates of group B bacteria, as well as group A bacteria, are able to isomerize linolenic acid (Harfoot and Hazlewood, 1997). The subsequent hydrogenation of the *cis*-Δ-9 bond yields *trans*-11, *cis*-15 18:2. Further hydrogenation by group A organisms yields VA, whereas hydrogenation by group B organisms results in the unique products of either *trans*-15 or *cis*-15 monoenes. The latter are not hydrogenated further and thus are true end-products (Harfoot and Hazlewood, 1997).

Other studies have shown that a strain of *Butyrivibrio hungatei* isolated from a cow grazing ryegrass-clover swards is capable of metabolizing both

linoleic and linolenic acids to stearic acid (van de Vossenberg and Joblin, 2003). Even though this bacterium produced transient intermediates according to established pathways, it is clear that in some cases complete BH of PUFAs may not necessarily require the involvement of two groups of complementary bacteria, as was previously thought (Kemp and Lander, 1984).

In addition to linoleic and α-linolenic acids, the BH of γ-linolenic acid (all *cis* 6, 9, 12 18:3) has been investigated using pure strains of *Butyrivibrio* S2 and *Fusocillus babrahamensis in vitro* (Kemp and Lander, 1983). The group B bacterium (*Fusocillus*) was able to metabolize γ-linolenic acid completely to stearic acid, whereas the group A bacterium (*Butyrivibrio*) produced *cis*-6, *trans*-11 octadecadienoic acid. It has been suggested that the BH of γ-linolenic acid is analogous to that of α-linolenic acid and proceeds via isomerization to a conjugate (*cis*-6, *cis*-9, *trans*-11 18:3) that is sequentially reduced to *cis*-6, *trans*-11 18:2, vaccenic acid, and stearic acid (Figure 5). Even though most conventional ruminant feeds do not contain γ-linolenic acid, this fatty acid is present in certain oilseeds including evening primrose and borage, but the effects of these lipids on ruminal BH *in vivo* or CLA concentrations in milk and meat have not been investigated.

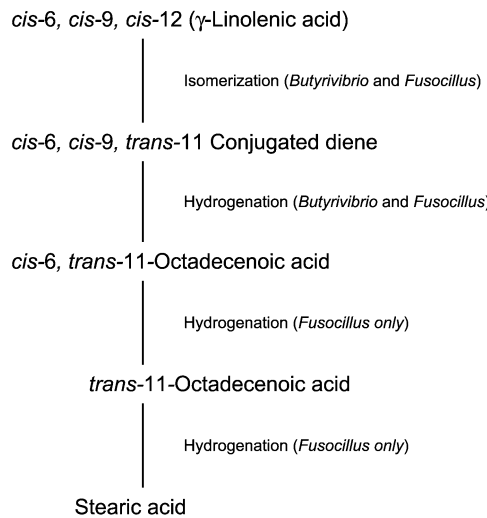

FIG. 5 Scheme for the biohydrogenation of γ-linolenic acid; the *Butyrivibrio* used in the study was a group A biohydrogenator, and the *Fusocillus* was a group B biohydrogenator (Kemp and Lander, 1983). Compare Figure 4 for biohydrogenation of α-linolenic acid. (Used, with permission, from Harfoot and Hazlewood, 1997.)

The effects of pH and amounts of linoleic and linolenic acids on extent of BH *in vitro* and proportions of BH intermediates, including monoenoic and dienoic isomers, were examined by Troegeler-Meynadier *et al.* (2003). When linoleic acid concentration was held constant, its disappearance declined when mean pH was less than 6.0, compared with a pH level of 6.5. Increasing concentration of linolenic acid decreased linoleic acid disappearance, suggesting an inhibition of isomerization. Lower pH levels decreased the ratio of *trans*-10:*trans*-11 18:1 intermediates. When linoleic acid concentration was increased, the proportion of linoleic acid disappearing declined, but the amount disappearing increased, without changing the *trans*-10:*trans*-11 ratio, suggesting a maximum capacity of isomerization rather than inhibition. Increasing initial concentrations of linoleic acid resulted in high amounts of VA and increasing stearic acid with time, suggesting a maximal capacity for the second reductive step of BH. High concentrations of linolenic acid did not affect amounts of RA and *trans*-18:1 formed or the *trans*-10:*trans*-11 ratio. Authors concluded that a ruminal pH near neutrality with high concentrations of linoleic acid should support maximal synthesis of VA and RA.

D. RUMINAL SYNTHESIS OF CLA

Relatively few measurements of ruminal CLA synthesis *in vivo* are available. Experiments across a range of diets have been conducted with sheep (Kucuk *et al.*, 2001), beef cattle (Duckett *et al.*, 2002, Lee *et al.*, 2003; Sackmann *et al.*, 2003), non-lactating cows (Lock and Garnsworthy, 2002), and lactating dairy cows (Piperova *et al.*, 2002; Shingfield *et al.*, 2003). Most studies indicate that RA is, in most cases, the most important CLA isomer formed in the rumen, but the amounts produced are relatively small compared with *trans*-18:1 BH intermediates. Flows of RA in the duodenum of sheep have been reported to vary between 0.12 and 0.20 g/day (Kucuk *et al.*, 2001) depending on the amount of forage in the diet. Comparison of RA synthesis in steers (0.1–2.7 g/day; Duckett *et al.*, 2002; Lee *et al.*, 2003; Sackmann *et al.*, 2003), non-lactating cows (0.3–0.5 g/day; Lock and Garnsworthy, 2002), and lactating cows (0.2–1.7 g/day; Piperova *et al.*, 2002; Shingfield *et al.*, 2003) indicate that diet type rather than level of intake is the major determinant of the amounts of RA synthesized in the rumen. Even though published reports indicate limited formation in the rumen, flows of RA entering the omasal canal can be as much as 9.9 g/day in cows fed grass silage–based diets supplemented with 750 g/day of sunflower oil (Shingfield *et al.*, unpublished observations).

RA is not the only CLA isomer formed in the rumen, and analysis of digesta by gas chromatography (Duckett *et al.*, 2002; Sackmann *et al.*, 2003)

TABLE II
DISTRIBUTION AND RUMINAL OUTFLOW OF *TRANS* 18:1 AND ISOMERS OF CONJUGATED 18:2 FATTY ACIDS IN GROWING AND LACTATING CATTLE[a,b]

Trans 18:1		Conjugated 18:2	
Isomer	Ruminal outflow (g/day)	Isomer	Ruminal outflow (g/day)
trans-4	0.5–0.7	*trans*-7, *cis*-9	<0.01
trans-5	0.4–0.6	*trans*-7, *trans*-9	<0.01–0.05
trans-6–8	0.4–6.7	*trans*-8, *cis*-10	0.01–0.02
trans-9	0.8–6.2	*trans*-8, *trans*-10	<0.01–0.10
trans-10	1.7–29.1	*cis*-9, *cis*-11	<0.01–0.01
trans-11	5.0–121.0	*cis*-9, *trans*-11	0.19–2.86
trans-12	0.5–9.5	*trans*-9, *trans*-11	0.22–0.55
trans-13 + 14	6.5–22.9	*trans*-10, *cis*-12	0.02–0.32
trans-15	3.2–8.5	*trans*-10, *trans*-12	0.05–0.06
trans-16	3.1–8.0	*cis*-11, *trans*-13	0.01–0.10
		trans-11, *cis*-13	0.01–0.46
		trans-11, *trans*-13	0.09–0.40
		cis-12, *trans*-14	<0.01–0.05
		trans-12, *trans*-14	0.08–0.19

[a]Adapted from Bauman *et al.* (2003).
[b]Data derived from three studies based on omasal (Shingfield *et al.*, 2003) or duodenal sampling (Duckett *et al.*, 2002; Piperova *et al.*, 2002).

or more comprehensive determinations using silver-ion HPLC (Corl *et al.*, 2002; Piperova *et al.*, 2002; Shingfield *et al.*, 2003) have shown that a range of isomers is formed (Table II). The latter have shown that *trans*-9, *trans*-11 CLA, *trans*-11, *cis*-13 CLA, and *trans*-11, *trans*-13 CLA are the most abundant after RA, with *trans*-10, *cis*-12 CLA being only a minor component. However, duodenal flow of the *trans*-10, *cis*-12 isomer has been reported to be higher than that of RA in steers fed corn–based diets supplemented with corn oil (Duckett *et al.*, 2002) or for low-forage diets containing sunflower oil (Sackmann *et al.*, 2003).

E. OTHER MONOENOIC ISOMERS

The established major pathways of BH describe the formation of VA but do not account for the occurrence of other 18:1 fatty acids identified in rumen digesta (Katz and Keeney, 1966) (Table II). Following detection of a large number of positional *trans* isomers of dienoic and monoenoic fatty acids, after linoleic acid was incubated with rumen contents, Ward *et al.* (1964)

suggested that BH of dienoic acid to monoenoic acids was associated with double-bond migration. However, as Griinari and Bauman (1999) noted, there is little evidence to suggest that processes of BH in the rumen are analogous to the extensive double-bond migration that occurs when vegetable oils are heated with metal catalysts. Furthermore, certain rumen bacteria isomerize *cis* monoenoic fatty acids, forming both *cis* and *trans* bonds (Kemp *et al.*, 1984a). Mosley *et al.* (2002) examined the BH of ^{13}C oleic acid by mixed ruminal microorganisms; extensive labeling was found in *trans* monoenes with double bonds from Δ-6 to Δ-16, except Δ-8, as well as in stearic acid. A similar distribution was observed when ^{13}C-labeled elaidic acid was the precursor (Proell *et al.*, 2002). Studies of a strain of *B. hungatei* have shown that this rumen bacterium produces a mixture of trans positional isomers during the BH of oleic acid (van de Vossenberg and Joblin, 2003). Formation of the mixed 18:1 isomers is not spontaneous but requires enzyme mediation (Mosley *et al.*, 2002); however, the enzymes and underlying mechanisms have not been identified.

F. SUMMARY OF THE MECHANISM OF BIOHYDROGENATION

Fatty acids with the *cis*-9, *cis*-12 pentadiene system are isomerized to the *cis*-9, *trans*-11 conjugated diene before BH takes place; the only other pentadiene that requires isomerization before BH is *cis*-2, *cis*-5. Other unsaturated bonds are hydrogenated directly. The predominant BH end-product is stearic acid; however, monoenoic, conjugated diene, and isolated dienes, with both *cis* and *trans* double bonds, have been described (Kemp *et al.*, 1975; Shingfield *et al.*, 2003).

G. FEEDING EFFECTS ON RUMINAL BH

In view of the potential benefits to human health, numerous studies have been conducted to examine nutritional strategies to enhance the CLA content of ruminant meat and milk. The diet of the ruminant animal is an important determinant of CLA content in milk and meat, and several generalizations can be made; levels of RA are higher in milk or meat from animals grazing pasture (Dhiman *et al.*, 1999; Kelly *et al.*, 1998b; Lawless *et al.*, 1998; Stanton *et al.*, 2003; Steen and Porter, 2003) and in milk from cows grazing at higher altitude, an effect that has been attributed to higher concentrations of PUFAs in alpine plants (Collomb *et al.*, 2002a,b); this response is lost as grass matures over a grazing season (Auldist *et al.*, 2002); feeding concentrates generally reduce milk CLA content in grazing cows (Stockdale *et al.*, 2003); levels of CLA can be enhanced by including

vegetable oils in the diet (Chouinard *et al.*, 2001; Dhiman *et al.*, 2000; Kelly *et al.*, 1998a; Lock and Garnsworthy, 2002), but greater responses occur when supplements of fish or marine oils are fed (Chilliard *et al.*, 2001; Chouinard *et al.*, 2001; Offer *et al.*, 1999, 2001; Whitlock *et al.*, 2002).

The effect of diet on the levels of CLA in milk and tissues has been reviewed extensively (Bauman *et al.*, 2001; Chilliard *et al.*, 2001; Griinari and Bauman, 1999; Lawson *et al.*, 2001; Stanton *et al.*, 2003). Nutritional strategies for enrichment of RA concentrations of ruminant foods can be grouped into four broad categories relative to potential modes of action: (1) provision of lipid substrates for ruminal VA and RA synthesis, (2) dietary factors that induce changes in microbial populations involved in BH either directly or via changes in rumen environment, (3) diets that provide both lipid substrates and induce changes in ruminal BH, and (4) feeding supplements of rumen-protected CLA and VA. A clear distinction among these approaches often is not possible because responses are usually related to more than a single factor. This section considers the impact of dietary changes on lipid metabolism in the rumen, because the effects of nutrition on CLA content of milk and meat can be related to the effects on ruminal BH and more specifically on the amounts of individual BH intermediates available for absorption. Griinari and Shingfield (2002) also argue that a simple description of dietary factors influencing CLA content in meat and milk does not provide sufficient insight for the development of nutritional strategies that optimize ruminal VA formation that is the major source of RA in ruminant foods, and therefore, *in vivo* assessments of ruminal BH are required.

Measurements of BH *in vivo* are relatively limited and can be summarized as studies examining the effect of forage type (Lee *et al.*, 2003), level of concentrate in the diet (Kalscheur *et al.*, 1997a; Kucuk *et al.*, 2001; Piperova *et al.*, 2002), lipid supplements (Duckett *et al.*, 2002; Kalscheur *et al.*, 1997b; Lock and Garnsworthy, 2002; Shingfield *et al.*, 2003), or the interaction between concentrate level and amount of oil in the diet (Sackmann *et al.*, 2003).

Because the metabolism of *trans*-18:1 to stearic acid is thought to be the rate-limiting step in complete BH (Keeney, 1970), the effects of diet on lipid metabolism in the rumen tend to have a more pronounced effect on the amount and relative proportions of *trans*-18:1 leaving the rumen than on other BH intermediates including isomers of CLA. With respect to enhancing RA content of ruminant foods, it could be argued that the most important aspects of dietary effects on ruminal BH relate to those on VA and the factors that regulate its synthesis.

In a comparison, Lee *et al.* (2003) reported that ruminal synthesis of RA and VA was higher in steers fed red or white clover silage than grass silage. However, dry matter intakes were markedly different among experimental

silage diets, so it is difficult to establish whether the positive effects on ruminal CLA and VA synthesis reflect the higher PUFA content, greater intake potential (and associated changes in digestion kinetics), or both attributes of legume silages. Relatively few studies have examined the effects of different forage sources on ruminal BH. Indirect comparisons of milk fatty acid composition have shown that VA is the predominant *trans*-18:1 from grass silage–based diets (Offer *et al.*, 1999), but *trans*-10 18:1 is the main isomer when corn silage is fed (Offer *et al.*, 2001). These findings suggest that the higher levels of starch, lower amounts of fiber, or both attributes of corn compared with grass silage promote shifts in BH pathways towards *trans*-10 18:1 formation at the expense of VA. Forage conservation method also appears to be important, following the observation that the rate and extent of BH of linoleic and α-linolenic acid *in vitro* is higher for fresh or ensiled grass than dried hay (Boufaïed *et al.*, 2003). However, whether this is a direct effect on BH per se or related to changes in the rate of lipolysis of forage lipids is unclear.

Increases in the amount of concentrate in the diet from 40 to 75% of DM decrease the reduction of *trans* monoenoic acids to stearic acid in the rumen, a change that also is associated with a lower ruminal pH level (Kalscheur *et al.*, 1997b). Adding a buffer to the diet normalized ruminal pH level (6.15 for high-forage diets; 6.02 vs. 5.83 for buffer vs. no buffer in high-concentrate diets) and decreased duodenal *trans*-18:1 flow (61 vs. 57 and 120 vs. 66 g/day for low- and high-concentrate diets, respectively). Further analysis of duodenal digesta from this experiment (Piperova *et al.*, 2002) indicates that high levels of concentrate increased ($P < .05$) all of the *trans* monoenes except *trans*-11 and *trans*-16. The most marked change for high-concentrate diets without buffer was seen in the flow of *trans*-10 18:1, which was fourfold higher compared with the high-forage diets. When buffer was added to the high-concentrate diet, flows of *trans*-9, *trans*-12, *trans*-13/14, and *trans*-15 monoenoic acids were comparable to the high-forage diet. Interactions of forage level and buffer effects were observed for *trans*-12, *trans*-13 + 14 monoenes, and total *trans* fatty acids. Amounts of CLA isomers were much lower compared with the *trans* monoenes, and the effects were highest for the high-concentrate diet without buffer; duodenal flows of *trans*-10, *cis*-12 18:2 increased fourfold, and those of RA, as well as *trans, trans* isomers were doubled ($P < .01$). Changes in the synthesis of individual BH intermediates associated with feeding high levels of concentrates were proposed to be mediated by the effects on ruminal pH. Presumably, changes in ruminal pH were associated with alterations in the balance of the growth and proliferation of specific bacteria capable of BH.

Measurements of duodenal fatty acid flows in the experiment of Kalscheur *et al.* (1997a) are consistent with concentrations of *trans*-10 18:1

in milk being increased fivefold in cows offered a high-concentrate diet supplemented with unsaturated fatty acids (Griinari *et al.*, 1998). Levels of *trans*-10 18:1 were comparable between high-concentrate diets supplemented with saturated fatty acids and high-forage diets containing either saturated or unsaturated fatty acids, indicating that shifts toward the formation of *trans*-10 18:1 were related to two factors: an altered rumen environment and a source of PUFA in the diet.

The effects of concentrate in the diet have also been examined in sheep. Flows of VA decreased linearly from 8.3 to 5.5 g/day as the proportion of forage in the diet increased from 18 to 60% of diet dry matter but increased inexplicably when diets contained proportionately 0.73 forage dry matter (Kucuk *et al.*, 2001). However, the variable addition of soybean oil to ensure diets contained 6% fat resulted in differences in dietary forage:concentrate ratio being confounded with oil supplementation so higher forage diets contained greater amounts of added oil. In spite of these concerns, high levels of concentrate in the diet also were shown to inhibit the final reduction of *trans*-18:1 to stearic acid; unfortunately, the occurrence of *trans*-18:1 other than VA was not reported. High levels of concentrate in the diet were also reported to reduce the formation of RA and increase the synthesis of *trans*-10, *cis*-12 CLA. Daniel *et al.* (2004) reported a near disappearance of VA, associated with large increases in *trans*-10 18:1, in abomasal digesta of lambs in response to a change in diet from dehydrated grass pellets to high-concentrate diets.

Increases in *trans*-18:1 formation also have been shown when diets are supplemented with vegetable oils. Kalscheur *et al.* (1997b) reported that duodenal flow of *trans*-18:1 was increased from 64 g/day for a control diet to 287 and 295 g/day when 500 ml of high oleic or high linoleic acid sunflower oils, respectively, were included in the diets. Unfortunately, flows of CLA or individual *trans*-18:1 isomers were not determined. In another study, duodenal flows of monoenoic and CLA isomers in steers fed corn silage, high oil corn silage, or corn silage supplemented with corn oil were reported (Duckett *et al.*, 2002). Under these feeding conditions, VA was the major BH intermediate for corn silage and high oil corn silage, but supplementing conventional corn silage with an equivalent amount of oil provided by the high oil corn silage enhanced *trans*-10 and *trans*-12 18:1 formation but had no effect on VA production. Thus, free oil was a more potent modifier of rumen BH in this experiment than the same oil contained in the matrix of the cereal grain.

More dramatic inhibition of the reduction of *trans*-18:1 in the rumen was observed when fish oil was included in the diet (Wonsil *et al.*, 1994). Studies have shown that feeding 250 g of fish oil/day to cows fed grass silage–based diets decreased ruminal outflow of stearic acid by 83% (Shingfield *et al.*,

2003) and enhanced the flow of most *trans* monoenes by twofold to fourfold, whereas *trans*-10 and *trans*-11 were increased by sevenfold and sixfold, respectively. Interestingly, whereas increased flows were observed also for nonconjugated dienes (*trans*-11, *cis*-15 increased nearly sixfold), both *cis*-9, *trans*-11 and *trans*-10, *cis*-12 conjugated isomers were decreased by feeding fish oil because of associated reductions in dry matter intake. This suggests that fish oil is a more potent inhibitor of bacteria involved in the hydrogenation of *trans*-18:1 and 18:2 than those responsible for the formation of conjugated dienes. Furthermore, the inhibition of complete BH of dietary PUFAs and accumulation of VA in the rumen could account for virtually all of the increase in milk RA content when fish oil was included in the diet.

Not only are alterations in BH dependent on the amount or type of lipid supplement or through changes in the basal diet, but a combination of factors are involved. For example, the production of BH intermediates in response to sunflower oil supplements was shown to depend on the proportion of concentrate in diet (Sackmann *et al.*, 2003). Duodenal flows of RA were higher in steers when supplements of sunflower were included in diets containing Bermuda hay forage at 12 or 24% of diet dry matter than 36% forage (0.16 and 0.21 vs. 0.07 g/day, respectively). Conversely, increases in the proportion of forage in the diet reduced *trans*-10, *cis*-12 CLA production (0.40, 0.26, and 0.22 g/day) and enhanced *cis*-11, *trans*-13 CLA formation (0.02, 0.04, and 0.06 g/day). Even though the composition of the basal diet altered the relative proportions of CLA isomers in response to sunflower oil, much greater changes were observed for duodenal flows of *trans*-18:1 BH intermediates. Increases in the proportion of concentrate in the diet reduced ruminal synthesis of VA (11.8, 7.0, and 3.5 g/day for diets containing 64, 76, and 88% concentrate dry matter, respectively), *trans*-9 18:1 (2.1, 2.3, and 0.7), and *trans*-12 18:1 (2.5, 2.5, and 1.3) but caused a marked increase in *trans*-10 18:1 (15.5, 29.8, and 41.4).

Griinari and Shingfield (2002) suggest that ruminal VA formation is dependent on three interdependent processes: (1) substrate supply, (2) inhibition of *trans*-18:1 reductase, and (3) prevention of a shift in ruminal BH toward *trans*-10 18:1 at the expense of *trans*-11 18:1 (Figure 6). This hypothesis, termed the "biohydrogenation balance model," attempted to characterize the impact of diet on the CLA content of ruminant foods by taking into account the effects of all three processes on ruminal BH simultaneously. The authors suggested that substrate supply has a typically permissive role in determining the extent of *trans*-18:1 fatty acid accumulation in the rumen in response to the other two processes, so the balance between *trans*-18:1 reductase inhibition and induction of the shift toward *trans*-10 18:1 at the expense of VA regulates the magnitude of the overall response.

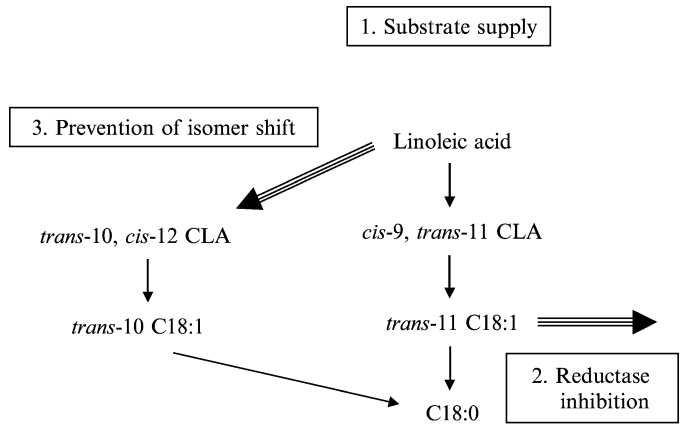

FIG. 6 Schematic of the "biohydrogenation balance model." (Used, with permission, from Griinari and Shingfield, 2002.)

To summarize the impact of diet, complete BH to stearic acid is most extensive when animals are fed diets containing high amounts of ensiled forages. Incomplete reduction to stearic acid, associated with the accumulation of BH intermediates, most notably *trans* 18:1, arises when diets contain high amounts of readily available unsaturated fatty acids, low amounts of fiber, or high levels of starch or cause low ruminal pH. Fish and marine oils are far more potent inhibitors of *trans*-18:1 reduction in the rumen than other sources of unsaturated fatty acids (Chilliard *et al.*, 2001; Offer *et al.*, 2001; Whitlock *et al.*, 2002). Changes from basal diets by a single dietary parameter in isolation have little effect on BH; simultaneous changes in interdependent processes are required to have an impact. It is this that explains the difficulties in predicting the effects of changes in the diet on the amount of ruminal BH intermediates available for absorption.

III. CLA SYNTHESIS BY NON-RUMINAL ORGANISMS

Alternatives to using ruminant foods to provide CLA in the diet are of great interest to the food-processing industry, perhaps most so in dairy processing. Sieber *et al.* (2004) reviewed the impact of microbial cultures on CLA in dairy products. Several strains of *Lactobacillus, Propionibacterium, Bifidobacterium,* and *Enterococcus* are able to form CLA from linoleic acid; lactic acid bacteria and propionibacteria appear to show promise to increase CLA during ripening of cheese. Presently, data are not convincing that this

approach significantly increases CLA content of dairy foods; further, effects are much smaller than can be accomplished by manipulating the animals' diets.

Of interest is a unique alternative biosynthetic pathway for CLA. Ogawa *et al.* (2001) reported that a strain of *Lactobacillus acidophilus,* under microaerobic conditions, produced 10-hydroxy-*cis*-12-octadecenoic acid and 10-hydroxy-*trans*-12-octadecenoic acid as intermediates in the synthesis of *cis*-9, *trans*-11 and *trans-9*, *cis*-11 18:2. The conversion was induced by presence of linoleic acid, and a high yield of CLA was reported. Hudson *et al.* (1998, 2000) showed that lactic acid bacteria, including *Lactobacillus, Pediococcus,* and *Streptococcus* species, are the major unsaturated fatty acid hydrating bacteria in the rumen, converting oleic acid to 10-hydroxy stearic acid and linoleic acid to 10-hydroxy-12-octadecenoic acid and 13-hydroxy-9 octadecenoic acid. Thus, potentially, CLA may be produced also in the rumen from linoleic acid by pathways other than the classic isomerase described by Kepler *et al.* (1966).

Finally, dairy products may be enriched with naturally occurring CLA by fat fractionation procedures. Fractionation of anhydrous milk fat by a supercritical carbon dioxide system (Romero *et al.*, 2000) or by controlled cooling and agitation (O'Shea *et al.*, 2000) resulted in both cases in a more than 60% increase in the CLA content as compared to the parent fat; also concentrations of PUFA and VA were increased.

IV. ENDOGENOUS SYNTHESIS OF CLA

A. BACKGROUND

As is outlined in the following section, it is now apparent that endogenous synthesis of RA occurs in most, if not all, animal species. The classic studies of Mahfouz *et al.* (1980) and Pollard *et al.* (1980) showed independently that positional isomers of trans-octadecenoic acids are desaturated by the enzyme Δ-9 desaturase in rat liver microsomal systems. All *trans* monoenes, Δ-4 to Δ-13, except Δ-8, 9, and 10, were substrates, with products being *trans*-Δ-x, *cis*-9 dienes. The rate of Δ-9 desaturation increased as the *trans* bond was removed further from the Δ-9 position, so that *trans*-Δ-4 and Δ-13 monoenes were most rapidly desaturated (Mahfouz *et al.*, 1980). The *trans*-5, *cis*-9 18:2 was isomerized rapidly to the cis, cis diene without changing bond positions; *trans*-4, *cis*-9 18:2 was isomerized similarly, but at a slower rate (Mahfouz *et al.*, 1980). Significant amounts of some of the *cis*/*trans* dienes were desaturated further at Δ-6, to yield *cis, trans, cis* trienes (Pollard *et al.*, 1980).

The end-products of rumen microbial metabolism of greatest interest with regard to CLA metabolism in the body are the various CLA isomers and the *trans*-11 monoenoic acid VA. The latter, being a substrate for Δ-9-desaturase, is converted to RA in animal tissues. Hay and Morrison (1970) reported the distribution of monoenoic isomers of milk fat, and the content of various CLA and other 18:2 isomers in the fat of ruminant products has been summarized by Parodi (2003).

B. CHARACTERISTICS OF Δ-9-DESATURASE

Although we refer to the enzyme as Δ-9-desaturase because it is active with numerous acyl-coenzyme A (acyl-CoA) substrates, its most common substrate is stearic acid, so it is most often identified as stearoyl-CoA desaturase (EC 1.14.99.5). The significance of endogenous synthesis as a source for RA emphasizes the critical role of Δ-9-desaturase in the production of RA in milk and meat fat in ruminants and in the conversion of dietary VA to RA in other species. Δ-9-Desaturase is a key regulatory enzyme for the biosynthesis of monounsaturated fatty acids that are in turn used for the synthesis of triacylglycerols, phospholipids, and cholesterol esters. Understanding of this enzyme in ruminants is limited, with current knowledge coming predominantly from investigations with rodents and rodent cell lines.

The oxidative reaction catalyzed by Δ-9-desaturase involves cytochrome b_5, NAD(P)-cytochrome b_5 reductase and molecular oxygen, and the CoA ester of fatty acids is required as the substrate (Ntambi, 1995). Thus, the CoA ester of VA is the substrate for formation of RA. Preferred substrates for Δ-9-desaturase are palmitoyl- and stearoyl-CoA, which are converted to palmitoleoyl- and oleoyl-CoA, respectively (Ntambi, 1999). Δ-9-Desaturase has no known allosteric or feedback inhibition involving its substrates or products. The regulation of this enzyme has been investigated extensively in rodent liver, and results indicate that gene expression and amount of enzyme is regulated by dietary factors such as glucose and PUFA and by hormones such as insulin, glucagon, and thyroid hormone (Ntambi and Miyazaki, 2004). Δ-9-Desaturase gene expression is down-regulated by both PUFA and *trans*-10, *cis*-12 CLA, but RA has no effect (Choi *et al.*, 2000; Lee *et al.*, 1998; Ntambi and Miyazaki, 2004). The point of regulation for the enzyme is gene transcription, which is effective because of the short half-life of the Δ-9-desaturase protein (~4 hours; Ozols, 1997). In mice, a significant proportion of the effects of leptin on metabolism may be mediated by inhibition of Δ-9-desaturase expression (Ntambi and Miyazaki, 2004). Interestingly, sterculic oil does not affect Δ-9-desaturase gene or protein expression, but it directly inhibits the activity of Δ-9-desaturase, possibly by a turnover-dependent

reaction (Gomez *et al.*, 2003). Both RA and *trans*-10, *cis*-12 CLA were reported to influence stearoyl-CoA desaturase activity in human breast cancer cell lines; however, mechanisms apparently differed between cell lines (Choi *et al.*, 2002). Both CLA isomers reduced stearoyl-CoA desaturase protein in MDA-MB-231 cells, whereas in MCF-7 cells, both isomers directly inhibited the enzyme activity. These results differ from other reports on effects of RA on desaturase activity; they have not been repeated and perhaps were unique to these cell types.

Three isoforms of the Δ-9-desaturase gene have been characterized in mice, whereas two have been characterized in rats and only one isoform has been identified in humans and ruminants (Ntambi and Miyazaki, 2004). Relatively few studies have involved ruminants or examined the regulation of Δ-9-desaturase in mammary tissue of any lactating mammal. Beswick and Kennelly (2000) reported that recombinant bovine growth hormone and bovine growth hormone–releasing factor decreased Δ-9-desaturase mRNA in adipose tissue, but not mammary tissue, of lactating cows. Δ-9-Desaturase activity has been reported in bovine milk microsomes (McCarthy *et al.*, 1965) and in adipose tissue, muscle, intestine, liver, and mammary gland of ruminants (Bickerstaffe and Annison, 1969, 1970; Chang *et al.*, 1992; St. John *et al.*, 1991). Abundance of mammary tissue Δ-9-desaturase decreases when milk fat depression is induced by diet (Peterson *et al.*, 2003a) or by supplements of *trans*-10, *cis*-12 CLA (Baumgard *et al.*, 2002). Regulation of this enzyme in mammary tissue probably involves sterol response element binding protein-1 (SREBP-1). PUFAs inhibit the processing of SREBP-1 and may decrease the abundance of the precursor protein, leading to reduction in transcription of many genes in the lipogenic pathways, including Δ-9-desaturase (Clarke, 2001; Horton *et al.*, 2002; Shimano, 2001). The addition of *trans*-10, *cis*-12 CLA to bovine mammary epithelial cells was shown to reduce the proteolytic activation of SREBP-1 (Peterson *et al.*, 2003b).

Ward *et al.* (1998) reported highest expression of Δ-9-desaturase mRNA in adipose tissue, liver, and mammary gland of lactating sheep, and expression was decreased by 80% in adipose tissue of late pregnant and lactating animals, a time when lipogenic activity is increased in mammary gland and decreased in adipose tissue (Bauman and Currie, 1980). Whereas Ward *et al.* (1998) reported significant Δ-9-desaturase mRNA expression in liver, St. John *et al.* (1991) found no enzymatic activity in liver of steers fed a diet high in corn. The same research group (Chang *et al.*, 1992) reported inconsistent desaturase activity (one of four animals) in liver of cattle fed a high-corn diet, whereas activity in liver was detected when a diet containing 8% oil from high oleate sunflower seeds was fed. The authors indicated that the unsaturated sunflower oil was extensively BH in the rumen, thus increasing the amount of stearic acid absorbed. Desaturase activity was also increased

in adipose tissue and intestinal mucosa and was induced sixfold ($P < .05$) in muscle. Although low desaturase activity in ruminant liver is consistent with low liver uptake of stearic acid (Bell, 1981), further studies are warranted to document induction of Δ-9-desaturase in ruminant tissues under conditions of high amounts of stearic acid absorption. Desaturase index has been shown to be different among adipose depots of sheep (Palmquist *et al.*, 2004) and growing Jersey cattle (Leat, 1975) but was not different in subcutaneous adipose tissues of two beef breeds, Angus and American Wagyu (Cameron *et al.*, 1994). A number of studies have reported breed differences in the CLA content of milk fat (Dhiman *et al.*, 2002; Lawless *et al.*, 1999; White *et al.*, 2001; Whitlock *et al.*, 2002), which may reflect differences in desaturase index among breeds. However, these studies have often involved very few animals or were confounded by diet or both. Using a larger data set, DePeters *et al.* (1995) reported breed differences in desaturase index in milk fat of dairy cows, consistent with the suggestion that the activity of Δ-9-desaturase is higher in Holstein than Jersey mammary tissue (Beaulieu and Palmquist, 1995). However, if breed differences exist, they are minor compared with the effect of diet and individual animal variation on the CLA content of milk fat and desaturase index (Bauman *et al.*, 2003).

C. ENDOGENOUS SYNTHESIS OF RA IN RUMINANTS

Griinari and Bauman (1999) proposed that endogenous synthesis could be an important source of the RA found in milk fat of ruminants, based on its transient occurrence as an intermediate in ruminal BH, as well as the observation that increases in the RA content of milk fat occurred when the dietary supply of linolenic acid was increased. As previously discussed, VA is an intermediate in the BH of both linoleic and linolenic acids and is the major unsaturated BH product passing from the rumen. Strong support for the hypothesis of endogenous synthesis was gained by abomasal infusion of VA (12.5 g/day to lactating dairy cows) that resulted in a 31% increase in milk fat content of RA (Griinari *et al.*, 2000). Although this study suggested that endogenous synthesis was a source of milk fat CLA, its contribution to total RA in milk fat was unknown.

Researchers have used one of two approaches to quantify endogenous synthesis. The first involves inhibiting the Δ-9-desaturase, thereby inhibiting conversion of VA to RA. Using abomasal infusion of sterculic oil to inhibit Δ-9-desaturase activity, combined with monoene/saturate pair ratios to correct for the extent of inhibition, Griinari *et al.* (2000) estimated that a minimum of 64% of RA in their milk fat samples was derived from desaturation of VA. Further experiments using sterculic oil also indicated that the

majority of the RA in milk fat was derived from endogenous synthesis via Δ-9-desaturation of VA. Corl *et al.* (2001) estimated that endogenous synthesis contributed more than 78% of milk fat RA when cows were fed a total mixed ration of hay and concentrate. Endogenous synthesis contributed an even greater extent when cows grazed fresh pasture, with more than 91% of RA in milk fat synthesized endogenously (Kay *et al.*, 2004).

A second approach to quantify endogenous synthesis of RA in milk fat uses indigestible markers and estimates ruminal outflow of RA. By comparing ruminal outflow of RA with milk output of RA, the maximum proportion of RA derived from ruminal production can be estimated, with endogenous synthesis representing the remainder. Assumptions and limitations to this approach and to inhibiting Δ-9-desaturase directly have been discussed (Bauman *et al.*, 2003). Piperova *et al.* (2002) fed high- and low-fiber diets with or without buffer and in all cases found that duodenal supply of RA was a very small percentage of the amount secreted in milk, whereas duodenal flow of VA provided more than adequate amounts of precursor. Calculations from their data show that endogenous synthesis accounted for more than 93–97% of milk RA, and this represented 20–33% of the duodenal supply of VA. Similar results have been reported by others. By extrapolating results from non-lactating dairy cows to lactating cattle on the basis of feed intake, Lock and Garnsworthy (2002) estimated that endogenous synthesis accounted for over 80% of the RA in milk fat when cows were fed a grass silage diet with concentrates differing in their content of linoleic and linolenic acids. Shingfield *et al.* (2003) used ruminal outflow measurements to calculate that endogenous synthesis accounted for more than 74% of the RA in milk fat when a grass silage plus concentrate diet was fed with or without fish oil.

Estimating the extent of endogenous RA synthesis in growing ruminants is inherently more difficult because RA in body fat accumulates throughout the lifespan of the animal. Nevertheless, ruminal output of VA ranged from 27- to 69-fold greater than output of RA for sheep fed diets that varied in forage content and supplementation with soybean oil (Kucuk *et al.*, 2001). Similar results were shown in cattle fed corn-based finishing rations with or without a corn oil supplement, in which ruminal outflow of VA ranged from 39- to 61-fold greater than rumen outflow of RA (Duckett *et al.*, 2002). When beef heifers were fed various diets (hay–concentrate mixtures, grazing pasture, and plant oil supplements), in all cases ruminal outflow of VA was substantially greater than outflow of RA (Carter *et al.*, 2002; Lake *et al.*, 2002; Scholljegerdes *et al.*, 2002). The only study to estimate endogenous synthesis of RA in growing ruminants used a mathematical modeling approach and estimated that 45–95% of RA in muscle and adipose tissues of lambs was synthesized endogenously (Palmquist *et al.*, 2004). They also

estimated that desaturation of VA ranged from 11 to 22% and concluded that the proportion of RA synthesized endogenously was inversely related to the amount of RA absorbed from the intestine and found in the tissues.

The relationship between substrate and product for Δ-9-desaturase is reflected by the desaturase index, defined as [RA ÷ (RA + VA)]. Various approaches to calculating desaturase index in milk fat are discussed by Kelsey *et al.* (2003). In the study by Corl *et al.* (2001), the desaturase index was 0.23 for the hay and concentrate diet and 0.20 when the diet was supplemented with PHVO. Kay *et al.* (2004) reported a desaturase index of 0.25 for the pasture diet and 0.22 when the diet was supplemented with sunflower oil. Piperova *et al.* (2002) observed desaturase indices for high- and low-fiber diets of 0.40 and 0.35, respectively. Shingfield *et al.* (2003) reported desaturase indices of 0.18 and 0.15 with a grass silage diet without or supplemented with fish oil; these values are probably lower than others because the analytical methods accounted for minor CLA isomers that typically co-elute with RA or because of inhibition of Δ-9-desaturase by the long-chain PUFA from the fish oil supplement. The desaturase index, as defined earlier, should approximate the proportion of VA desaturated in the tissues. A summary of endogenous RA synthesis estimates and the proportion of VA desaturated in the tissues is in Table III.

Although diet is the major determinant of milk fat RA content, there is also a twofold to threefold range in milk fat content of RA among individual cows within a herd consuming the same diet (Bauman *et al.*, 2003). A similar level of variation also has been shown in the desaturase index, with a several-fold range among cows (Kelsey *et al.*, 2003; Lock and Garnsworthy, 2002, 2003; Peterson *et al.*, 2002a). Peterson *et al.* (2002a) demonstrated a consistency in the hierarchy among cows in desaturase index over time when cows were fed the same diet and when cows were switched between diets. In the largest study of this type, Kelsey *et al.* (2003) demonstrated that the variation in milk fat content of RA, and the desaturase index, was about threefold among individuals consuming the same diet. The effect of breed (Holstein vs. Brown Swiss), parity, and days in milk had no relationship to the individual variation in desaturase index, and neither did milk yield, milk fat percentage, or milk fat yield (Kelsey *et al.*, 2003; Lock *et al.*, 2003).

Overall, investigations in both lactating and non-lactating ruminants have shown that the major source of RA in milk fat and adipose tissue is endogenous synthesis, with the precursor being VA derived from rumen production. The relatively constant ratio observed between VA and RA in milk fat reflects the substrate–product relationship for Δ-9-desaturase; therefore, successful approaches to increase the milk fat content of RA will involve enhancing rumen output of VA and increasing tissue activity of Δ-9-desaturase, as discussed in a following section.

TABLE III
QUANTITATIVE STUDIES OF ENDOGENOUS CLA SYNTHESIS IN LACTATING COWS

Source	Diet	Duodenal flow (g/day)		Content in milk fat (%)		Endogenous (%)		
		VA	RA	VA	RA	Desaturase inhibition[a]	Rumen outflow[b]	VA to RA (%)[c]
				(%)				
Griinari *et al.*, 2000	Hay/concentrate TMR	—	—	1.4	0.43	64	—	<20
Corl *et al.*, 2001	Hay/concentrate TMR	—	—	2.18	0.65	78	—	23.4
	+ partially hyd. veg. oil	—	—	3.03	0.76	78	—	16.4
Kay *et al.*, 2004	Pasture	—	—	3.56	1.21	91	—	24
	+ sunflower oil	—	—	4.20	1.16	91	—	20
				g/day				
Piperova *et al.*, 2002	High forage	21.4	0.35	10.9	7.3	—	97	39
	High forage + buffer	20.7	0.3	11.0	6.6	—	97	37
	Low forage	34.6	0.5	14.4	7.6	—	96	34
	Low forage + buffer	21.5	0.2	9.7	6.0	—	98	38
Shingfield *et al.*, 2003	Grass silage/concentrate	17	2.9	12.8	2.8	—	39	8
	+ fish oil	121	2.1	50.9	9.0	—	86	13

Note: VA, vaccenic acid; RA, rumenic acid.
[a]Endogenous synthesis estimated by sterculic acid inhibition of Δ-9-desaturase combined with saturate/monoene pair ratios to correct for extent of inhibition.
[b]Calculated from respective authors' data; endogenous synthesis estimated using markers to estimate rumen outflow and assuming that 60% of duodenal fatty acid flow is incorporated into milk fat (80% absorption [Palmquist, 1991]; 75% of absorbed fatty acids incorporated into milk fat [Palmquist and Mattos, 1978]).
[c]Percentage of VA desaturated to RA. See text for calculation.

The second most prevalent CLA isomer in ruminant fat is *trans*-7, *cis*-9 CLA, representing 3–16% of total CLA in ruminant fat (Corl *et al.*, 2002; Parodi, 2003; Piperova *et al.*, 2000, 2002; Shingfield *et al.*, 2003; Yurawecz *et al.*, 1998). A number of the studies previously described have determined the source of *trans*-7, *cis*-9 CLA in ruminant fat. Corl *et al.* (2002) showed that the *trans*-7, *cis*-9 CLA in milk fat was derived almost exclusively from endogenous synthesis by using both sterculic acid and *trans*-10, *cis*-12 CLA to inhibit Δ-9-desaturase; they also found that *trans*-7, *cis*-9 CLA concentration in rumen fluid was very low and at the limit of detection. Similarly, Piperova *et al.* (2002) found that virtually all of the *trans*-7, *cis*-9 CLA in milk fat was produced post-ruminally. As mentioned previously, *trans*-7 18:1, a minor BH intermediate in ruminal contents, also is a substrate for Δ-9-desaturase (Mahfouz *et al.*, 1980; Pollard *et al.*, 1980).

D. ENDOGENOUS SYNTHESIS OF RA IN HUMANS AND OTHER SPECIES

In addition to ruminants, the conversion of dietary VA to RA occurs in humans. Fogerty *et al.* (1988) recognized that dietary VA might be converted to RA in human tissues, quoting earlier work of Pollard (1980) with Δ-9-desaturase in rat liver microsomes. However, based on results from their study, they concluded that nondietary RA came from "free radical isomerization of linoleic acid *in vivo*." Parodi (1994) also recognized the possibility of endogenous synthesis and suggested further that another non-dietary source of CLA could be production by bacteria in the digestive tract. Chin *et al.* (1994) examined this and concluded that intestinal bacteria were capable of converting linoleic acid to CLA based on comparisons between conventional and germ-free rats.

Emken *et al.* (1986) fed deuterium-labeled VA as triacylglycerol to two young adult male subjects and found no evidence in plasma lipids of VA desaturation. Subsequently, they reanalyzed the samples from one subject and reported a CLA enrichment of about 30% (Adlof *et al.*, 2000). Salminen *et al.* (1998) showed that humans consuming a diet high in trans fatty acids (25% of dietary fatty acids) had higher serum concentrations of CLA than those consuming diets low in trans fatty acids. The addition of VA to human mammary and colon cancer cell lines resulted in an increased cell content of RA (Miller *et al.*, 2003). Turpeinen *et al.* (2002) used slope response to increasing dietary VA to estimate the extent of VA desaturation to RA in humans. Thirty healthy subjects consumed a baseline diet rich in oleic acid for 2 weeks, followed by diets containing 1.5, 3.0, or 4.5 g of VA/day for 9 days. Test diets contained no CLA. The change in RA in the serum

very-low-density lipoprotein (VLDL) triacylglycerol was plotted versus the change in VA plus the change in RA in the serum VLDL triacylglycerol. The slope of the regression indicated that 19% of the supplementary VA was converted to RA as a mean response. Based on this work, Parodi (2003) has suggested that CLA intake multiplied by 1.4 would provide an estimate of the effective physiological dose of CLA derived from ruminant products.

Endogenous synthesis of RA from VA also has been shown in other species including rats, mice, and pigs. In a study that compared CLA-enriched butter to commercial CLA sources as anticancer agents in rats, Ip *et al.* (1999) found that when provided at equal dietary CLA levels, butter was a more effective agent to suppress proliferation of mammary terminal end bud cells and mammary tumor yield. Rats consuming butter accumulated twice as much RA in the mammary fat pad and other tissues as those fed free CLA, and authors suggested that this was due to the VA in butter being converted to RA. This was confirmed when feeding rats increasing amounts of pure VA resulted in a progressive increase in the tissue concentration of RA (Banni *et al.*, 2001). Further, increasing dietary supply of a VA-enriched butter resulted in a dose-dependent decrease in mammary tumors and an increase in the accumulation of RA in liver, plasma, and mammary fat pad, with the ratio of VA to RA in the mammary fat pad approaching 1:1 (Corl *et al.*, 2003). Inhibiting Δ-9-desaturase in the same rodent mammary cancer model by feeding sterculic oil reduced the accumulation of RA in tissues with a corresponding reduction in the suppression of mammary premalignant lesions; there was a 39% reduction in the RA content of the mammary fat pad with the addition of sterculic oil to diets containing 1.6% VA (Lock *et al.*, 2004). Considering that RA has been shown to be effective in reducing plasma cholesterol and cholesterol-induced atherogenesis, it seems logical that dietary VA supplied by dairy products also may have beneficial effects on variables associated with increased risk for atherosclerosis, and that this will relate to its use for endogenous synthesis of RA in a manner similar to the ability of dietary VA to reduce mammary cancer risk.

Santora *et al.* (2000) showed that 50% of VA in tissues of mice was desaturated to RA; increasing intake of unsaturated corn oil inhibited the desaturase and decreased desaturation by 30%. VA fed to lactating mice increased VA and RA in the plasma, tissue lipids, and milk lipids of the dams, and in liver of the suckling pups (Loor *et al.*, 2002). However, RA was not found in the tissue lipids of the nursing pups. Finally, Gläser *et al.* (2000) reported that feeding partially hydrogenated vegetable fat as a source of VA to fattening pigs increased CLA content (0.44% of total fatty acids) of the back fat.

V. CONCLUDING SUMMARY

Because of its potential to improve human health, there is great interest to increase the amount of CLA in the human food supply. This has caused a great deal of effort to be expended toward increasing the concentration of CLA, and more specifically RA, in the milk and tissues of ruminant foods because these are the predominant source of CLA in human diets. RA is the predominant CLA isomer present in ruminant products, and the major source of its occurrence is endogenous synthesis via desaturation of VA by Δ-9-desaturase. The central effort of this research has been directed toward improving the understanding of biohydrogenation in the rumen and examining milk and tissue CLA responses to a range of diets. Even though the major metabolic pathways are well documented, the diversity of various BH intermediates in digesta, milk, and tissues indicates the complexity of the BH processes as a whole and the population dynamics of the ruminal bacteria involved. Predicting the outcome of changes in the diet is complicated by the interactions of the ruminal environment, substrate supply, and forms of dietary lipids, all of which influence the BH process simultaneously. Development of nutritional strategies for enhancing the RA content of ruminant foods has been focused on increasing ruminal supply of VA and preventing shifts in BH toward other *trans*-18:1 isomers. The predominant source of *trans*-7, *cis*-9 CLA and RA in ruminant tissues is clearly via endogenous synthesis from *trans*-7 and *trans*-11 C18:1 as precursors, by the actions of Δ-9-desaturase. Other isomers of CLA found in ruminant foods are related directly to their synthesis in the rumen. Given the importance attached to RA, understanding the variation in activity of Δ-9-desaturase in tissues also has become a major focus of research. Research indicates that it is possible through dietary means to increase the concentration of RA in milk by 5- to 10-fold, but the increases reported for tissues appear to be lower. In all cases, use of nutrition to enrich ruminant foods with RA is associated with an unavoidable increase in *trans*-18:1 content. Levels of *trans*-10, *cis*-12 CLA in ruminant foods are extremely low and, therefore, are not significant sources of this isomer in the human diet. Finally, humans and other species also are able to convert VA to RA via Δ-9-desaturase, thereby further increasing availability of RA in the human diet from ruminant products.

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SAFETY ISSUES ASSOCIATED WITH HERBAL INGREDIENTS

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I. INTRODUCTION

To understand safety issues associated with herbal ingredients, it is important to appreciate how they were derived, what regulatory policies (or not) dictate all aspects of their production, and what options are available to making rational choices as to whether they are what they claim to be, do what they contend, and are safe in the context of their use. As globalization of herbal medicine progresses and reports of adverse events continue to escalate, it is recognized that the status quo can no longer ensure consumer confidence and protection of these types of treatments. This is the reason that both international and national policies are being evolved and implemented to address these concerns.

Since the evolution of traditional pharmacopeias has been predicated upon time-honored empirical testing, customary herbal remedies continue to be used by a vast majority of the world's population who depend on traditional medicine for their primary needs. Worldwide, the practice of herbalism continues to expand, and it has been estimated that the industry estimated at $60 billion today will significantly expand during this century (World Health Organization [WHO], 2004). To prove parameters of efficacy, more and more are being clinically evaluated in the context of use. Many have also been found to possess specific bioreactive compounds linked to their medical value, which in turn not only has verified their therapeutic worth, but also has led to new and significant drug discoveries (Lewis and

Elvin-Lewis, 2003). Current surveys are indicating that even in countries where pharmaceutical uses are favored, the popularity of herbal medicine, which began in the early 1970s, has risen markedly in the last decade (Block and Mead, 2003, Eisenberg *et al.*, 1998, Elvin-Lewis, 2001). This generational shift toward herbal use is related to the diminishing trust in conventional medicine that relies primarily on promoting synthetic mechanistic-based drugs for treatments. This is because in spite of their rational design, potency, and rapid action, concerns continue to surface regarding the serious adverse reactions, which are linked to their use. There is a growing belief that herbal medicines are better and safer than modern alternatives. This attitude is further fueled by an increased self-reliance in these populations, the tendency to self-medicate, particularly among the better educated (Klepser *et al.*, 2000), and the lack of knowledge that some of these practices can be harmful. Unfortunately, only a proportion of those using dietary supplements can appropriately interpret use and disclaimer claims (Cupp, 2003), and these claims may not be distributed with the products but as other materials. Also, herbal remedies are not always used alone and may be combined with pharmaceuticals with the notion of enhancing the positive attributes of the treatment or diminishing side effects that are known for the conventional drug (Vuckovic and Nichter, 1997). For this latter reason, the U.S. National Institutes of Health (NIH) has been obliged to introduce new policies involving use of alternative supplements by patients enrolled in clinical trials (Sparber *et al.*, 2004).

Designer "foods" are becoming increasingly popular within the "health food" industry, and they are formulated in ways to supposedly enhance the health benefits of the product by incorporating a wide variety of medicinal herbs, their extracts, and other ingredients in the mixture. These secondary additives are frequently listed on the packaging, but usually in a diminished format, without providing any sense to the consumer as to how each ingredient has been apportioned, what value they might be providing, or if any adverse effects can be expected. A noteworthy example is the wide variety of soy-powder products promoted as a health drink or for weight loss, which can include, among other ingredients, undefined amounts of bulking agents like psyllium from *Plantago ovata.* Psyllium can affect intestinal absorption in both positive and negative ways. While providing a feeling of satiety, improving glycemic control in patients with type 2 diabetes and having the potential to lower cholesterol especially with cholestyramine, it can also decrease the absorption of important nutritional minerals such as zinc, copper, iron, calcium, and magnesium in addition to important therapeutic agents such as lithium, carbamazepine, digoxin, and warfarin (Lewis and Elvin-Lewis, 2003).

Another disconcerting practice is the widely used ploy of substituting the term *cane juice* for that of sugar or sucrose in a broad variety of "health"

foods and breakfast cereal products. While this epithet implies that the sweetening agent is in its "natural" rather than "refined" state, there is no proof that this is the case because the starting point for refined sugar is "cane juice." It remains an allusive device to hide the fact that these products contain sucrose, which in any form should be avoided by many needing to either monitor or restrict their intake of this sweetener.

II. WHAT IS AN HERB?

The WHO defines an *herb* as being fresh or dried, fragmented or powdered plant material, which can be used in this crude state or further processed and formulated to become the final herbal product. Treatment of herbs by squeezing, steaming, roasting, decocting or infusing in water, extracting with alcohol, or sweetening and baking with honey can create "herbal products" such as juices, tinctures, decoctions, infusions, gums, fixed oils, essential oils, and resins. These may be used medically or as the starting material for additional processing and as food ingredients. Depending on the sophistication of the "herbal preparation," these products may be subject to any number of physical, chemical, or biological processes, including pulverization, extraction, distillation, expression, fractionation, purification, concentration, or fermentation. Formulation of the "final product" may require mixing one or more plant preparations with minerals or animal products and constituents isolated from herbal materials or synthetic compounds. These phytotherapeutic formulations may also be referred to as *drugs* or *botanicals,* or when taken orally to provide health benefits, they may be called *dietary supplements,* or even food ingredients in some cases. Additional standardization may include incorporating particular ingredients in amounts consistent with the bioreactivity of certain of their chemotaxonomic markers. These types of herbal products, which are usually clinically validated, are either referred to as *phytopharmaceuticals* or *botanical drugs* (Raskin *et al.*, 2002; WHO, 2000; Wyllie, 2003).

III. SOURCE OF HERBAL INGREDIENTS

The selection of plants to be used in herbal remedies can depend on many factors, and their quality is primarily dependent on the taxonomic expertise of individuals that gather them and the skill of those who prepare them for use. Misidentifying or even selecting the incorrect plant part is often the source of other than expected efficacies and adverse reactions. Those who wild-craft may be able to recognize the plants at only the family or generic

level, whereas other collectors are able to discern specific species and even certain chemotypes. For example, wild crafters collecting "cats' claw" in Amazonia are unable to differentiate between the more common *Uncaria guianensis* and the less common *Uncaria tomentosa* but usually refer to their collections as being the latter species. This places in doubt a number of scientific studies that have been generated on *U. tomentosa*, because the species actually tested is unclear. Without taxonomic verification to suggest otherwise, a more accurate citation for these test materials should have been *U. tomentosa/U guianensis* (Lewis, personal communication, 2005). Individuals who harvest medicinal plants may also be aware of what time of year to collect, store, and prepare the plant or one or several of its parts (leaves, flowers, stems, seeds, roots, rhizomes, fruits, bark, sap, etc.) to ensure optimal potency, flavor, and/or aroma. Also, because types of bioreactive components are often shared between closely related plants (although their concentrations may vary), knowledge of this type may be only of relative importance to the healer who uses them. This is also the reason that many examples exist of related medicinal plants or those possessing similar biosynthetic pathways being selected for comparable uses by populations that do not have any contact with each other or live in disparate parts of world (Elvin-Lewis, 2003, Lewis and Elvin-Lewis, 2003).

Today, to meet the needs of expanding herbal markets, many popular herbs are no longer totally wild-crafted. Depending on national laws, regulatory policies may control the amounts to be harvested from the wild, how they might be propagated for commercial harvest, or if restrictions of export of live material is allowed. Applying resource management to preserve rare or threatened medicinal species is a matter of concern for many countries where such taxa exist, and there is a need to rigidly enforce national regulations and international policies to ensure future availability. Guidelines for the Conservation of Medicinal Plants formulated by the WHO, the International Union for Conservation of Nature and Natural Resources (IUCN), and the World Wide Fund for Nature (WWF) (WHO/IUCN/WWF, 1993) is regularly updated (e.g., July 28, 2004) and forms the basis for providing a framework for the conservation and sustainable use of plants in medicine. Also, to safeguard the world's biological diversity, a recommendation of the Global Diversity Strategy, jointly produced by the World Resources Institute (WRI), IUCN, and the United Nations Environment Program (UNEP), is included in the document.

The following example indicates how appropriate resource management of a rare and endangered medicinal taxa can be accomplished. Chinese ginseng (*Panax ginseng*) has been essentially extirpated in the wild, and export of its seed from China for cultivation elsewhere is prohibited. Also, harvesting of its American counterpart (*Panax quinquefolius*), from its

natural habitat, is carefully regulated to ensure sustainable wild populations (Lewis, 1990), and because it is highly favored over cultivated forms and can vary in composition depending on where it is harvested (Jackson *et al.*, 2003), it is extremely costly (e.g., up to U.S. $500/pound). Though fastidious in its growth requirements, cultivation of several *Panax* species (*P. ginseng, Panax notoginseng, Panax zingerberensis,* in Asia, and *P. quinquefolius* in North America) has become the only viable alterative for accommodating sourcing needs because of its popularity in herbal medicine. In addition, other species may be locally available but are rarely sold commercially (Lewis and Elvin-Lewis, 2003). These species may differ in the presence of certain ginsenosides, and within the commercial arena, there are broad chemical disparities in the quality of products that are available and labeled "ginseng" (Asafu-Adjaye and Wong, 2003; Cui, 1995; Lau, 2003; Liberti and Marderosian, 1978). Similar disparities have been found for Siberian or Russian ginseng (*Eleutherococcus senticosus*) products and their eleutherosides (Harkey *et al.*, 2001).

Resource management of other American species has evolved depending on their ease of cultivation or the need to protect wild populations that have become endangered. Some herbs are more easily grown from seed than harvested from the wild, and today *Echinacea* species are primarily obtained from cultivated rather than natural sources (Anonymous, 2000; APHA, 2004b). Others have become so seriously depauperated by wild crafting that they are now classified as endangered and are listed in Appendix II of the Convention for International Trade on Endangered Species of Wild Fauna and Flora (CITES). Sale of wild-crafted plants Goldenseal (*Hydrastis canadensis*) is disallowed, and not only are permits required for its cultivation and propagation, but when sold, documentation must be provided that roots, rhizomes, or seeds came from legally acquired parental stock, and that the plants were cultivated for 4 years or more without augmentation from the wild (Davis and McCoy, 2000).

Until conservation practices are realistically established, certain medicinal plants will continue to be harvested in a non-sustainable way. Examples can be found wherever guidance or laws are not available to regulate harvesting procedures or where strategies have not evolved to overcome challenges to cultivation. For example, in Africa, there is a tendency to inappropriately harvest the bark of *Prunus africana,* so the tree dies. To prevent depletion of wild stocks in the mountain forests of Africa and Madagascar, conservation practices and breeding programs are being instituted. Similar approaches are being employed for Devil's Claw, *Harpagophytum procumbens,* which grows in southern Africa and Namibia. In Namibia, certified organically grown material is now available (WHO, 2004). In Amazonia, because harvesting *Croton lechleri* sap by tapping the trunk is time consuming and labor

intensive, these trees are usually cut down to obtain maximum amounts of the product. This is a weedy fast-growing tree and plantations are being established in Peru to accommodate commercial needs.

There is a wide variation in the types of cultivars that are grown for commercial purposes, with propagating stock or seeds being derived from customarily available sources or carefully selected to meet commercial standards needed to provide optimal potency of a desired bioreactive ingredient or ingredients. By applying chemical and ethnomedical dereplication methods, it is also possible to identify additional medicinal plants, which share similar or identical metabolic pathways and comparable medicinal uses. Although this knowledge may be used to introduce new marketable products, there are risks in promoting these as replacements unless their parameters of use and safety are well understood and comparable.

The amount of certain bioreactive compounds may be enhanced or transferred to another species by applying current genetic technologies, namely genetic modification (GM) (hence, the protectiveness of certain countries for its genetic resources). Although the utilization of GM may have its detractors (Uzogara, 2000), these techniques when applied correctly have the ability to increase a medicinal plant's potency or provide alternate sources for a rare and desired compound. The potential for the evolution of suitable plant crops as functional/medicinal foods has exciting possibilities such as in the creation of foods with enhanced vitamin content (e.g., yellow rice) or in the evolution of edible vaccines. However, altering medicinal plants in similar ways could also induce unexpected effects, and so their utilization as herbal medicines will have to be cautiously pursued (Littleton *et al.*, 2003; Raskin *et al.*, 2002; Tatlow, 2003; Wiley, 2003).

Regardless of the genetic resource, sourcing of quality raw medicinal plant materials depends on how appropriate agriculture and field practices are applied, the oversight policies in place in the country of origin, and how the manufacturer of the final product might detect overt problems. Nuances in cultivation, collection, harvest, post-harvest processing, transport, and storage can all have an impact on the value of the final product. Whatever the source, the worth and safety of a product may also be affected by overt or inadvertent adulteration with noxious plant species and inappropriate contamination with microbial, chemical (insecticides, herbicides, heavy metals, radio active substances), and insect and animal parts and excreta. Regional guidelines for the production of herbal products following standards of good agricultural practices (GAP), as recommended by the WHO (2003), are in the process of being instituted by the European Union (E.U.), China, and Japan. Also, to prevent insecticide and herbicide contamination, certain producers will claim to grow their herbs organically. The issue of permitting this labeling on products within the United States is now being

addressed under the province of the U.S. Department of Agriculture. The goal is to permit labeling that designates that production of certain herbs or finished "dietary supplements" has conformed to the National Organic Program (NOP). Presently, there is no assurance that similarly labeled products, produced elsewhere, conform to American standards outlined in the Organic Foods Production Act of 1990 (OFPA) (AHPA, 2004b).

Because cultural differences often dictate how diagnosis and efficacy are determined, regional variations in herbal remedies using the same flora are also likely to occur. Much depends on the origins of the medicinal systems that are being applied, the distribution and availability of particular plant species, and the outside influences that are affecting both formulation and application. Clearly, employing a remedy with optimal efficacy is the goal of any competent healer, and certain traditional treatments are often carefully designed for this purpose. Under ideal conditions, care is taken to correctly identify each ingredient, to harvest plants for optimal potency, to prepare the remedies under strict guidelines, and to prescribe them to achieve the best clinical response. For example, conventional Asian pharmacopeias are replete with formulations planned to specifically address this need by not only incorporating several plants that share the same bioreactivity, but also including other groups of plants with complementary activities. These practices are meant to accommodate expected variations in potency, to potentiate the efficacy of the treatment, and to prevent or anticipate any adverse reactions that may occur. However, this does not ensure that every practitioner will adhere to formulatory guidelines set out in these time-honored manuscripts. In both India and China, if certain species are unavailable, usually because of regional disparities, replacements with local taxa are not atypical (Elvin-Lewis, 2001). Also, a practitioner, without changing the name of a remedy, may alter the contents of a traditional formulation to accommodate the patient's specific needs. Such is the case with herbal preparations called "eternal life" (Sanders *et al.*, 1995). These practices can fundamentally alter the parameters of efficacy and make it challenging to understand the basis for any unusual or unfavorable events that may accompany these established treatments.

IV. REGULATORY ASPECTS

Globally, a wide range of conventional policies control the availability of herbal products to the general public. How this is accomplished depends on their derivation and whether they are categorized as medicinals, drugs, botanicals, or dietary supplements. The status of the regulatory situation as viewed worldwide in 1998 is available online (WHO, 1998). However, as

herbalism in all its forms continues to expand, it has become evident that the additional workable regulatory policies and guidelines are needed to ensure that commercial products are reliable, safe, and efficacious. Furthermore, the international community is becoming aware that the accelerated export of herbal medicines and medicinal plants between cultures and continents is creating the need to address these issues in a more proactive manner. In response to these concerns, the WHO (2004) has set forth guidelines to provide national regulatory bodies with appropriate advice. Although this achievement can be applauded, a universal consensus on how these principles might be applied has yet to be realistically achieved. Today, even in developed countries such as the United States, resources are insufficient to provide the surveillance and enforcement aspects that are necessary to prevent many of the scurrilous activities leading to negative health experiences that are inherent to today's practices in the herb trade industry.

A. WHO GUIDELINES

WHO guidelines on good agricultural and collection practices (GACP) for medicinal plants (WHO, 2004) is a comprehensive document that covers appropriate recommendations for good agricultural practices for growing medicinal plants. It addresses issues of plant identification/authentication, the nature of propagation materials, parameters of cultivation and protection, methods of harvest, and types of personnel that are needed. Included are acceptable practices for the appropriate collection of medicinal plants from the wild or cultivated plots. The need to comply with national and international laws regarding obtaining permits for collection and export, in addition to phytosanitation certificates, is stressed. It also outlines technical aspects governing post-harvest processing, bulk packaging and labeling, storage and transportation, the equipment required, quality assurance, documentation, and other relevant ethical and legal considerations associated with intellectual property rights, benefits sharing, protection of threatened and endangered species, and research needs. China, Japan, and United Europe (UE) are actively engaged in adopting these recommendations into their national policies.

In response to the need to better understand the parameters of safety and efficacy of traditional medical treatments, the WHO has prepared general guidelines to aid in the research and evaluation of these methods. This document presents useful methods that can be applied to the study of herbal medicines, procedure-based therapies, and clinical studies. It also includes discussions associated with pragmatic research issues, the ethics involved, the education and training of personnel, and the surveillance systems that might be imposed. It is emphasized that these studies be conducted in a

manner that ensures adequate safety for the subjects, and that prior to initiating the study every effort is made to identify any adverse events that might be encountered. These clinical trials must also be performed within the framework of the prevailing law in a given country or state, and whenever applicable, rescue treatments should be provided to patients given a placebo or unproven treatment (WHO, 2000).

To facilitate needed information exchange, the WHO is also generating monographs on selected medicinal plants. The format is designed to provide information on safety, efficacy, and quality control and to assist in the development of national monographs and formularies. These monographs are not pharmacopeial based but are designed to be "comprehensive scientific references for drug regulatory authorities, physicians, traditional health practitioners, pharmacists, manufacturers, research scientists and the general public" (WHO, 1999). In a regional context, a number of countries are attempting to "harmonize" their regulations to make common marketing of herbal products compatible. This is underway within UE nations and between Australia and New Zealand in what is referred to as the *Trans-Tasman Therapeutics Products Agency*. Challenges still remain because Australia regulates supplements as medicines-therapeutic goods, and New Zealand may consider the same products as traditional foods, enactment is not expected until mid 2006 (Anonymous, 2003).

B. UNITED STATES

In 1994, in response to concerns regarding lack of regulatory mechanisms to protect the public using herbal products, the Dietary Supplement Health and Education Act (DSHEA) was inaugurated. In its regulatory framework and enforcement mechanism, jurisdiction is shared between the U.S. Food Drug Administration (FDA) and the Federal Trade Commission (FTC). The FDA was provided with increased authority to remove unsafe products and regulate advertising claims regarding health benefits but was not required to approve the product before marketing unless it contained substances not grandfathered by use before 1994 (McGuffin and Young, 2004). The FTC was given the mandate to enforce deceptive practices of manufacturing and advertising, to investigate complaints, and to litigate against those violating trade regulations rules (McGuffin, 2002; Soller, 2000). However, by classifying medicinal herbs as dietary supplements, the DSHEA substantially altered the definitions, standards, and mechanisms under which claims of safety and efficacy could be enforced (Talalay and Talalay, 2000). Moreover, this definition tended to confuse the public about the herbs' safety and efficacy and failed to convey that appropriate oversight was lacking (Harris, 2003). As time passed, these policies have evoked

considerable consternation within the professional medical community who are concerned about the vague or oblique claims for health maintenance that are allowed, as well as the increasing number of serious adverse effects, including deaths, being reported. (Bent *et al.*, 2003). Added to this unease is their recognition that regulatory agencies have lacked the resources to respond in a timely manner to safety issues and counter misleading health claims. Many consider that the DSHEA has been ill conceived and that current regulations are limited and ineffectual. They propose that if the current system is to have any value for the consumer, radical changes in policies must take place, even to classifying some dietary supplements as drugs (Angell and Kassirer, 1998; Lipman, 2004; Marcus and Grollman, 2002).

Furthermore, appropriate regulatory oversight of Internet and media marketing practices continues to be a problem in controlling the sale of certain types of potentially problematic dietary supplements or herbal products. Consumers seem to be unaware that many vendors, regardless of their origins, are in violation of DSHEA guidelines or FDA rulings evoked to protect them. To promote these goods, inappropriate health claims are conveyed in such a way that it is obvious, regardless of the verbiage employed, that they are to be used to treat, prevent, diagnose, or cure specific diseases, including cancer (Bonakdar, 2002; Morris and Avorn, 2003). The widespread and inappropriate marketing of *Ephedra* products is but one example (Ashar *et al.*, 2003; Haller *et al.*, 2004) in which the FDA (2004c), should have acted more expeditiously but was bound by interference from lobbying and other interest groups from doing so until 2004 (Marcus and Grollman, 2002; Talalay and Talalay, 2000). Also, certain scurrilous vendors, in spite of notification from the FDA to discontinue these activities, will persist in aggressively promoting forbidden herbal products. Apparently they are willing to take this risk, to clear out inventories, because they believe that litigation is unlikely. There is a growing consensus that to curtail these illegal activities, substantial reform in advertising, regulation, and enforcement is overdue.

The FDA (2004a) is responding to these concerns by recognizing that it must be more proactive regarding issues of safety and enforcement of dietary supplements. Safety alerts for drugs, biologics, medical devices, and dietary supplements are posted through their MedWatch online system (http://www.vmcfscan.fda.gov/~dms/aems.html). The number of cases reported is but the "tip of the iceberg" and in all probability only represents about one-tenth of those actually existing (Marcus and Grollman, 2002). There is also acknowledgment that the overall safety profiles may be unreliable because certain types of unfavorable events tend to be reported more often than others. Nonetheless, it is its intention to improve evidentiary base FDA uses and implement these in a transparent, systematic, and predictable

process. Within this context, any new ''dietary ingredient'' must reach reasonable expectations of safety, which are based upon evidence other than history of use. Although current law requires that any new dietary supplement, not marketed before October 15, 1994, entail a pre-market safety notification to the FDA (AHPA Report, 2004; McGuffin and Young, 2004), improvements in regulatory oversight and enforcement, to cover all herbal products are a welcome addition.

The FDA is also reviewing certain botanicals via the Investigational New Drug/New Drug Application (IND/NDA) process. Priority will be given to those with a long history of safety, particularly for short-term use, because information is unlikely to be adequate to support claims of efficacy for long-term use (Elvin-Lewis, 2001; FDA, 2004b). This process in quite prolonged, and as of the end of 2004, about 228 pre-IND and IND applications have been received. Only half are active, and none of these are in review or have been accepted (Casper, personal communication, 2004).

They are also aware that quality control for manufacturers of dietary supplements must be established so that good manufacturing practices (cGMPs) are applied to ensure the identity, purity, strength, and composition of products that are sold. To expedite this process in 2002, the Dietary Supplements Methods and Reference Materials Program (DSMRMP) was established within the Office of Dietary Supplements (ODS) at the National Institutes of Health (NIH). Their mandate is to provide industry, researchers, and regulators with validated analytical methods and appropriate reference materials so that appropriate quality standards are developed to meet labeling claims and regulatory compliance directives, and that quality test materials are used in research (Saldanha *et al.*, 2004). Their strategic plan for achieving this goal by 2009 is now available (ODS, 2004). Unfortunately, it will take time to see whether these titular proposals do in fact serve their intended purpose. To complement these activities within the NIH, the Office of Alternative Medicine was established in 1992 and upgraded into the National Center for Complementary and Alternative Medicine (NCCAM) in 1998. This center is ''dedicated to exploring complementary and alternative healing practices in the context of rigorous science, training complementary and alternative medicine (CAM) researchers, and disseminating authoritative information to the public and professionals.'' It actively supports basic and clinical research to evaluate herbal treatments, as does other institutes at the NIH and other funding agencies. In this way, pharmacognosists, natural products chemists, and toxicologists are able to explore the therapeutic basis and safety parameters of herbal products. Computer Access to Research on Dietary Supplements (CARDS) identifies ongoing federally funded research being conducted on dietary supplements and

TABLE I
PROPOSED CLINICAL EVALUATION PROTOCOL FOR THE DEVELOPMENT OF AN HERBAL DRUG[a]

- Confirm ethnomedical value in country of origin
- Note all parameters of use, particularly among children, the elderly, or others with underlying disease states
- Review traditional formulations to understand rationale of use
- Know variations to standard formulations and reasons for additions or substitutions
- Identify bioreactive components to ensure standardization of content
- Conduct toxicological studies to understand safe parameters of use
- Conduct placebo-based clinical trials with formulation believed to be the best following appropriate guidelines for patient entry, evaluations of efficacy, and so on to comply with regulations where product is sold

[a]Amended from Table I in Elvin-Lewis (2001).

individual nutrients. Clinical evaluation protocols should include those parameters cited in Table I (Elvin-Lewis, 2001). To support these activities, another NIH database, the International Bibliographic Information on Dietary Supplements (IBIDS), can be accessed at the ODS web site (http://odp.od.nih.gov/ods) and contains several hundreds of thousands of citations and abstracts of published international scientific literature on dietary supplements including vitamins, minerals, and botanicals and is updated quarterly. In addition, the American Products Herbal Association (AHPA) has generated a number of useful reference books, namely *The Botanical Safety Handbook,* 2nd edition (McGuffin *et al.*, 1998), and *Herbs of Commerce,* 2nd edition (McGuffin *et al.*, 2000). In the first book, 600 commonly used phytomedicines are described in terms of their medicinal safety, common toxicities, international regulatory status, and standard dosage. The FDA accepts the *Herbs of Commerce* as the authoritative text for label nomenclature related to herbal products. The American Herbal Pharmacopeia and Therapeutic Compendium is preparing monographs of at least 2000 medicinal plants. Also, to aid pharmacists in understanding risks and benefits of herbal products, the U.S. Pharmacopeia (USP) (www.U.S.p.org) is also compiling standard monographs for herbal dietary supplements and dispensatory information (DI). Other references include the National Formulary (NF) (AAHP, 1998), the Homeopathic Pharmacopeia of the United States, the *Physicians' Desk Reference for Herbal Medicines* (PDR, 1998), and the *Encyclopedia of Dietary Supplements* (2005) (Elvin-Lewis, 2001). Following a review of available databases, Walker (2002) considers the Natural Medicines Comprehensive Database, AltMedDex, and the Natural Pharmacist

to be the best in providing suitable information regarding herbal products posed in clinical practice.

C. CANADA

On January 1, 2004, the Natural Health Products Regulations (NHP Regulations), which falls under the Food and Drugs Act, came into effect. These regulations are intended to standardize substances that are safe for over-the-counter (OTC) use by ensuring that these are appropriately manufactured, packaged, labeled, imported, and distributed according to the category of natural medicine to which they belong (e.g., traditional, homeopathic, botanical, or herbal). They will be allowed to make health claims according to structure function, risk reduction, and treatment, or in the case of vitamins and minerals, nutritional support. A health claim requires proof of a causal relationship established by careful scientific evaluations and unambiguous conclusions between a nutrient, drug, or other compound and a disease condition. In cases in which long-term uses of traditional products are known to be safe, Standards of Evidence (SOEs) may be supported solely by traditional references, and most so state on the labeling. There is no distinction between these natural products and a synthetic product manufactured in a facility. Because of either a medical claim, a pharmacological effect, or both, they are classified as drugs and a drug identification number (DIN) will be required. Some may be available only on prescription. Product labeling should be clear and consistent, outlining the contents, source of materials, parameters of storage, its recommended use or purpose, routes of administration, and dosage, in addition to stating any known potential health risks, cautions, warnings, contraindications, or possible adverse effects. Product license holders are required to report all serious adverse effects within 15 days of the event, whether they occurred in Canada or elsewhere.

It is compulsory that new products be licensed with respect to safety, efficacy, and quality. Supporting evidence, based on a risk management approach, may be derived from information in the Canadian Compendium of Monographs or elsewhere. The format of these monographs will follow those already established by the WHO and European Scientific Cooperative on Phytotherapy (ESCOP) and the European community. Understanding the entrepreneurial nature of the industry, Health Canada is willing to provide guidance to small and medium-sized businesses engaged in these endeavors. It will also support original research and capacity building. To enable companies to develop SOEs, supporting safety and efficacy of their products, a transition period ranging from 2 years (for site licensing) to 6 years (for licensing of products already issued a DIN) is being allowed.

The National Health Products Directorate and Health Products and Food Branch of Health Canada believe these new policies, when fully enacted, will serve the consumer well. By meeting quality and safety standards based on good manufacturing practices and only allowing health claims supported by appropriate levels of evidence to accompany natural health products, the basis for rational use has been established (Health Canada, 2004).

D. AUSTRALIA AND NEW ZEALAND

Similar strategies that generally follow European Economic Community guidelines for registration, production, evaluation, and marketing of safe natural health products was established in Australia in 2004 and follow the Australian Regulatory Guidelines for Complementary Medicines. In New Zealand, there are two ways herbal medicines may be manufactured and sold according to the Medicines Act of 1981. Licensing is not required for individualized formulations designed for a specific purpose, which are identified only by their content, but are not accompanied by information regarding use. Alternatively, marketing approval is required for herbal medicines not in that category, and these must meet assessment standards outlined for proprietary medicines by the New Zealand Therapeutics Assessment and Utilization Section of the Department of Health (WHO, 1998a). Harmonization of these two policies through the TTTPA is currently underway (Anonymous, 2003).

E. EUROPEAN ECONOMIC COMMUNITY

Many countries within the UE do not distinguish between medicinal products made from chemical substances and those made from plants or natural material and consider them drugs or medicines, whereas others have a more liberal approach to how uses of herbal remedies may be applied and sold. When defined by presentation or function as a medicinal product and manufactured for commercial purposes, they must now be authorized for marketing. Some form of reconciliation will have to be made so that categories, which are in existence, are appropriately integrated into the system. For example, in Britain three categories of herbal products are available: those that are licensed, others that are exempt from licensing, and dietary supplements, which are unlicensed (Shaw *et al.*, 1997). Germany, Sweden, Denmark, and Switzerland have established regulatory policies regarding the evaluation of herbal products, and the Netherlands, and Portugal regulate them as pharmaceuticals and require GMPs and Good Agriculture Practices to be applied (Bent *et al.*, 2004). The Committee of Human Medicinal Products working with the ESCOP is empowered to harmonize

these inconsistencies and to identify problematic formulations that are likely to cause adverse reactions or promote carcinogenicity. Over the last few years, they have become aware of the meteoric increase in adverse reactions being reported and the need to educate the public more effectively as to the parameters of use of herbal products. The WHO has suggested that information on efficacy, safety, and contraindications be widely disseminated by the mass media and on the Internet (WHO, 1999, 2004).

To aid in these endeavors, the E.U. parliamentary Directive (2004/24/EC) has established a code related to the traditional use of safe herbal medicinal products. This code is being transformed into national law by the 25 E.U. member countries. It introduces a simplified registration system that differentiates between herbal medicinal products that have been marketed in Europe for more than 30 years from others that have 15-year history of use in Europe, and another 15-year use elsewhere. These products are designed to be available to the public without medical supervision, and thus, parameters of preparation, labeling, safety, and use are carefully circumscribed through the generation of community herbal monographs. During the process of establishing these community-generated documents, it is permitted to use other suitable monographs (e.g., German Commission E), publications, or data. For possible extension, the European Parliament will assess this traditional-use registration in 2007 (Cox and Roche, 2004). Long-established use in Europe is a precondition for registration, so it has yet to allow registration of traditional herbal medicines derived from other well-known systems, particularly of Asian origins (e.g., Ayurvedic and chinese traditional formulas [Hasslberger, 2004]).

F. ASIA

Regulatory and surveillance methods are far from consistent within countries using Asian traditional medicines, and significant risks on populations dependent on any of these alternative methods of phytotherapy still exist.

1. India

In India, the Department of Indian Medicine has been renamed the Department of Ayush, covering the traditional practices of Ayurveda, Yoga, Unani, Siddha, and Homoeopathy. This is the first step toward establishing mechanisms by which appropriate mechanisms of evaluation and standardization of herbal products can be achieved so that safety, efficacy, and quality can be ensured and markets elsewhere logically established (Anonymous, 2004; Kurvilla, 2002; Ravinder *et al.*, 2003).

2. *People's Republic of China*

In 1984, the People's Republic of China implemented a Drug Administration Law, which classified traditional Chinese medicine (TCM) herbal remedies as "old drugs," and except for new uses, were exempt from testing for efficacy or side effects. Efficacy is evaluated on "empirical facts or experience" as exemplified by reference data, clinical test reports, and so on, rather than by the pharmacological action of each ingredient (WHO, 1998a). Crude materials and medications recorded in the Chinese Pharmacopeia are approved for safe use if applied according to Chinese medical philosophy and practice (Chan, 2003; the Pharmacopoeia Commission of PRC, 2000), and new herbal products are regulated through the Chinese Ministry of Public Health (Gilhooley, 1989). OTC licenses are required only for Chinese medicinal materials that are in proprietary form, not in their raw or processed state. OTC sale and export of Chinese medicines is common, but supervision is lacking to ensure that they are being ethically conceived. Within this context, it is believed that risks of adverse reactions can be minimized when qualified practitioners use quality products (Hohmann and Koffler, 2002), and ongoing surveillance of adverse reactions identifies problematic practices so that preventive measures can be instituted (Chan, 1997). However, to ensure that consistent parameters of safety and efficacy are known, it has been proposed that quality control of these remedies or new herbal products be achieved by using modern analytical and chemical techniques (Feng and Xie, 2003; Lee, 2000). In addition, quantitative and qualitative data will be needed if evidence-based studies are to have any value (Critchley *et al.*, 2000).

There is a wide variation on how Chinese medicinal formulas are manufactured or formulated outside of China. For example, in Malaysia, the Drug Control Authority requires registration of herbal medicines, which must be prepared according to standards of conformity and safety. In Singapore, regulatory control of Chinese proprietary medicines is governed under several state statutes regarding their appropriate manufacturing, promotion, and sale. These rules carefully outline which ingredients are prohibited and impose limits on the amounts of other substances such as heavy metals that are permitted. Licensing is required for all who are involved in their production and sale. Surveillance is also conducted to ensure that formulas containing toxic heavy metals and undeclared drugs are excluded from the market place (Koh and Woo, 2000). In contrast, there is little control over the manufacturing of Chinese formulas in the Philippines (Hartigan-Go, 2002). In Australia, to provide guidance on the level of anticipated toxicity associated with their use, the development of a TCM toxicology database and monographs is underway (Bensoussan *et al.*, 2000). Health Canada is examining the issues of recognizing TCM as a profession

and identifying practices that would be permitted. It differentiates between TCM products manufactured and requires a DIN and those formulated by an individual practitioner. In the latter case, it recommends that provincial regulations be established to determine which substances with safety concerns can be dispensed by health practitioners in compliance with provincial legislation. To aid in this endeavor, it has developed a list of candidates where safety concerns have been identified and for which an acceptable benefit–risk ratio for non-prescription sale has not been demonstrated (Chisholm *et al.*, 1998). Concerns have also been raised in the United States regarding the inadequacy of laws and regulations to ensure that these products are manufactured under the highest standards, that manufacturers are appropriately licensed, their products appropriately evaluated, and that surveillance systems are in place to ensure that consumers are adequately protected (Ko, 2004). The E.U. has yet to address the issue, because it is involved with harmonizing its policies regarding the use of regional pharmacopeias (Hasslberger, 2004).

3. Japan

Japan is also addressing regulatory issues and is actively engaged in developing a Japanese Pharmacopoeia so that the quality, efficacy, and safety of each product, regardless of its source, are appropriately delineated. Quality control is regulated under Japanese government's policies for "Manufacturing Control and Quality Control of Drugs," and its regulations regarding developing countries' herbal medicines are widely regarded. Claims and rules of combinations are determined on the basis of the pharmacological actions of each ingredient. The Pharmaceutical Affairs Law, designed for prescription and OTC drugs, regulates both herbal medicines designated "quasi-drugs" or "food medicines," as well as Kampo medicines, which are categorized "prescriptions/ethical" and sometimes called "Shosaiko-To" (Anonymous, 1998). Depending on their components, doses, use efficacy, and directions of use, some of these "Shosaiko-To" formulations and Chinese herbal formulations meeting appropriate safety standards may also be sold OTC. The Ministry of Health and Welfare (MHW) registers Kampo drugs, and its acceptance has taken place without clinical validation studies. Nonetheless, about half of conventionally trained Japanese physicians prescribe Kampo medicines, and many more do so for Chinese formulations (Tsumara, 1991; WHO, 1998a). Within Japan's regulatory format, herbal–pharmaceutical combinations for a wide range of products are also allowed (Saito, 2000). Adverse drug reactions are reported voluntarily, through a Pharmacy Monitoring System, and an Adverse Reaction reporting from Manufacturers (WHO, 1998a).

V. WHAT IS AN HERBAL REMEDY?

In definition, herbal remedies used as medicines may be traditionally or serendipitously derived, varying in formulation, preparation, and standardization, sometimes unreliable as to plant identification or to chemical composition, and depending on their cultural source, infrequently validated, in conventional ways, as to efficacy or safety. They may be prescribed by a healer of experience and training or of questionable skill, or they may be used in self-medication. As exemplified in American and African indigenous populations, prayers, mantras, or other forms of healing ceremonies may be used as an adjunct to phytotherapy. The applications of "energy medicine" to potentiate the curative process are still poorly understood (Elvin-Lewis, 2003, 2004).

Depending on its traditional origin, a traditional phytotherapeutic remedy may consist of individual or mixed formulations of plant material and sometimes other products used together or sequentially as infusions, decoctions, tinctures, poultices and salves, eye, ear and nose drops, enemas, purgatives, suppositories, emetics, snuffs, vapor baths, inhalants, oral hygienic or healing aids, and injectables. One plant itself may contain a significant bioreactive compound or a host of bioreactive molecules related or otherwise that will circumscribe its usefulness as a phytotherapeutic agent (Elvin-Lewis, 2001). Only rarely will any medicinal plant yield a compound such as quinine, which is low in toxicity, potent, and unique enough to be worthy of becoming a candidate for pharmaceutical development (Elvin-Lewis, 2003; Lewis and Elvin-Lewis, 2003). Most often, a medicinal plant is likely to contain several useful but less active molecules that can work together to potentiate the totality of its efficacy and safety profile. If used as an anti-infective herbal, for example, several related anti-infective compounds may prevent the development of resistance (Lewis *et al.*, 2004b). Traditionally, uses of plants with known toxic characteristics are carefully circumscribed. It follows that when several plants are employed in combination, usually representing complementary activities, additional optimal therapeutic responses are achieved. This form of combination therapy may elicit additive or synergistic effects that are not apparent when the bioreactive compounds are used independently of one another. The methods described in the ancient pharmacopeias of Asia exemplify this latter approach. Using plants in a sequential manner to affect the same goal is also traditionally applied, and many examples of this nature can be found in certain African and South American medicinal systems (Burkill, 1985, 1994, 1995, 1997, 2000; Elvin-Lewis, 2001, 2004).

It should be emphasized that pharmacopeias are not static entities but are constantly evolving. Those of current Neo-Western origin may incorporate

both traditional and novel uses of phytomedicines, including combining plants from a number of disparate medicinal systems, as well as pharmaceutically active ingredients. A similar trend is occurring in countries that use traditional Asian formulations. Also, as contact increases between isolated indigenous groups and the outside world, modifications to the uses of their medicinal plants are taking place. In addition, some scurrilous entrepreneurs may "invent" a traditional use to promote the sale of a particular plant product. They may go as far as introducing the idea into communities still depending on traditional herbal medicines, so in a few months or years, it appears that it has been conceived in the traditional manner. In a similar fashion, the media may be responsible for promulgating the same sort of fictitious concepts (Lewis *et al.*, 2004). Within this developing global context, one can expect to find deviations in appropriate plant identification, nuances of formula preparation, dosage, and types of treatment regimens, as well as the introduction of herbal remedies lacking authenticated traditional uses. Valuable information regarding safe parameters of use and related adverse reactions might also get lost during this transition. Depending on the regulatory systems imposed, the packaging information accompanying the commercial product may not accurately reflect the quantity and quality of the ingredients or verify its medicinal value. Unless there is a rational reason to believe otherwise, the safety and efficacy of any newly evolved phytotherapy, particularly if it represents a novel polyherbal mixture, must be considered circumspect, until appropriate clinical evaluations can be made.

However, when traditionally applied, most customary formulations derived from recognized pharmacopeias are usually moderate in potency and toxicity, are not fast acting, and when compounded and used appropriately, are unlikely to cause many life-threatening events. Because they are not physiologically inert, adverse reactions can be expected and are probably underreported, especially in parts of the world where mechanisms to acquire such data are not in place or when reporting methods are not user friendly. Generally, these events are unworthy of eliciting major concerns and are an accepted consequence of treatment. Most of these undesirable conditions are considered mild and transitory, causing rashes, headaches, or gastrointestinal (GI) upsets. Linkages to use are more difficult if unfavorable responses are rare, develop gradually, or occur after protracted use. Predictable risks are likely to occur when appropriate regulations do not mandate the proper formulation and labeling of herbal products or when self-medication fosters abuse. Acute toxicity is readily detectable, and accumulating evidence has linked a number of serious or life-threatening events to certain noxious ingredients in traditional (mostly Asian) formulations, to inappropriate or prolonged use, and to the incorporation of adulterants, undocumented pharmaceuticals, or interactions with other medications (Elvin-Lewis, 2001).

In addition, unwanted side reactions are likely to be more prevalent among those that tend to prefer the plethora of new untested and unregulated products available. Unfortunately, these individuals, who frequently self-medicate, are unlikely to report any undesirable incident because it would impugn their judgment and beliefs that herbal products are safe, that the contents and packaging claims regarding use and value are accurate, and that the benefits they hope to obtain are otherwise unavailable.

What is herbalism? Herbal medicine or phytotherapy as practiced today can represent the unique and still private knowledge of a group of indigenous people, the widespread use of traditional knowledge recorded in ancient pharmacopeias, modern monographs, and various ethnobotanical sources, as well as the employment of novel formulations that can incorporate one or more known or unknown medicinal herbs or other ingredients. Knowledge and self-medication may be widespread within a population, known to only a few specialized healers through oral tradition, or applied by trained practitioners using well-documented formulations outlined in the long-established pharmacopeias of Asia or current Western monographs. Today, herbalism is categorized as belonging to four major types: Indigenous, Asian, European, or neo-Western (De Smet, 1993; Elvin-Lewis, 2001).

A. INDIGENOUS MEDICINE

Practices of indigenous medicines occur in circumscribed parts of the world where traditional knowledge is still honored (Amazonia, sub-Saharan Africa, Oceania, etc.). Phytotherapy is very much a part of these customary practices, and it usually follows that when a remedy is widespread in acceptance, its efficacy and safety has a sound therapeutic basis. As evidence of this worth spreads, it is not unusual for it to be incorporated into other established forms of herbalism or phytotherapy. Neo-Western herbalism is particularly prone to adopting these remedies.

B. EUROPEAN TRADITIONAL MEDICINE

European traditional medicine evolved from practices known to ancient Mediterranean civilizations of Egypt, Greece, and Rome, as well as the Muslim world. Their use of medicinal plants is reflected in several noteworthy manuscripts derived from these times, such as the Ebers Papyrus (1500 BC), the De Materia Medica of Disocorides (first century AD), and the text of Jami of Ibn Baiar (eleventh century AD) (Ackernecht, 1973). These phytotherapies and others incorporated from regional indigenous uses throughout Europe, and elsewhere, represent the broad spectrum of practices

represented in the ways modern-day European herbalism is practiced. Although self-medication is widespread, reliance on professionals to provide guidance in diagnosis and treatment is also common. Within the countries that encompass the E.U., practicing herbalists are more likely to undergo formal training, belong to guilds or like organizations, and are required to be registered. Similarly, phytotherapy is also a part of the practice of the professions of Naturopathy, Homeopathy, Chiropractic Medicine, and in some countries, Allopathy (also called *conventional medicine*). For example, in Naturopathy, formulations containing plant extracts or phytochemicals, primary derived from well-known ''gentle herbs,'' are prescribed at pharmacognotically determined levels of efficacy. Uses of herbs by conventional physicians are likewise prescribed, particularly if the treatment requires close surveillance. This is particularly true in Germany, where the Commission E Monographs are used for guidance (Blumenthal *et al.*, 1998, 2000). Homeopathic formulations differ in that they are likely to contain very dilute concentrations of plant extracts, which in higher doses may excite the opposite physiological effect (Steinberg and Beal, 2003). They are far from inert and are known to have the potential to cause a broad spectrum of minor adverse reactions (Franklin, 1999; Glisson *et al.*, 1999; Shaw *et al.*, 1997).

C. NEO-WESTERN HERBALISM

In its totality, European herbal practices have matured, along with American herbal introductions into neo-Western herbalism. The herbal practices that have evolved may represent an amalgamation of many philosophies of treatment and include the use of a variety of novel formulations containing herbal products from disparate cultures and parts of the world. The adoption of herbal uses derived from indigenous medicines is widespread. However, the inventiveness that is inherent to this ever-evolving system continues to challenge regulatory bodies wanting to ensure that these products have value and are not dangerous. Many of these types of products, often disguised as dietary supplements, are available to the self-medicating consumer as unregulated OTC products or on the Internet. Unfortunately, this group of users, often with limited knowledge of herbal medicine, is particularly vulnerable to unsubstantiated promotional claims. Moreover, many in this category have a tendency to rely on unqualified sales personnel or self-appointed ''herbalists'' to guide them, rather than to seek help from appropriately trained and knowledgeable individuals who are professionally recognized and licensed to practice this art.

Unlike their European counterparts, North American herbalists are more likely to be self-taught or to be trained in an array of apprenticeship

programs, many are associated with the American Herbalists Guild or North American Herbalists Guild. These organizations are working toward certification processes so that standards of practice are involved in membership. Several schools of Naturopathy exist in the Unites States and Canada, who train their practitioners to be primary care physicians with knowledge of phytotherapy, homeopathy, and acupuncture. Their holistic approach uses conventional medical diagnostic procedures and therapeutic modalities, as well as natural methods of diagnosis and treatment in an integrative approach to health care delivery. Currently, 12 states and the U.S. territories of Puerto Rico and the U.S. Virgin Islands and 8 Canadian provinces have licensing laws for naturopathic doctors. North American chiropractors also promote uses of certain herbal products. In the nineteenth century, American Eclectic Physicians used phytotherapy in their practices. Many of their therapies were derived from Amerindian herbalism, including the use of *Echinacea.* Today, this herb, which is highly regarded for immune stimulating purposes and to treat colds, is the most popular remedy in North America and Europe (Elvin-Lewis, 2001; Lewis and Elvin-Lewis, 2003).

D. ASIAN TRADITIONAL PHYTOTHERAPEUTIC MEDICINES

Asian traditional medicines are based on ancient pharmacopeias such as the Characka Samhita and Sushruta Samhita of Aryuveda, the Classic of Materia Medica of First Century China, and the Daido Ruiju-ho of Japan, which were written about 2000 years ago and are derived in part from religious healing philosophies of that time, which included the Upanishads (Saito, 2000). Contact between these cultures occurred very early and is reflected in similar concepts of diagnosis, formulation, and treatment that are outlined in these treaties. Noteworthy are the uses of complex formulations, oftentimes only varying in nuance from one system to the other, the presence of plants from disparate parts of Asia, as well as the inclusion of minerals and animal parts as a part of some of the mixtures. Within the Asian context, these medicines are referred to as *drugs.* The components of these mixtures are designed to complement each other so that a satisfactory therapeutic outcome is achieved. What makes these medicinal systems distinctive are the ways regional herbs are incorporated or how methods of diagnosis and treatment are employed.

Throughout Asia, a variety of traditional systems coexist along with conventional medicine, and it is not unusual for patients to rely on several of these methods for maintenance of health or treatment of a particular ailment. In India, for example, several types of practitioners may conjointly share the same hospitals and certain types of training. Like their

conventional counterparts, practitioners of these systems are carefully schooled in the art and science of their profession and are trained to formulate their remedies under strict rules, to prescribe them with care so that therapeutic outcome is predictable, and to anticipate the types of adverse reactions that might arise. Predominant traditional and alternative systems of medicine in India include Ayurveda (Hindu), Siddha (Jain), Unani (Arabic), Yoga, as well as the Tibetan system and those of European origins such as Naturopathy and Homeopathy; together, these systems are referred to under the acronym "AYUSH." Similar choices are available in neighboring countries, and their prevalence is often influenced by the dominant religion of the nation (e.g., Pakistan and the Unani system).

The Chinese Medicinal System continues to dominate traditional medical practices throughout Eastern Asia, Southeast Asia, and the South Pacific regions. Its early spread to Korea and then to Japan occurred between the fifth and seventh centuries AD, respectively. Kampo medicine of Japan is but a simplified derivation of this system, but also includes native plants from indigenous origins. The incorporation and/or substitution of local species into many of these remedies also occur elsewhere. Because modern Chinese traditional medicine is not a static entity, new formulations may include medicinal plants favored throughout the world. These changes are reflected in the 1977 version of the *Chinese Materia Medica* as compiled in the *Encyclopedia of Traditional Chinese Medicine Substances* (Zhong yao da ci dian) (Bensky and Gamble, 1993), and the Pharmacopoeia Commission of PRC (2000). Other sources are also available (Leung, 1990; WHO, 1989). The WHO monographic series is also targeting related Asian pharmacopeias, and several volumes describing these medicinal plants are available for Korea (WHO, 1998b), the South Pacific (WHO, 1998c), and Viet Nam (WHO, 1990, 1999); Japan is developing a pharmacopeia as well (Saito, 2000).

VI. ADULTERATIONS

Within the current global regulatory environment, the detection of adulterated or contaminated herbal products is not always an easy task, particularly because many policies to prevent these events have yet to be fully implemented and ways to identify the problems are not always easy to achieve. The presence of undocumented pharmaceuticals, heavy metals, misidentified plants, noxious plant compounds, and so on is still widespread, particularly in those formulations of Asian origins (Chan, 2003; Elvin-Lewis, 2001; Ernst, 2002b). Also, many Asian medicines traditionally incorporate substances such as heavy metals, which under today's standards of safety should

probably be disallowed or restricted to very low levels. For example, in Singapore legal limits have been set for arsenic at 5 ppm; for copper, 150 ppm; for lead, 20 ppm; and for mercury, 0.5 ppm. They also provide guidelines for permissible numbers of bacteria, yeasts, and molds (Koh and Woo, 2000). Malaysia has also adopted similar guidelines (Ang, 2004). Unfortunately, because of poor quality control during the manufacturing process of air-drying and preservation, certain heavy metals may be present in unusually high concentrations, further compromising the health of those who use them (Chan, 2003; Wong *et al.*, 1993). Other contaminants may be introduced advertently or inadvertently during the manufacturing process, or when storage permits insect infestations or growth of microorganisms. Ideally, herbal remedies should be free of undeclared substances such as pharmaceuticals and plant substitutions, toxic botanicals, metals, and radioactive agents, excessive levels of microorganisms or their toxins, as well as insects, pesticides, herbicides, or fumigation agents.

A. HEAVY METALS

Incorporation of certain heavy metals into Asian traditional medicines is based on the common time-honored belief that metals such as lead, copper, gold, iron, mercury, silver, tin, and zinc are needed for appropriate bodily functioning and that any imbalance can result in disease (Ernst, 2002a). A number of beneficial effects are considered to be associated with their use. Formulators understand that some of these metals are noxious, and therefore, elaborate ways of "detoxification" have been evolved so that they can be "safely" incorporated into their formulas. According to these formulary philosophies, this might be achieved by the combination of other ingredients in the herbal remedy or by subjecting the metal to a series of multifaceted treatments. For example, in Aryuveda, processed lead or "nagabhasma" is produced by subjecting it to a pharmaceutical procedure that involves repeatedly heating the metal until it glows and alternately dipping it in several herbal mixtures before it is combined with arsenic sulfide (Thatte, 1993). Unfortunately, these heavy metals are often present in traditional formulations in excessive amounts due to inherent problems in preparation, deliberate inclusion, or through contamination during the manufacturing process such as the use of metal pots (Yu and Yeung, 1987) or grinding weights (Chia *et al.*, 1973). Another source of adulteration may be via herbs grown in metal-rich soils (Schilcher, 1983).

Clinical poisonings have been reported with herbal or ethnic remedies rich in mercury and lead and other heavy metals from the Far East, India, the Middle East, South Africa, Australia, Western Europe, and North America. Symptoms related to heavy metal intoxication are not always distinctive,

many of which are constitutional, and blood levels may have to be determined to identify the problem. A linkage to the use of a suspect herbal medicine is imperative. For example, lead poisonings may cause abdominal and GI distress, constipation, chest pain, anemia, or seizures; arsenic may lead to hyperkeratosis and muscle wasting; and mercury to weight loss, diarrhea, skin rash, motor and vocal tics, and paraesthesias (Ernst, 2002a).

Mercury poisonings are particularly prevalent among those consuming TCM formulations containing excessive amounts of either red mercuric sulfide (cinnabar, cinnabaris, zhu sha) or mercurous chloride (calomel). Lead can exist as Mi Tuo Seng (Lithargyrum) and has also been found in an Malaysian herbal preparation containing *Smilax mysotiflora* (Ang *et al.*, 2004), in a Loaotian preparation known as Pay-loo-ah, a Korean remedy, Hai ge fen, containing clamshell powder (Borins, 1998), in Taiwan a TCM Cordyceps remedy (Wu *et al.*, 1996), in Indian traditional cosmetics used as eyeliners (surma) (Shaw *et al.*, 1997) and lead containers for toothpaste (Elvin-Lewis, 2001); arsenic compounds are incorporated in various herbal remedies including Xiong Huang (Realgar); and copper as Dan Fan (Chalcanthitum) (Koh and Wood, 2000). Adulteration with cadmium and thallium is also disconcerting, as evidenced by the significant number cases of cadmium and lead intoxication in Texas consumers of "chuifong tokuwan," who had imported the product from Hong Kong (Anonymous, 1989). Alopecia and sensory polyneuropathy was associated with thallium in a Chinese herbal (Schaumburg and Berger, 1992). Manganese poisoning with severe chorea followed the ingestion of Chien Pu Wan in Holland (De Krom *et al.*, 1994; Wauters, 1995). Lists of patented Chinese medicines containing heavy metals, sometimes in excess, have been generated in a number of studies and case reports (Au *et al.*, 2000; Kang-Yum and Oransky, 1992; Koh and Wood, 2000; Liang *et al.*, 1998; Wong *et al.*, 1993) and their toxic effects described (Dukes, 1996; Ennever, 1994; Klaassen *et al.*, 1996; Olson, 1999; Ueng *et al.*, 1997; Wu, 1992). A case of severe dyserythropoiesis and autoimmune thrombocytopenia was associated with ingestion of kelp supplements for slimming, contaminated with arsenic (Pye *et al.*, 1992).

Within Indian traditional pharmacopeias, there is also a preponderance of lead- and mercury-containing formulations, although significant amounts of arsenic and to a lesser degree cadmium may also be found (Kew *et al.*, 1993; McElvaine *et al.*, 1991). Symptoms may represent that of mixed intoxication or reflect one of the heavy metals that are present (Kew *et al.*, 1993; Sheerin *et al.*, 1994). Lead poisonings are particularly prevalent, in part because these toxicities are more readily diagnosed, and because children are especially vulnerable to experiencing serious reactions or life-threatening events (Ernst, 2002a). Unfortunately, parents who have provided Indian remedies to their children are often reticent to reveal the source of

the intoxication in time to provide needed chelation therapy (Eisenberg *et al.*, 1998). Use of local traditional medicines has also been linked to acute lead encephalopathies among infants in Middle East (al Khayat *et al.*, 1997) and to other types of lead poisonings in South America (Levitt *et al.*, 1984). The seriousness of this widespread problem is outlined in a number of interesting case reports (Ernst, 2002a,b).

B. DOCUMENTED AND UNDOCUMENTED PHARMACEUTICALS

In Asian markets, it is not unusual to find traditional herbal remedies containing undocumented pharmaceuticals. However, these illicit practices are now widespread, and serious adverse effects linked to their use have become commonplace (Bury *et al.*, 1987; De Smet, 2002, 2004; Ernst, 2002c; Koh and Woo, 2000). Table II indicates the wide range of undocumented drugs that have been identified, but these are only representative of the possibilities that likely exist. In the current regulatory environment, it has become a monumental task to detect these types of adulterations, and constant surveillance is required to ensure the safety of traditional medicinal products that can be adulterated in this fashion. This concept is compounded by the fact that in certain countries, such as Japan, combinations

TABLE II
EXAMPLES OF UNDOCUMENTED DRUGS FOUND IN HERBAL REMEDIES

Category	Type
Analgesic, antiinflammatory	Aminophenazone, aminopyrine, diclofenac, dexamethasone, dipyrone, phenylbutazone, phenylbutazone, indomethacin, fluocinolone acetonide, diclofenac, mefenamic acid, methyl salicylate, paracetamol, (acetaminophen) ibuprofen
Corticosteroids antiinflammatory	Clobetasol propionate, fluocinonide
Antihistamine decongestant	Chlorpheniramine, cyproheptadine, promethazine
Weight loss	Fenfluramine
Depressants	Barbiturates, bromide
Antipsychotic	Benzodiazepines diazepam
Diuretic and hypertensive	Hydrochlorothiazide
Glucose lowering	Glyburide (INN, glibenclamide) phenformin
Blood thinner	Warfarin
Erectile dysfunction	Sildenafil (Viagra) and tadalafil (Cialis)
Sedative	Estazolam (benzodiazepine)

of herbal drugs with vitamins or pharmaceuticals may be sold legally as OTC preparations. This is only allowed if they are appropriately labeled and their formulations are described in *Drugs in Japan, OTC Drugs (2000–2001),* 12th edition (Japan Pharmaceutical Information Center, 1998). Various adverse reactions can be associated with these drugs, particularly if they are being taken for prolonged periods before they are detected. For example, agranulocytosis with septic shock was a common symptom of a number of cases exposed to aminopyrine, phenylbutazone, and dipyrone; Cushing's syndrome with dexamethasone, indomethacin, hydrochlorothiazide, diclofenac, mefenamic acid, and diazepam; eczema with fluocortolone; coma with phenytoin; excessive increase of International Normalized Ratio (INR) or prothrombin time (PT) with oil containing 50% methyl salicylate; GI tract bleeding, ulcer, and somnolence with mefenamic acid and diazepam; and ecchymotic/purpuric skin lesions with prednisolone and indomethacin; and fatal lactic acidosis with phenformin as described in detail by Koh and Woo (2000) and Ernst (2002c). In addition, hepatic failure leading to liver transplantation or death has been linked to the *N*-nitroso-fenfluramine content in "chaso" and "onshido," marketed in Japan for weight loss (Adachi *et al.*, 2003). The FDA has also issued warnings regarding maternal consumption of "Sleeping Buddha" because this herbal preparation also contains estazolam, a known teratogenic agent. Also a current spate of so-called herbal sexual enhancement products (Vinarol, Viga, Sigra, Stamina Rx, Stamina Rx for Women, Y-Y, Spontane ES, Uroprin, Acta Rx and Yilishen) has been promoted (primarily on the Internet) for erectile dysfunction or as a female equivalent in North America and China (Wooltorton, 2002). Many of these have been found to contain significant levels of prescription phosphodiesterase inhibitors: sildenafil (Viagra) and tadalafil (Cialis), which affect blood flow and thus present serious risks to consumers who have underlying medical conditions such as cardiovascular disease and are on medication with drugs such as nitrates (Mitka, 2003; Thurairaja *et al.*, 2004). The FDA has issued several warnings in 2003 and 2004 regarding their use.

C. PESTICIDES AND FUMIGATION AGENTS

The clinical relevance of the presence of pesticides and fumigation agents in herbal products is unknown. Many may be present in excessive amounts and thus present a potential risk to the consumer. These include the chlorinated and organic pesticides, carbamate insecticides and herbicides, dithiocarbamate fungicide, and triazine herbicides. For example, exposure to Baygon and other carbamate-based insecticides *in utero* has the potential of causing MLL gene fusions (translocation of MLL gene on chromosome

11q23 and its fusion with AF4 on chromosome 4 and ENL on chromosome 19) leading to the development of infant acute leukemia (Alexander *et al.*, 2001). Fumigation agents include ethylene oxide, methyl bromide, phosphine, and sulfide-yielding agents (De Smet, 2002, 2004). The character of many of these substances is described in detail in Lewis and Elvin-Lewis (2003).

D. PATHOGENIC MICROORGANISMS (BACTERIA, FUNGI, VIRUSES) AND TOXINS

It is the nature of raw herbal products or unprocessed food products not to be sterile. Most of the organisms present are saprophytic; however, there is always the possibility that potentially pathogenic organisms can be acquired from a wide variety of sources from the environment, during cultivation, and preparation (Kneifel *et al.*, 2002). Also, if storage is improper or prolonged, certain organisms may multiply or elicit toxins to dangerous levels (Lewis and Elvin-Lewis, 2003). A number of studies on herbal teas and/or medicinal plants in Europe (Holt, 1998), Nigeria (Efunotoye, 1997–98), Japan (Hitokoto *et al.*, 1978), and Argentina (Rizzo *et al.*, 2004) indicate that a wide range of fungal contaminants may be present, and that in some cases mycotoxins (aflatoxin, sterigmatocystin, and ochratoxin A) can also be found. In all these studies, a significant proportion of the samples contained species of *Aspergillus* (with their mycotoxin-eliciting potentials), *Mucor,* and *Penicillium.* A few samples also contained *Rhizopus, Absidia, Alternaria, Aureobasidium*, *Cladosporium, Fusarium,* and *Trichoderma.*

Inhalation of these spores during preparation of these products can be problematic for certain predisposed individuals. For example, *Alternaria, Aspergillus, Cladosporium, Fusarium,* and *Penicillium* are important fungal allergens (Lewis and Elvin-Lewis, 2003), and sensitized atopic individuals are potentially at risk from these sources. However, because of their ubiquity, linkage to an adverse event would be difficult unless appropriate and accurate forms of molecular analyses were applied. In one case, using polymerase chain reaction (PCR) techniques, a naturopathic remedy contaminated with a specific strain of *Mucor indicus* was implicated in the user's development of hepatitic mucormycosis (Oliver *et al.*, 1996). Workers working in the dusty environment of preparing dried herbs are also subject to developing allergies through exposure to bacterial and fungal contaminants. One study among employees working with thyme indicates they were significantly sensitive to the gram-negative bacteria *Pantoea agglomerans,* in addition to the usual fungal allergens (Golec *et al.*, 2004).

Human or animal fecal contamination is always possible when the herbs have been harvested from small rural farms still using as fertilizer either

"night soil" or animal dung or when the workers themselves prepare the products under unsanitary conditions. Although survival of bacterial enteropathogens for any length of time is unlikely in dried material, these and other organisms have the potential of causing GI tract and nosocomial infections (Czech *et al.*, 2001; Wilson *et al.*, 2004). Natural medicinal infusions can also be the source of a number of potentially pathogenic microorganisms (Martins *et al.*, 2001). Because hepatitis E is generally a waterborne disease and can be found in stool, it is possible that it is within this category that a Japanese man acquired hepatitis E from ingesting a virus-contaminated Chinese herbal preparation. Although only one serotype of hepatitis E exists, there are regional variations in nucleotide sequencing of the 3′ terminal region of its complementary DNA (cDNA). Using PCR amplification, Ishikawa *et al.* (1995) found that the hepatitis E virus from the patient's serum showed a high degree of homology (99.8% of 752 nucleotides) with the Chinese strain retrieved from the remedy. Bacterial spores or parasitic ova are more likely to be present, although acquisition of parasites in this manner has yet to be established. Bacterial spore contamination can express itself in a number of interesting clinical ways. For example, an outbreak of cutaneous anthrax (malignant pustule) was reported among Iranian patients applying *Kimbucha* mushrooms to treat their skin lesions (Sadjadi, 1998). Also, multiple cases of infant botulism are known, and though reasonably rare, children younger than 1 year are particularly vulnerable to this disease, which results in blockade of voluntary motor and autonomic functions. It is acquired from ingesting honey contaminated with the spores of *Clostridium botulinum* and the toxin that is produced. Because some herbal products are made with honey, any honey-containing product or supplement should be suspect (Cox and Hinkle, 2002; Tanzi and Gabay, 2002).

E. BOTANICAL SUBSTANCES

Inadvertent or advertent adulteration with plant products is a continuing global concern, and constant vigilance is needed to prevent the serious consequences related to these practices. Table III provides some noteworthy examples. For instance, confusion of Japanese star anise with that of Chinese star anise in herbal teas sold in Europe for infant colic resulted in an epidemic of convulsive disorders among Dutch, Spanish, and French users. Japanese star anise contains a sesquiterpene lactone anisatin, which is neurotoxic and, by being a noncompetitive γ-aminobutyric acid antagonist, can cause symptoms of tremors or spasms, hypertonia, and hyperexcitability with crying, nystagmus, and vomiting (De Smet, 2004; Ize-Ludlow *et al.*, 2004; Johanns *et al.*, 2002; Minodier *et al.*, 2003).

TABLE III
BOTANICAL ADULTERANTS

Adulterant	Instead of	In	Reference
Podophyllum hexandrum	*Clematis Gentiana* sp. (londanco)	Wai-Ling-Sin Lung-Dam-Cho	DeSmet, 2004
Podophyllum emodi	*Gentiana* sp.		But, 1994; Chan, 1997; Drew and Myers, 1997
Digitalis lanata	*Plantago* sp.	U.S. Dietary supplement	De Smet, 2004
Illicium anisatum; I. religiosum (Japanese star anise)	*Illicium verum* (Chinese star anise)	Several European products, herbal tea, etc.	De Smet, 2004; Ize-Ludlow *et al.*, 2004; Johanns *et al.*, 2002; Minodier *et al.*, 2003
Mandragora officinarum	*Panax ginseng*	Chinese herbals	Chan, 1995
Rauwolfia serpentina	*Panax ginseng*	Chinese herbals	Drew and Myers, 1997
Cola spp.	*Panax ginseng*	Chinese herbals	Drew and Myers, 1997
Datura metel Rhododendrum molle	*Innocuous herbs*	Chinese herbals	Tomlinson *et al.*, 2000
Atropa belladona	*Vaccinium myrtillus*	European products	De Smet, 2004
Periploca sepium	*Eleutherococcus senticosus*	Chinese herbals	Miller *et al.*, 2004

Numerous reports of serious herbal poisonings in China, Malaysia, and Taiwan have been linked to adulterations with either *Datura metel* or *Podophyllum emodi* (But, 1994). The great majority of cases involving anticholinergic effects, causing reduced visceral activity, are related to the use of yangjinhua, the dried flower of *Datura metel,* for treating bronchial asthma, chronic bronchitis, pains, and flu symptoms (Chan, 1995). The incorporation of *Podophyllum* into Asian formulations may be related to several Chinese pharmacopeias erroneously referring to xiaoyelian as *P. emodi* (Shang *et al.*, 1994). *Podophyllum* intoxications have elicited severe life-threatening events (Chan, 1997; Drew and Myers, 1997). Adulterations can come from unexpected sources and underscore the wisdom of pregnant women not taking herbal preparations during pregnancy. For example, colchicine has been found in human placental blood of a number of mothers

claiming to have taken Ginkgo or other herbal supplements during pregnancy (Petty *et al.*, 2001).

Ginseng preparations should be particularly suspect because not only is there a wide variation in their ginsenosides content (Asafu-Adjaye and Wong, 2003; Cui, 1995; Lau, 2003; Liberti and Marderosian, 1978), but adulterations with dangerously bioreactive plants have been reported. Noteworthy has been the inclusion of mandrake (*Mandragora officinarum*) containing scopolamine, *Rauwolfia serpentina* containing reserpine, and *Cola* species with its high caffeine content (Drew and Myers, 1997). In the latter case, the stimulatory effects of caffeine could potentiate or mimic those seen with the use of ginseng.

Adulterations are also likely to take place when natural disasters affect the supply of a particular product. Apparently, this has been the case in the aftermath of the 2004 Florida hurricanes when the saw palmetto harvest was adversely affected and prices of available sources trebled. According to a number of established suppliers, the market has been flooded with material contaminated with palm and other types of fatty oil complexes, events that could directly affect the quality of certain available products (Blumenthal, 2005a,b).

VII. PHARMACOKINETIC BEHAVIOR OF PLANT-DERIVED DRUGS

There are many reasons that variable responses to herbal use are seen within human populations. Studies on the pharmacokinetic behavior of quercetin and rutin (Erlund *et al.*, 2000) and quinine and sparteine have been invaluable to understanding this phenomenon. Genetic and racial differences may mediate how certain compounds are metabolized, just as storage and clearance rates may be affected by the age of the individual. When internal organs such as the kidney and liver are affected by disease, their ability to function within normal parameters is affected so that clearance of certain compounds is altered or underlying conditions are exacerbated. For example, plasma levels of quinine are raised during infections such as malaria, and renal clearance is slowed when the urine is alkaline due to low-protein diets. Oral or metabolic clearance rates may also be affected by smoking or by certain drug interactions (De Smet and Brouwers, 1997). In addition, *Vites agnus castus* use has been found to alter normal ovarian function (Cahill *et al.*, 1994).

A number of drug interactions with herbal remedies are noteworthy. A sudden remarkable decrease in cyclosporine trough concentrations can occur when a patient is co-medicated with St. John's wort (SJW) (*Hypericum perforatum*), which is a dangerous event for patients undergoing

transplant therapy (Mai *et al.*, 2000). This herb causes an upregulation of intestinal P-glycoprotein and cytochrome P450 (CYP) 3A4 in the liver and intestine, resulting in impaired absorption and stimulated metabolism of cyclosporine, which leads to subtherapeutic plasma levels. Other CYP 3A4 substrates are also affected so that the protease inhibitors indinavir and nevirapine, oral contraceptives, and tricyclic antidepressants such as amitriptyline are more rapidly metabolized.

The potential for additional drug interactions is also possible with herbs such as milk thistle (*Silymarum marianum*), *Angelica dahurica, Ginseng,* and garlic (*Allium sativum*) preparations, Danshen (*Salvia miltiorrhiza*), and licorice (*Glycyrrhiza glabra*), which have the ability to modulate CYP activity (Ioannides, 2000). A potential clinical benefit may exist for licorice, because oral administration of glycyrrhizin increases plasma prednisolone concentrations and influences its pharmacokinetics by inhibiting its metabolism, but not by affecting its distribution (Chen *et al.*, 1991). With hydrocortisone, its glycyrrhetinic acid potentiates the cutaneous vasoconstrictor response (Teelucksingh *et al.*, 1990).

By demonstrating the presence of compounds or their metabolites in urine, it is possible to study the pharmacokinetics of polyherbal remedies. This was accomplished in renal clearance studies conducted by Homma *et al.* (1992) with the Japanese remedy for bronchial asthma, saiboku-to.

VIII. PROBLEMATIC HERBS AND THEIR ADVERSE EFFECTS

A. TRADITIONAL CHINESE HERBS

1. General

As adverse reactions are reported and studies reveal the source of the problems, the potential danger of using certain botanicals or their compounds is beginning to be recognized. For example, TCM remedies containing *Isatis tinctoria* are commonly associated with adverse reactions (Ko *et al.*, 1999). Also, because of their toxic potential, Taiwan and China control the use of tubers of *Arisaema* species, unprocessed tubers of *Typhonium giganteium* and *Pinellia ternata,* the unprocessed resin of *Garcinia morella,* seeds of *Impatiens balsamina, Pharbitis nil,* and *P. purpurea,* roots of *Knoxia valerianoides,* and flowers of *Rhododendron molle* (De Smet, 2004). Other potent or toxic Chinese medicinal materials meriting some form of regulatory control include those derived from roots of *Aconitum brachypodum, A. carmichaeli, A. coreanum,* and *A. kusnezoffiii,* tubers of *Arisaema erubescens, A. heterophyllum,* and *A. amurense,* fruits of *Croton tiglium,*

flower buds of *Daphne genkwa,* flowers of *Datura metel,* roots of *Euphorbia fischeriana, E. ebracteolata,* and *E. kansui,* seeds of *E. lathyris* and *Hyoscyamus niger,* stem/root bark of *Melia azadarach* and *M. toosendan,* roots of *Phytolacca acinosa* and *P. americana,* resin *of Rhus verniciflua,* and seeds of *Ricinus communis* and *Strychnos nux-vomica* (Tomlinson *et al.*, 2000). In Germany, Madder root (*Rubia tinctorium*) is banned because it contains lucidin, which is mutagenic and carcinogenic (Blumethal *et al.*, 1998). However, *Rubia cordifolia* and other Asian rubiacious plants (*Morinda umbellata, Hymendodictyon excelsum,* and *Damnacanthus indicus*) containing related anthranoids have yet to be similarly categorized and controlled (De Smet, 2004; Kawasaki *et al.*, 1992).

2. *Jin Bu Huan*

Jin Bu Huan, a Chinese patent medicine sold in pill form as an anodyne and painkiller, is perceived as innocuous despite that it contains, levo-tetrahydropalmatine, which is present in *Stephania* and *Cordylis* species and is a potent neuroactive substance. Regrettably, the packaging is often mislabeled, delaying the identification of the offending alkaloid and its association with the distinct serious undesirable conditions, which develop in children or adults. In children, acute life-threatening cardiovascular and neurological manifestations have been reported, whereas prolonged adult use can induce various symptoms from acute toxicity, lethargy (due to low blood pressure, heart rate, and respiratory function), muscle weakness, bradycardia, and coma, to ''extreme fatigue,'' fever, jaundice, and hepatitis. Hepatotoxicity and the development of chronic hepatitis are particularly prevalent (Brent, 1998; Elvin-Lewis, 1991; Horowitz *et al.*, 1996; Ko and Woo, 2000; Piciotto *et al.*, 1998).

3. *Chuen-Lin*

Berberine-containing phytomedicines are valued for a wide variety of medicinal purposes (Lewis and Elvin-Lewis, 2003). The alkaloid can be found in numerous medicinal plants such as goldenseal (*Hydrastis canadensis*) and golden thread *Coptis sinensis* (Huanglian). In Singapore berberine is banned, primarily because of the use in neonates of the Chinese herbal preparation ''Chuen-Lin'' containing *Coptis chinensus/japonicum.* This formulation was found to cause neonatal jaundice and increase the risk of brain damage (Ko and Woo, 2000; Yeung *et al.*, 1990).

4. *Ma Huang (Ephedra)*

The medicinal use of ephedra has been traced back to Neanderthal times and the value of *Ephedra sinica* (Ma Huang) in TCM is well known. In

traditional Asian remedies, it is used to treat fever, chills, and cough, improve the circulation, and promote sweating. All Eurasian species contain ephedrine alkaloids, with ephedrine and pseudoephedrine predominating, in addition to norephedrine, norpseudoephrine, methyl ephedrine, and normethypseudoephedrine. Species in North and South America lack ephedrine, and these may contain only traces of pseudoephedrine. Ephedrine acts as a sympathomimetic (agonistic) on α_1, β_1, and β_2 adrenoreceptors, increasing blood pressure through vasoconstriction, raising heart rate, and stimulating the central nervous system (CNS), whereas pseudoephedrine causes bronchodilation through its β_2-receptor, thus relieving the symptoms of nasal congestion through its α_1-agonist effects (Lewis and Elvin-Lewis, 2003; White *et al.*, 1997). In the dietary supplement industry, *Ephedra* was valued primarily for its stimulatory effects, and until only recently, *Ephedra* and its alkaloids were included in a wide range of products sold to enhance energy, for bodybuilding, or for weight loss. Ingredient panels on these ephedrine-containing products listed ma huang, Chinese *Ephedra*, ma huang extract, *Ephedra* herb powder, *Ephedra* extract, *Ephedra*, *Ephedra sinica,* epidonine, ephedrine, or ephedrine alkaloids (FDA, 2004c). Unfortunately, some of the labeling was targeted to adolescents and young adults, implying their use could provide a "high." To enhance the stimulatory effects and promote this property, a number of these formulations also included caffeine-containing herbs, the alkaloid itself, or other stimulating compounds. Several studies have lauded its merits or demerits for weight loss and bodybuilding (Shekelle *et al.*, 2003a,b) or have considered its adverse effects (Bent *et al.*, 2003).

Its banning by the FDA in 2004 occurred after a long and protracted evaluation of the numerous adverse effects and deaths (including notable sports figures) resulting from the use of these products. Indications under consideration included reports of hypertension, palpitations, tachycardia, myocardial infarctions, and important neurological and psychiatric events (brain hemorrhage, seizure, or psychiatric symptoms). Most disconcerting were the number of sentinel events that occurred in persons aged 30 years or younger, These studies were thorough and extensive, and scrutiny of a large number of cases was made before the final decision was accomplished. The concluding ruling of Section 119.1 dietary supplements containing ephedrine alkaloids reads as follows: "Dietary supplements containing ephedrine alkaloids present an unreasonable risk of illness or injury under conditions of use recommended or suggested in the labeling, or if no conditions of use are recommended or suggested in the labeling, under ordinary conditions of use. Therefore, dietary supplements containing ephedrine alkaloids are adulterated under section 402(f)(1)(A) of the Federal Food, Drug, and Cosmetic Act" (FDA, 2004c). Saudi Arabia has followed

the U.S. banning of ephedra products. Rulings from the E.U. and elsewhere have yet to evolve.

Of course, this restriction has caused some consternation among herbalists or Chinese practitioners who still believe that within the context of traditional use, Ma Huang has its merits. In Canada, the ephedrine alkaloid is limited to levels well below the excesses seen in U.S. products, a policy that allows the continued sale of traditional Chinese *Ephedra* products. Canada also allows *Ephedra* to be included in products used for nasal congestion in the following small doses: 8 mg/dose or 32 mg/day of ephedrine and 400 mg/dose or 1600 mg/day. However, Health Canada has issued several warnings regarding the illegal sale of *Ephedra* and the potential risks that are involved, particularly when it is combined with caffeine or other stimulants.

B. KAVA: A NEO-WESTERN REMEDY WITH POLYNESIAN ROOTS

Use of kava (*Piper methysticum*) extracts as a safe alternative to anxiolytics drugs has been banned in the entire E.U. and Canada, and in Australia the products have been voluntarily removed from the market. In the United States, kava has been the subject of cautions and advisories by the FDA, and its status is still pending depending on the outcome of current investigations (Blumenthal, 2002). The reason for this concern is the seriousness of the adverse events that began to be reported in 1988, when heavy kava use in an Australian aboriginal community triggered unease about its effects on health, its induction of scaly rashes, and its potential for hepatotoxicity in populations already infected with hepatitis B (Mathews *et al.*, 1988). Subsequently, kava dermopathy was also seen in heavy kava drinkers in the Tonga Islands (Ruze, 1900). At that time, effects on impaired vision were also observed (Garner and Klinger, 1985) and were included in the Commission E monographs (Blumenthal, 1998). However, beginning in 1998, a number of serious adverse reactions were reported in Europe in patients taking kava extracts for anxiety. Most of these cases involved liver-related adverse effects, leading to hepatic failure and subsequent liver transplantations (Clouatre, 2004; Moulds and Malani, 2003) and even death (Brauer *et al.*, 2001). In many instances, this linkage to hepatotoxicity remains in dispute because of other potentially hepatotoxic medications being taken concomitantly. However, a number of cases still exist in which kava consumption alone has been implicated (Clouatre, 2004). Other adverse events have been associated with neurological complications, including the exacerbation of Parkinson's disease and interactions with CNS depressants, such as benzodiazepines causing coma (Almeida and Grimsle, 1996; Stevinson *et al.*, 2002). Although its status in the United States remains uncertain, it is agreed that kava drinking is contraindicated if underlying conditions that might

adversely effect liver function exist such as the concomitant intake of prescription drugs associated with liver damage, excessive alcohol use, or preexisting liver disease or infections (Blumenthal, 2002; Waller, 2002).

IX. INADVERTENT OVERDOSING

The use in TCM of "chuanwu" (the main root of *Aconitum carmichaeli*) and "caowu" (the root of *A. kusnezoffii*) in the treatment of various musculoskeletal disorders has led to serious *Aconitum* poisonings, leading to tachyarrhythmias, including tachycardia and fibrillation ventricular arrhythmias and sometimes, death (Chan *et al.*, 1994; Dickens *et al.*, 1994; Tai *et al.*, 1992). A case of tetraplegia has also been reported (Chan *et al.*, 1994). These aconite roots contain aconitine, mesaconitine, and hypaconitine, which are neurotoxins and cardiotoxins. In Hong Kong, awareness of this problem of overdosing has led to stricter adherence to recommended doses, with a subsequent lowering of adverse reactions being reported (Chan, 2002). Shandoguen (root of *Sophora tonkinensis*) is another herb with a low therapeutic ratio. Excessive doses can cause vomiting, diarrhea, headache, dizziness, and in some cases, death. Overdosing with upper respiratory preparations such as lushenwan, containing "chansu," the venom of the toad *Bufo bufo gargarizans* and *B. melanostictus,* can cause cardiac arrhythmias, which can be fatal in children. Local skin inflammations have been linked to its unwarranted use as a topical medicament to treat skin infections (Tomlinson *et al.*, 2000).

Though less toxic, certain herbs used inappropriately for long periods or in excessive amounts can also be problematic. Ginseng (*Panax ginseng; Panax. quinquefolius*) is widely valued as a panacea and adaptogen (Lewis and Elvin-Lewis, 2003), but when taken in high doses, toxic effects similar to caffeine toxicity such as nervousness, mood elevation, insomnia, rash, depression, and morning diarrhea have been described in what is referred to as the "ginseng abuse syndrome" (Siegel, 1979). It has been known to induce manic states in depressive patients (Gonzalez-Seijo *et al.*, 1995) and to cause palpitations, nausea, vomiting, amenorrhea, and blurred vision. Taken in cigarettes, it can exacerbate symptoms in schizophrenics. These effects are possibly due to its dammarenetriol glycoside, which has strong excitatory effects on the nervous system and other components (D'Arcy, 1991; Elvin-Lewis, 2001; Ernst, 1998; Kassler *et al.*, 1991; O'Hara *et al.*, 1998; Saxe, 1987; Wilkie and Cordess, 1994). Because of these stimulatory effects and its link to increasing blood pressure, the British Herbal Compendium does not recommend its use with stimulants, including excessive amounts of caffeine (Bradley, 1992). Estrogenic affects such as mastalgia (Palmer *et al.*, 1978)

and unexplained vaginal bleeding following use of a ginseng face cream (Hopkins *et al.*, 1988) have been reported. It also reduces the effects of warfarin (Yuan *et al.*, 2004). Numerous OTC products claim to contain ginseng, primarily because the word promotes sales, and consumers should be aware of unexpected events that are associated with the use of these products. Its effects on the developing fetus have been discussed elsewhere.

Several Asian remedies contain *Glycyrrhiza glabra* (licorice) or *Glycyrrhiza uralensis* (gancao) (Lewis and Elvin-Lewis, 2003), and these are generally considered safe. Their content of glycyrrhizic and glycyrrhetinic acids, with aldosterone-like properties, can in large doses induce hypokalemia and sodium and water retention (Tomlinson *et al.*, 2000).

X. HERBAL DRUG TRANSMISSION *IN UTERO* OR THROUGH MOTHER'S MILK

Use of herbal remedies by pregnant or nursing mothers can result in the transmission of certain phytochemicals to the fetus or infant. Effects can be transient, grave, or fatal, and much is still unknown about residual effects (Conover, 2003; Pinn and Pallett, 2002). The fetus is particularly vulnerable to herbs, which are toxic, teratogenic, carcinogenic, or possess abortifacient properties. For example, salicylates are potentially teratogenic or embryocidal even when applied externally in Oil of Wintergreen. Ingestion of *Sassafras albidum* as an herbal tea also poses concerns, because transplacental carcinogenesis has been demonstrated in mice. This phenomenon is possibly due to its major carcinogenic component safrole (De Smet, 1992). Care should be taken to avoid other herbal remedies during pregnancy because they may induce menses, infant death, low birth weights or neonatal jaundice. For example, the use of feverfew (*Tanacetum parthenium*) for headaches may induce menses (another traditional use) and terminate the pregnancy (O'Hara *et al.*, 1998); similar abortifacient risks have been linked to rue, parsley, (Blumenthal, 1998; Ciganda and Laborde, 2003), Goldenseal, and sparteine (Benssousan *et al.*, 2000). Herbs and foods rich in unsaturated pyrrolizidine alkaloids may cause fetotoxic or embryotoxic effects (Rasenack *et al.*, 2003), and infant fatalities have resulted from maternal drinking of teas or cough remedies containing these alkaloids (Roulet *et al.*, 1988; Winship, 1991). Ginseng use during pregnancy is also inadvisable (Anonymous, 2003), because neonatal androgenization has been observed (Anonymous, 1991); and during the critical period of organogenesis, its major ginsenosides, Rb_1, has been found to cause teratogenesis in rat embryo cultures (Chan *et al.*, 2003). The chewing of the stimulant Khat (*Catha endulis*) can result in lower birth weights (Eriksson *et al.*, 1991; Ghani *et al.*,

1987), and hydrastine in Goldenseal (*Hydrastis canadensis*) and barberry (*Berberis)* can cause neonatal jaundice. A mother should also avoid taking heparin-containing herbs, because either her fetus or her nursing infant is at risk of developing bleeding disorders (Ernst, 1997). Use of blue cohosh (*Caulophyllum thalictroides*) to promote uterine contractions at parturition is unwise because the plant contains vasoactive glycosides, a toxic alkaloid, and sparteine. In one instance, this practice caused a newborn to develop an acute myocardial infarction, congestive heart failure, and shock, and although the neonate survived, it was ill for several weeks (Jones and Lawson, 1998). A case of fetal alcoholic syndrome may also be related to maternal use for 2 months during the antenatal period of an "Herbal Health Tonic" containing 14% alcohol (Pradeepkumar *et al.*, 1996). Severe congenital lead poisoning in a preterm infant was linked to the mother's long-term ingestion of a lead-containing herbal pill (Tait *et al.*, 2002).

There are a number of reasons nursing mothers should also avoid herbal use (Conover and Buehler, 2004). Should Chasteberry fruit (*Vitex agnus-castus*), be taken at this time, its dopaminergic compounds can reduce pituitary prolactin reserves and thus affect lactation (Wuttke *et al.*, 2003). Other harmful phytochemicals may be found in colostrum and transmitted to the nursing infant, causing serious adverse effects. Examples include evoking catharsis in a nursing infant following the use of Rhein-containing senna laxatives by its mother; the exposure of a nursing infant to hepatotoxic, genotoxic, and carcinogenic echimidine through maternal drinking of Comfrey tea (Winship, 1991); the development of a venoocclusive hepatic illness resembling Budd-Chiari syndrome following the mother's use of a tea containing flowers of *Tussilago farfara* (Tussilaginis Farfare, Flos) and roots of *Petasites officinalis* (Radix petasidis) (Roulet *et al.*, 1988; Spang, 1989); and the development of a fatal illness mimicking Reye's syndrome, through the maternal use of an herbal cough remedy containing the pyrrolizidine alkaloid senecionine (Fox *et al.*, 1978). Hypertension in both an infant and its mother has also been linked to the use of a Chinese herbal medicine (Nambiar *et al.*, 1999).

XI. HERBAL USE IN CHILDREN

The capacity of children to absorb, distribute, metabolize, and excrete certain substances differs from that of adults. With larger livers, children are generally more efficient in their ability to detoxify; however, their developing nervous and immune systems can also make them more sensitive to the adverse effects of certain botanicals. Children are particularly vulnerable to developing significant dehydration and loss of electrolyte balance with the

use of powerful herbal cathartics or diuretics. They are especially at risk in taking herbs containing pyrrolizidine alkaloids, because no safe dose or duration has been determined. Also, even the first introduction of an atopic child to certain herbs has the potential of sensitizing or eliciting a wide range of allergic reactions (Woolf, 2003). Of particular concern is the use of TCM within the pediatric age-group because numerous life-threatening reports have been associated with their use and have been discussed in context throughout the chapter (Chan, 1994; Ize-Ludlow *et al.*, 2004; Minodier *et al.*, 2003; Tomassoni and Simone, 2001).

XII. ALLERGIC REACTIONS

A. TYPE I (IMMEDIATE) HYPERSENSITIVITY

Herbal use can induce allergic reactions in many ways (Lewis and Elvin-Lewis, 2003; Reider, 1994). Those most frequently observed are the symptoms of rhinitis, headache, dermatitis (hives), and/or anaphylactic shock typical of type I reactions, and erosive and weeping lesions of contact dermatitis, typical of delayed hypersensitivity, type IV reactions. The most common incitants are members of the Asteraceous (daisy family), and the cross reactions that occur among them. In the United States, the widespread occurrence of ragweed (*Ambrosia* species) is a major cause of sensitization to large portions of eastern and midwestern populations. Atopic individuals in this category that drink chamomile (*Chamaemelum nobile*) herbal teas (Reider *et al.*, 2000) or use herbal preparations of *Echinachea* (Mullins, 1998; Myer and Wohlmuth, 1998) or *Tanacetum parthenium* (feverfew) (Hausen, 1981) are at risk of developing headaches, exacerbating symptoms of allergy or asthma, or developing other type I reactions including anaphylaxis. Unfortunately, most American chamomile drinkers are unaware of these hazards of potential cross reactivity, because their European counterparts do not share the same prospect of sensitization, unless they are allergic to wormwoods (de la Torre *et al.*, 2001; Subiza *et al.*, 1989) or sensitized during the commercial preparation of the herbal product (Dutkiewicz *et al.*, 2001). Most disconcerting are the increasing number of cases of anaphylaxis (Benner and Lee, 1973; Subiza *et al.*, 1989) and some fatalities related to chamomile use in teas or as enemas (Jensen-Jarolim *et al.*, 1998; Thein, 2001). Allergic conjunctivitis can also be induced by using the tea as an eye wash (Subiza *et al.*, 1990). Several cases of *Echinacea*-associated anaphylaxis have been reported in Australia (Mullins and Heddle, 2001). A case of anaphylaxis has also been reported following the use of milk thistle (*Silybum marianum*) (De Smet, 2004).

B. TYPE IV DELAYED CONTACT HYPERSENSITIVITY

1. Asteraceous plants

Exposure to Asteraceous plants may also result in the development of contact dermatitis. One Serbian study has indicated that it is not unusual to detect sensitization to chamomile (*Chamomilla recutita*), arnica (*Arnica montana*), tansy (*Tanacetum vulgare*), and feverfew (*Tanacetum parthenium*) (Jovanovic *et al.*, 2004). Contact dermatitis, along with asthma and rhinitis, may also accompany occupational exposure to chamomile (Rudzki *et al.*, 2003) and contact dermatitis to feverfew (Hausen, 1981). Similarly, chamomile in cosmetic products can also be a cause of dermatitis (Paulsen, 2002; Rycroft, 2003). Because chamomile-containing products, particularly in shampoos and other OTC products, are so widespread, the linkage to these types of adverse events are likely underreported. Also, use of royal jelly, a thick mixture of honey, pollen, and their allergens, has been associated with several cases of bronchospasm, and topical application of concentrated forms of bee pollen (propalis) to contact dermatitis (Perharic, 1993). Milk thistle has also been known to cause urticaria (De Smet, 2004).

2. Essential oils

Essential oils are found in foods, chewing gums, flavorings, fragrances, and medicinal products and may be ingested, chewed, used in dental products, inhaled as perfumes or in aromatherapy, or applied to the skin for medicinal or other purposes. Whatever the source, they have the capacity to sensitize and to elicit allergic reactions, particularly of the type IV variety. Canker sores (Elvin-Lewis, 2001; Elvin-Lewis *et al.*, 1985; Nolan *et al.*, 1991) and cheilitis (Corazza *et al.*, 2002; Francalanci *et al.*, 2000; Hausen, 1984; Holmes and Freeman, 2001) are examples of their oral manifestations. Eugenol is a primary offender, possibly because its occurrence is so widespread in spices (oil of cloves), herbs, foods (e.g., artichokes), flavorings, cosmetics, fragrances, dental products, and medicines. Similarly, L-carvone in many mint and peppermint oils has been implicated in contact allergies (Anderson, 1978; Corazza *et al.*, 2002; Holmes and Freeman, 2001; Paulsen *et al.*, 1993; Worm *et al.*, 1989), as has cinnamaldehyde found in cinnamon (Drake and Maibach, 1976; Thyne, 1989).

Lavender, jasmine, and rosewood oils used in perfumes and as ingredients of aromatherapy can elicit on inhalation similar allergic reactions in the nasal passages and respiratory tract (Schaller and Korting, 1995; Selvaag *et al.*, 1995a; Sugiura *et al.*, 2000), and with skin contact, dermatitis is possible for these oils, as well as French marigold, cedarwood oil,

peppermint oil, tea tree oil, and Tylang-ylang (Ernst, 2000; Selvaag *et al.*, 1995b). Dermatological conditions can also result from handling allergenic plants and their products (Sasseville, 1999; Simpson *et al.*, 2004). Noteworthy among these examples are the adverse effects associated with the widespread use of tea tree oil (extracted oil of *Melaleuca alternifolia*) in aromatherapy, and its antimicrobial and therapeutic properties are causes for growing concerns. Several of its components, namely D-limonene (and its oxidative derivative D-carvone), α-terpinene, aromadendrene, terpinen-4-ol, p-cymene, and α-phellandrene have been found to be sensitizing agents (Knight and Hausen, 1994). It has been found to have the potential to also elicit allergic contact dermatitis, systemic contact dermatitis, linear immunoglobulin A disease, erythema multiforme-like id reactions, and systemic hypersensitivity reactions (Crawford *et al.*, 2004).

A review by Ernst (2000) considers additional herbal remedies and aromatherapy oils known to elicit allergic skin reactions and other dermatological disorders. Examples of those used as topical medicaments include aloe vera, black cumin oil, camomile, camphor, citrus hystrix, curcumin, bergamot oil, and *Inula helium* massage oil (Ernst, 2000). Tincture of benzoin or balsam of Peru obtained from *Myroxylon pereirae*, used for cosmetic wound healing, is a common allergen (Scardamaglia *et al.*, 2003). An exudative erythema may appear on exposed areas of skin, following airborne exposure to jasmine (Schaller and Korting, 1995). Skin rashes may also be caused by a variety of phytoirritants or compounds that can elicit pruritus and wheals such as histamine-containing nettles (Lewis and Elvin-Lewis, 2003).

Contact dermatitis can also result from the use of a number of Chinese topical formulations, and studies have shown that many of the ingredients have the potential of being sensitizing agents (Chen *et al.*, 2003). Garlic (*Allium sativum*) with its allergen, diallyl disulfide, can cause irritant contact dermatitis, with the rare variant of zosteriform dermatitis, as well as contact urticaria, including the hematogenic variant (Jappe *et al.*, 1999)

3. *Oral medications*

Oral medications may also elicit dermatological reactions. For example, Stevens-Johnson syndrome has been associated with drinking Mai-Meu-Dong-Tang, a Chinese health drink (Mochitomi *et al.*, 1998) or ingesting opiopogonas tubers (Mochitomi *et al.*, 1998b). A lupus-like syndrome can result through the use of yohimbe (*Coryanthe yohimbe*) (Sandler and Aronson, 1993). Garlic is also known to elicit pemphigus, allergic asthma and rhinitis, angioedema (Jappe *et al.*, 1999), and photosensitivity reactions

(Alvarez *et al.*, 2003). Similarly, photosensitivity may be elicited by taking *Psorolea corylifolia* (Maurice and Cream, 1989) or *Hypericum perforatum* (SJW) (Cocks and Wilson, 1998). Widespread skin eruptions have resulted from taking Fang Feng Tong Sheng Wan and Bi Yan Pian (Mather *et al.*, 2000). The use of the Indian remedy guggulipid, derived from the guggul tree, *Commiphora mukul,* and used for the treatment of hypercholesterolemia, is associated with the development of a skin rash in some patients (Szapary *et al.*, 2003) and headache, mild nausea, eructation, and hiccup in others (Singh *et al.*, 1994). Delayed hypersensitivity reactions have been linked to the use of Hexilor (extract of European mistletoe, *Viscum album*), when it is used for adjuvant cancer therapy (Stein and Berg, 1999). Eczema can result from ingestion of *Krameria triandra,* found in a number of Asian traditional remedies (Goday *et al.*, 1999). Stevens-Johnson syndrome and diffuse morbilliform skin reactions have been linked to ginkgo supplement ingestion (*Ginkgo biloba*) (Chiu *et al.*, 2002; De Smet, 2004). Lupus-like syndromes are associated with use of yohimbe (*Coryanthe yohimbe)* (De Smet, 2004).

XIII. DENTAL PRODUCTS

Aside from allergic manifestations elicited by essential oils, adverse reactions related to use of dental products are rare (Ocasio *et al.*, 1999). However, so-called ''natural'' dentifrices always present a risk of damaging the enamel due to the variability in abrasivity of their calcium carbonate sources or contributing to heavy metal toxicity should this compound be derived from seashells that could contain high amounts of mercury (Elvin-Lewis, 2001). Caries rates are always higher in communities that do not fluoridate their water supplies to euflorotic (1 pmm) levels. Many brands of ''bottled'' water lack fluoride, so consumers are at risk of losing the protective effects to enamel that is conferred by this trace element. Some accommodation to this loss is possible, if black or green tea is drunk. Enamel mottling only occurs when fluoride exceeds 2–3 ppm and excessive amounts are drunk (Elvin-Lewis *et al.*, 1980; Lewis and Elvin-Lewis, 2003).

Because oral hygienic products are used on a daily basis and some of their contents may be swallowed or absorbed locally, there is always a concern that long-term effects may occur. One noteworthy example was the association of the use of *Sanguinaria canadensis* root-extract mouthwashes with the development of hyperorthokeratosis, epithelial atrophy, and epithelial atypia/mild dysplasia and an associated squamous cell carcinoma (Allen, 1999; Damm *et al.*, 1999). This phenomenon has been called the *sanguinaria-associated leukoplakia syndrome,* and evaluation of the 143 known cases

indicates that use of this product was a risk factor for the development of these types of lesions (Mascarenhas *et al.*, 2002). The product has been reformulated and the offending extract, with its potentially carcinogenic isoquinoline alkaloids, removed. However, these incidents could have been prevented had the manufacturer heeded the warnings for more than 15 years of leading pharmacologists and toxicologists who counseled them that regular use of sanguinarine, even in small amounts, would be unwise (Elvin-Lewis, 2001). Of current concern is the use of neem products derived from the oil, leaves, and part of *Azadirachta indica* for dental purposes. Though long being valued as chewing-stick, and in crude extract dental formulations for teeth cleaning, as well as for a variety of medicinal purposes, current research is indicating that its bioreactivity profile is not conducive to long-term uses as a dental product. This is because many of its components are used as insect deterrents and anti-feedents, as well as being immunomodulatory, contraceptive, hypoglycemic, and abortifacient (Elvin-Lewis, 2002).

XIV. OCULAR SIDE EFFECTS FROM HERBAL MEDICINES AND VITAMIN SUPPLEMENTS

A recent comprehensive review discusses numerous types of adverse ocular manifestations associated with use herbal and nutritional supplements either topically or systemically (Fraunfelder, 2004). Noteworthy among these are numerous reports linking the use of canthaxanthin used in cosmetics and ingested as a food coloring or for purposes of artificial suntanning. This compound can deposit in all layers of the retina, especially in the superficial layers of the macula causing retinopathy, visual changes such as abnormalities in static threshold perimetry, electroretinography, and dark adaptation. Most individuals are asymptomatic, whereas others may experience diminished visual acuity. These retinal findings are slowly reversible.

Allergic conjunctivitis and/or eye irritation has been associated with the medicinal use of chamomile (*Matricaria chamomilla*) (Subiza *et al.*, 1990) and *Echinacea purpurea* (Fraunfelder, 2004). Hyphema (bloodshot eyes) or retinal hemorrhage has been linked to use of *Ginkgo biloba* (Fraunfelder, 2004) and transient visual loss due to vasospasm with licorice ingestion.

Use of vitamin supplements such as niacin and vitamin A (retinal) in excess of recommended doses has been associated with several cases of cystoid macular edema and intracranial hypertension, respectively (Fraunfelder, 2004).

XV. PROBLEMS ASSOCIATED WITH LONG-TERM USE

The current habit of using herbal remedies to maintain or enhance good health or prevent certain conditions from occurring has resulted in numerous unexpected consequences. Because they are often promoted as safe and efficacious, the consumer is usually unaware that these practices can be harmful, particularly if they are taken for indeterminate periods. In many cases, their endorsed uses may fundamentally differ from how they were valued traditionally, so safety parameters are unknown. This is particularly true in the United States where their sale as a dietary supplement does not require prior FDA approval and appropriate oversight practices, particularly regarding labeling, are not consistently applied. This classification infers that there is little danger to their use, although there is no penalty for withholding reports of adverse effects, and withdrawal by the FDA is only initiated after a prolonged evaluation process (Elvin-Lewis, 2001; Marcus and Grollman, 2002).

What is so disconcerting are the number of problems associated with well-known medicinal herbs such as ginseng, golden seal, ginkgo, milk thistle, cassia, saw-palmetto, valerian, and a variety of stimulants (De Smet, 2004; Elvin-Lewis, 2001; Ernst, 1998; Lewis and Elvin-Lewis, 2003; O'Hara *et al.*, 1998). Symptoms of misuse can vary from trivial to extreme and may include life-threatening events affecting the heart, blood pressure, liver, GI tract, as well as nervous and endocrine systems. Dermatological reactions are considered elsewhere in the chapter.

Goldenseal root (*Hydrastis canadensis*), used as an anti-infective, is generally well tolerated but can when taken in large amounts induce various GI complaints, cause damage to the stomach lining, promote liver swelling and anorexia, and induce hypertension, seizures, respiratory failure, cardiac spasms, psychoses, and death (O'Hara *et al.*, 1998). Leaves of Ginkgo (*Ginkgo biloba*), favored to enhance mental functioning, can induce headache and nausea, and in rare cases cause cerebral and extracerebral hemorrhage, thrombocytopenia, seizures, and ventricular arrhythmia. In addition to causing a number of allergic reactions, single cases of intraoperative hemorrhage, leukopenia, and hypokalemic renal tubular acidosis have been cited following ingestion of *Echinachea* preparations containing its leaf, root, or stalk. As an immune stimulant, it is contraindicated in patients with hyperimmune diseases (e.g., multiple sclerosis). Saw palmetto (*Serenoa repens*) used to control benign prostatic hyperplasia may cause various GI complaints, headache, and erectile dysfunction; a single case of intraoperative hemorrhage has been reported. SJW (*H. perforatum*) used as an antidepressant can cause a multitude of adverse effects from dry mouth, GI upset, dizziness or confusion, restlessness, headache, sexual dysfunction, and frequent urination.

It can elicit dangerous neurological effects by inducing serotonin syndrome–like events, hypomania/mania, and psychotic relapse in schizophrenia, as well as promoting cardiovascular collapse during anesthesia and delaying the emergence from anesthesia. Use of black cohosh (*Cimicifuga racemosa*) for menopausal symptoms and to treat rheumatism can cause nausea, vomiting, and gastroenteritis (Saxe, 1987) and has been associated with cases of hepatitis and hepatic failure. Blue cohosh (*Caulophyllum thalictroides*) may also cause similar symptoms (Sax, 1987). Valerian (*Valeriana officinalis*) used as a sedative can also promote dizziness, nausea, headache, and somnolence and may be hepatotoxic with long-term heavy use. Evening primrose oil (*Oenothera biennis*) used to treat eczema is known to promote weight gain, cause GI upset, and elicit skin complaints; one case of anaphylaxis has been reported. Yohimbe (*Corynanthe yohimbe*), considered an aphrodisiac, can elicit additional excitatory symptoms such as nervousness, anxiety, panic, maniclike symptoms, hypertension, tachycardia, angina pectoris, increased urinary frequency, renal failure, and bronchospasm (De smet, 2004; Elvin-Lewis, 2001; Lewis and Elvin-Lewis, 2003). A reverse of the immunostimulating effects of *Echinacea* has been observed, with leukopenia developing after long-term use (Kemp and Franco, 2002).

Caffeine in guarana (*Paullinia cupana*), mate (*Ilex paraguariensis*), or concentrated green tea (*Camelia sinensis*) elicits a wide range of excitatory symptoms, particularly if it is present in excess. These plant products are frequently present in slimming preparations, and consumers should be aware of their potential to cause palpitations, sweating, insomnia, restlessness, agitations, tremors, headache, polydipsia and polyuria (DeSmet, 2004; Elvin-Lewis, 2001).

Anthranoid laxatives such as aloe (*Aloe vera*), cascara (*Rhamnus purshiana*), and senna (*Senna alexandrina*) can be used in moderation safely, but when used in excess can promote abdominal pain, diarrhea, and severe catharsis. Due to their chronic irritating properties, if used on a long-term basis, they may be a risk factor for colorectal cancer (Siegers *et al.*, 1992). Also, abuse of these laxatives can increase the loss of serum K, thereby potentiating the effects of cardiac glycosides and antiarrhythmic agents (Blumenthal, 2000). Using common names, these data are summarized in Table IV.

XVI. EFFECTS ON INTERNAL ORGANS

A primary function of the liver and kidneys is to detoxify and clear poisonous substances from the body. For these reasons, they are often the first to be affected by the use of toxic herbs. A number of reviews address the global

TABLE IV
HERBS THAT CAN CAUSE PROBLEMS WITH LONG-TERM USE

Herb: common name	GI	Hepatotoxic	CNS	Headache	Cardiovascular	Blood thinning	Allergy	Other	Death
Aloe	X							X	
Black cohosh	X	X							
Blue cohosh	X	X							
Echinacea							X	X	
Evening primrose	X						X	X	
Goldenseal	X		X		X				X
Ginkgo	X		X		X	X	X		
Guarana			X						
Mate			X						
St. John's wort	X		X	X				X	
Tea, green/black			X					X	
Valerian	X								
Yohimbe			X		X			X	

seriousness of this problem and offer explanations as to which mechanisms may be involved. Most of these examine the subject of hepatotoxicity-associated herbal use (Chitturi and Farrel, 2000; Larrey, 1994, 1997; Pak *et al.*, 2004; Pittler and Ernst, 2003; Stedman, 2002; Stickel *et al.*, 2000; Zhou *et al.*, 2004). Herbal effects on the kidney are addressed by Isnard *et al.* (2004), and that of cardiotoxicity by Ernst (2002). The capacity to injure these organs may be associated with one or more plants or other ingredients that are found in offending formulations. Some of these events may be elicited by traditionally formulated phytomedicines, whereas others have been associated with adulterants they might contain. Various herbal teas have also been implicated. Initial linkage to herbal use is not always easy, because many patients, unless solicited, are unlikely to reveal the source. Using common names, these data are summarized in Table V.

A. LIVER

Unfortunately, any diagnostic delay with those herbal remedies that are hepatotoxic can lead to the perpetuation or exacerbation of liver injury (Chitturi and Farrel, 2000). Depending on the level of toxicity, the range of liver damage may be transient and mild so only minor transaminase elevations are detected, whereas in other cases the development of steatosis (fatty liver), cholestasis (suppressed or stoppage of bile flow), acute and chronic hepatitis, zonal or diffuse hepatitic necrosis, hepatic fibrosis and cirrhosis, bile duct injury, venoocclusive disease (VOD) (obstruction of the veins), and acute liver failure causing death or requiring transplantation and carcinogenesis may arise (Stedman, 2002). Other underlying conditions such as alcoholism, liver infections, or concomitant use of other remedies known to adversely affect the liver such as those that induce CYP enzymes may also influence the course of the toxic event and the degree of permanent damage or recovery, that results. Also, females are more predisposed to develop hepatotoxicity, particularly during pregnancy. In addition, during an adverse hepatic episode, the functional integrity of the liver may be altered in ways that the action of conventional drugs is encumbered (Stedman, 2002).

1. Traditional Asian Medicines

Because of their herbal contents, certain Asian traditional medicines have been consistently linked to undesirable hepatic events (Benoussan, 2000; Pittler and Ernst, 2003). It has been estimated that from 1 to 8% of patients using these "drugs" develop elevated liver enzyme levels, which tend to return to normal once therapy is completed (Al-Khaafi, 2000; Dasgupta, 2003; Melchart *et al.*, 1999a,b). In a Taiwanese study, a number of cases of

TABLE V
HERBS WITH ADVERSE EFFECTS ON INTERNAL ORGANS

Common name	Liver	GI	Kidney	Heart and CVS	Death
Aloe			X		
Artichoke				X	
Cascara		X		X	
Chinese foxglove	X				
Celandine (greater)	X	X			
Celandine (lesser)	X	X			
Chaparral	X			X	X
Cloves				X	
Bajialoan	X			X	
Garlic					
Gas Plant	X				X
Germander	X				
Ginseng, Siberian				X	
European mistletoe				X	
Fo ti	X				
Fructus Aristolochiae			X[a]		
Hawthorn				X	
Hyayruro			X		
Impili	X		X		
Licorice				X	
Ma Huang	X				
May Apple	X				
Meadow-sweet				X	
North African thistle	X				
Papaya				X	
Pennyroyal	X				X
Peony	X	X			
Pineapple				X	
Sassafras	X				
Senna	X	X		X	
Skullcap	X				
Sweet birch				X	
Sweet melilot				X	
Sweet woodruff					
Tonka beans				X	
Willow				X	
Wintergreen				X	

GI, gastrointestinal system; CVS, cardiovascular system.
[a]Urothelial carcinoma also.

abdominal pain, elevated liver enzymes, and neurological complications were reported following the use of Bajialoan (*Dysoma pleianthum*), a Chinese remedy used for snake bite, condyloma acuminata, tumors, and lymphadenopathy (Kao *et al.*, 1992). The related taxa, May apple (*Podophyllum*

peltatum), used as a liver tonic is also known to elevate liver enzymes, in addition to evoking symptoms of nausea, vomiting, inflammation, edema of the bowel, and hematological abnormalities (Saxe, 1987).

Serious liver damage may ensue from taking other herbal remedies. Noteworthy are cases linked to Ma Huang with its ephedrine alkaloids. Predominant symptoms include jaundice and liver necrosis (Nadir *et al.*, 1996) including autoimmune hepatitis (Borum, 2001). Hepatotoxicity associated with compound heterozygosity for hereditary hemochromatosis has also been reported (Bajaj *et al.*, 2003). Polyherbal mixtures used to treat psoriasis and eczema are particularly unpleasant tasting, and their use possibly causes nausea and vomiting on their own. However, in some cases, these events may be followed by abdominal pain, jaundice, and liver necrosis (Kane *et al.*, 1995; Perharic *et al.*, 1995). Those cases reviewed by Sticet *et al.* (2000) all contained species of *Paeonia* (Paeoniae Rubrae Radix), and most, the Chinese foxglove *Dictamnus* and the *Rhemannia*. The gas plant or Burning bush, *Dictamnus albus* subspecies *dasycarpus* has been implicated in two fatal cases of severe hepatitis (McRae *et al.*, 2002). Cases of malaise, nausea, vomiting, and jaundice following use of Shou-wu-pian for graying hair and alopecia have been reported. This remedy contains Fo ti, *Polygonum multiflorum*, with its laxative anthraquinones, emodin and pyscin (But *et al.*, 1996; Mazzanti *et al.*, 2004; Park *et al.*, 2001). Chan Su used for heart disease has also been linked to fatal cases of hepatitis (Dasgupta, 2003).

Kampo medicines may also cause liver injuries, although the incidence of these events is considered low (<1%) (Mantani *et al.*, 2002). Those implicated include Saiko-keishi-kankyo-to (Hanawa, 2000), Sai-rei-to (Nishioji *et al.*, 1994), Bukuryo-in-go-hange-koboku-to (Yoshikubo *et al.*, 1997), and Syo-saiko-to (also known in China as Xiao-chai-hu-tang) (Itoh *et al.*, 1995).

Cases of acute hepatitis linked to the use of Jin Bu Huan are less clear, because adulteration with levo-tetrahydropalmatine, an alkaloid present in *Stephania* and *Corydalis,* has been implicated (Brent, 1998; Woolf *et al.*, 1994). Similarly, adulteration may be the cause of a fatal case of VOD attributed to skullcap (*Scutellaria lateriflora*), because this plant does not contain pyrrolizidine alkaloids associated with this syndrome. Nonetheless, in one instance, skullcap was implicated in a case of mild flulike symptoms associated with jaundice and abnormal liver function test results (Enlow, 1996).

Though generally considered harmless and present in a number of remedies for weight loss or to lower cholesterol, adverse effects have also been reported for use of the mints germander (*Teucrium chamaedrys*) and *T. polium.* For example, acute hepatitis, nausea, and asthenia (loss of energy and strength) were common symptoms in nine cases associated with their use

(Dourakis *et al.*, 2002; Pittler and Ernst, 2003). A diterpenoid teucrin A is considered responsible for these symptoms (Larry *et al.*, 1992) possibly due to the oxidation by CYP 3A4 of its furan ring, to reactive epoxide, which reacts with proteins such as CYP 3A and epoxide hydrolase (Zhou *et al.*, 2004).

2. *Neo-western herbalism*

Nausea and jaundice related to the development of necrotizing hepatitis, associated in some patients with marked cholestasis without liver failure, was characteristic of 10 patients using greater celandine (*Chelidonium majus*) (Benninger *et al.*, 1999) or lesser celandine (Strahl *et al.*, 1998) for the treatment of biliary, gastric disorders, and atopic eczema (Pittler and Ernst, 2004). It contains several alkaloids including chelidonine, chelerythrine, sanguinarine, and protopine, some of which are potentially carcinogenic (Lewis and Elvin-Lewis, 2004). Hepatotoxic reactions have also been observed after the ingestion of *Atractylis gummifera, Senna,* and pulegium. The monoterpene pulegium in pennyroyal (*Hedeoma pulegoides* and *Mentha pulegium*) is an abortifacient but can also cause liver damage, shock, and death (Anderson *et al.*, 1996). The generation of p-cresol from extensive pulegone metabolism acts as a glutathione depletory (Zhou *et al.*, 2004). *Cassia angustifolia* (senna) contains a number of bioreactive alkaloids, including anthren. *Cassia* is used as a laxative and is found in slimming formulas and in TCM as *folia sennae.* When taken in excessive amounts, it has been known to cause toxic hepatitis (Beuers *et al.*, 1991; De Smet *et al.*, 1996; Ghali and Lindor, 2004). A number of cases of severe and chronic liver damage, including periportal inflammation and necrosis and fulminant hepatitic failure, requiring liver transplantation, have been associated with the use of Chaparral or creosote bush (*Larrea tridentata*) (Anonymous, 1992; Grant *et al.*, 1997; Sheikh *et al.*, 1997). It is used to treat respiratory tract infections and in short-term and moderate doses is not considered dangerous; however, its use in capsule form is not recommended because there is a danger of overdose (Heron and Yarnell, 2001). *Sassafras albidum* is a popular Appalachian spring tonic; it contains safrole, which is considered weakly hepatocarcinogenic, so its incorporation as a beverage flavoring and root canal antiseptic is no longer allowed in the United States (Lewis and Elvin-Lewis, 2003).

3. *African indigenous medicine*

Hepatitic necrosis has been seen following the use of the North African thistle *Atractylis gummifera* and the Zulu medicinal plant, impila, *Callilepsis laureola.* The glucosides of *A. gummifera* such as atractylate can elicit severe

liver failure (Hamouda *et al.*, 2004; Kairis, 1996). Impila in KwaZulu-Natal has been responsible for several deaths among the Zulu population (Chitturi and Farrell, 2000).

4. *Pyrrolizidine alkaloids and venoocclusive disease*

Hepatotoxic pyrrolizidine alkaloids are widely represented within species of the Asteraceae, Borginaceae, and Fabaceae and can be found in medicinal plants, foods (including honey), or as contaminants. More than 300 plant species have been implicated in the development of VOD, which is particularly harmful to the liver and lungs (Winship, 1991). Abdominal pain, vomiting, hepatomegaly, raised liver enzyme levels, and the development of ascites characterize this condition, and deaths in 20–30% of cases may ensue if intake of the offending substance is not discontinued. Noteworthy cases are those related to use of *Crotalaria, Heliotropium, Senecio,* and *Symphytum.* Mass human poisoning has occurred from ingestion of *Heliotropium lasocarpium* seeds contaminating cereal crops in Afghanistan (Tanden, 1978), Tadjikistan (Chauvin *et al.*, 1994; Drew and Myers, 1997), and *Crotalaria* seeds in India (Tanden *et al.*, 1976). Likewise, outbreaks among sheep have occurred in Australia (Harris and Nowara, 1995) with *H. europaeum* and among horses with *H. indicum* in Costa Rica (Van Weeren *et al.*, 1999). In Africa or Central America, intoxication is sometimes endemic because these plants are often used for making tea. Episodes of "bush tea"–associated VOD have occurred among the Zulus of Natal following ingestion of *Callilepis laureola* (Stickel *et al.*, 2000), in Jamaica, to consumption of *Senecio* or *Crotalaria* species (Bras *et al.*, 1954, Hill *et al.*, 1951), in Argentina following the ingestion of herbal infusions containing *Senecio vulgaris* (Vilar *et al.*, 2000), and in Ecuador from using an herbal tea containing *Crotalaria* (Lyford *et al.*, 1976). Herbal remedies containing *Senecio* have been implicated in numerous cases of VOD reported among children and infants such as a Hispanic infant in Arizona taking "gordo lobo" (Fox *et al.*, 1978; Stillman *et al.*, 1977), and another infant in Brazil, which consumed the herbal tea "maria-mole" (*Senecio brasiliensis*) (Magnobosco *et al.*, 1997). Similarly, an 18-month-old Austrian boy who was mistakenly given Alpendost (*Adenostyles alliariae*) instead of coltsfoot (*Tussilago farfara*) in an herbal tea prepared by his parents developed reversible hepatitic VOD (Sperl *et al.*, 1995). If drunk during pregnancy, herbal teas containing senecionine or other pyrrolizidine alkaloids have also been shown to be the cause of fatal VOD among neonates (Rasenack *et al.*, 2003; Roulet *et al.*, 1987).

Pyrrolizidine alkaloids can be found in numerous plants used in Indian and Chinese medicine (Roeder, 2000; Zhao *et al.*, 1989). For example, a medicinal herbal preparation containing *Heliotropium eichwaldii* and its

pyrrolizidine alkaloid heliotrine (Datta *et al.*, 1978) and an Indian remedy for psoriasis containing pyrrolizine alkaloids have been associated with VOD in a number of Asian patients (Kumana *et al.*, 1985). Comfrey teas, once popular as a European/Western remedy for treatments of inflammatory disorders such as arthritis, thrombophlebitis, gout, and as a treatment for diarrhea, are no longer permitted in Germany, Canada, and the United States (FDA, 2001; Stickel and Seitz, 2000). This is related to their hepatotoxicity in humans and carcinogenicity in animals. These effects are most likely due to various hepatotoxic pyrrolizidine alkaloids such as lasiocarpine and symphytine, as well as their related *N*-oxides (Stickel and Seitz, 2000), which may react with CYP enzymes to form pyrroles that are highly reactive hepatocarcinogens (Huxtable, 1990).

B. THE KIDNEY

Nephrotoxicity is associated with the use of several medicinal herbs (Nortier *et al.*, 1999; Wojcikowski *et al.*, 2004), and various renal syndromes can develop including tubular necrosis, acute interstitial nephritis, Fanconi's syndrome, hypokalemia or hyperkalemia, hypertension, papillary necrosis, chronic interstitial nephritis, nephrolithiasis, urinary retention, and cancer of the urinary tract (Isnard *et al.*, 2004). For example, in South Africa, an herbal remedy containing Cape aloes caused acute oliguric renal failure and liver dysfunction (Luyckx *et al.*, 2002), and another, *Callilepsis laureola,* produced acute renal failure (Seedat and Hitchcock, 1971). In Peru, a remedy containing seeds of *Ormosia coccinea* called "hyayruro" or the "lucky bean," proved to be nephrotoxic and required the patient to be placed on hemodialysis (Guerra, personal communication, 2005). In India, additional nephrotoxins are encountered both in common edible plants (djenkol beans, mushrooms) and in medicinal herbs (impila, cat's claw) (Jha and Chugh, 2003). Flavonoids such as sciadopitysin can cause severe nephropathy (Lin and Ho, 1994). A number of *Aristolochia* species commonly found in TCM have also been associated with undesirable effects on the kidney (Sun *et al.*, 2002) and liver. Herbal metabolites formed during the process of bioactivation can covalently bind to cellular proteins and DNA, leading to toxicity via multiple mechanisms such as direct cytotoxicity, oncogene activation, and hypersensitivity reactions. Aristolochic acids can undergo reduction of the nitro group by hepatic CYP 1A1/2 or peroxidases in extrahepatic tissues to reactive cyclic nitrenium ion, which is capable of reacting with DNA and proteins, resulting in activation of H-*ras* oncogene, gene mutation, and finally leading to mutagenesis (Zhou *et al.*, 2004). A urothelial carcinoma was reported following use of a Chinese herbal medicine containing *Aristolochia fangchi* (Nortier *et al.*, 2000).

C. HEART AND CARDIOVASCULAR SYSTEM

Use of herbal medications for the treatment of cardiovascular problems must be done with care. Although some may be useful, many contain natural glycosides or blood thinners or affect blood pressure. They are not only bioreactive on their own but also serve to potentiate or diminish the action of prescribed medications. Concomitant uses of conventional treatments for cardiovascular problems along with traditional remedies for the same purpose are inadvisable because these combinations can seriously compromise the treatment outcome. Potentially grave adverse effects associated with herbal use are arrhythmias, arteritis, cardiac glycosides overdose, chest pain, congestive heart failure, hypertension, hypotension, myocardial infarction, over-anticoagulation, pericarditis, and death (Ernst, 2003). Effects on the coagulation are particularly noteworthy, and few patients on chronic anticoagulant therapy have completely stable PT/INR values because of interactions with certain drugs and foods, particularly those that contain anticoagulant coumarins or additional like substances (Schulman, 2003). The flavonoid cyanidanol has also elicited hemolytic anemia in several patients (Gandolfo *et al.*, 1992), and in one instance, use of an herbal medicine precipitated massive hemolysis (Baker and Thomas, 1987).

Licorice can also induce hypokalemia and hypertension. These symptoms have been associated with the use of liquorice-flavored chewing gum (DeKlerk *et al.*, 1997), and following its use as a tea sweetener along with the long-term consumption of licorice candy, hypokalemic paralysis developed (Elinav and Chajek-Shaul, 2003). In the kampo medicine "shakuyaku kanzou tou," it induced symptoms of pseudoaldosteronism (Kanda *et al.*, 2004).

European mistletoe (*Viscum album*) berries, present in tonics for the cardiovascular and nervous systems, are also cardiotoxic and neurotoxic and can cause GI tract bleeding (Stein and Berg, 1999). Similarly, Hawthorn (*Crataegus monogyna*) berries or flowers used similarly, can potentiate digitalis activity, increase coronary dilatation effects of theophylline, caffeine, papaverine, sodium nitrate, adenosine, and epinephrine, and increase barbiturate-induced sleeping times (ESCOP, 1997, 1999; Mawrey, 1993; Tyler, l994; Upton, 1999). In addition, excessive use of herbal laxatives such as senna (leaves and pods of *Cassia senna* and other *Cassia* species) and cascara (*Rhamnus purshiana*) can deplete blood electrolytes, particularly potassium, further contributing to the toxicity of digoxin (Miller and Murray, 1998). Digoxin also shares structural similarities to compounds in *Periplocia sepium.* Significantly increased digoxin blood levels in a patient taking Siberian ginseng (*Eleutherococcus senticosus*) were likely due to adulteration with this plant (Awang, 1996). Cases have been described in which increased INR levels have been evoked in patients ingesting herbs such as Wintergreen

leaf (*Gaultheria procumbens*), sweet birch bark (*Betula lenta*), willow bark (*Salix alba*), and meadow sweet (*Filipendula ulmaria*); all contain salicylates whose significant antiplatelet activities can surpass those of aspirin and indomethacin (Samuels, 2005; Teng *et al.*, 1991). In addition, coumarin derivatives found in sweet melilot (*Melilotus officinalis*), tonka beans (*Dipteryx odorata*), and sweet woodruff (*Galium odoratum*) can potentiate the anticoagulating effects of warfarin (Williamson, 2003). Chaparral (*Larrea tridentata*), as a folk tea, can cause hypotension in patients with cancer under treatment (Anonymous, 1992).

Common foods may also potentiate antiplatelet effects. For example, the allicin in garlic (*Allium sativum*) can enhance fibrinolytic activity and inhibit platelet aggregation, as does capsicin in red pepper. Eugenol and acetyl eugenol found in cloves (*Syzygium aromaticum*) (Srivastava 1993), other spices, and artichokes (*Cynara scolymus*) (Lewis and Elvin-Lewis, 2003) can inhibit platelet thromboxane formation and increase formation of 12-HPETE (Wang *et al.*, 1984), in addition to enhancing fibrinolytic activity (Wasantapruek *et al.*, 1974). Because of the bromelian content of either papaya (*Carica papaya)* or pineapple (*Ananus comosus*), there is an increased tendency for bleeding in patients eating excessive amounts of these fruits. Patients should also guard against using garlic as a dietary supplement to lower cholesterol when taking ACE inhibitors to treat hypertension such as lisinopril, because their interactions can cause symptoms of hypotension and fainting (McCoubrie, 1996).

D. GASTROINTESTINAL SYSTEM

Flavonoids in herbal preparations may also affect the GI tract (e.g., cirkan causes chronic diarrhea [Maechel, 1992]); and a phlebotonic French drug, cyclo-3 fort containing *Ruscus aculeatus*, herperidin methyl chalcone, ascorbic acid can elicit colitis (Beaugerie *et al.*, 1994). Reports of nausea and vomiting induced by other herbal remedies are represented throughout the chapter.

XVII. DIABETES

Numerous herbal remedies exist for the treatment of diabetes, and many, which are used ethnopharmacologically to treat symptoms of diabetes mellitus (I and II), have been studied experimentally. Of those traditional remedies examined for hypoglycemic activity, more than 80% were found to possess this potential (Lewis and Elvin-Lewis, 2003; Yeh *et al.*, 2003). Among these is the fruit of karela (*Momordica charantia*), which grows in tropical regions of Asia, the West Indies, and Africa and is used as a food or

bush tea. In one report, a poorly controlled patient taking chlorpropamide experienced much better diabetic control after ingesting karela along with this medication (Aslam and Stockley, 1979). Although this was a positive outcome, this may not always be the case, and care should be taken in using any of these natural therapies or hypoglycemic foods, especially as an adjunct to conventional medicines for diabetic control. There is always the risk that their ability to potentiate hypoglycemic effects could be additive and act adversely. For example, popular hypoglycemic plants with the potential of eliciting additive effects include *Aloe vera* gel and juice (known to India and Central America) (Vogler and Ernst, 1999), gumar leaves (*Gymnema sylvestre*) (used in Asia) (Grover *et al.*, 2002), and the prickly pear, *Opuntia streptocantha* (used by Hispanic populations in the United States and Central America) (Coronodo *et al.*, 2004). A clinical trial among patients with type II diabetes indicates that Pycnogenol, a proprietary mixture of water-soluble bioflavonoids extracted from the French maritime pine (*Pinus maritima*) used as a supplement to standard antidiabetic treatment, can significantly lower plasma glucose levels for about 1 month and improve endothelial function, which retards diabetic retinopathy and improves visual acuity (Liu *et al.*, 2004; Schonlau and Rohdewald, 2001). However, a number of patients also experienced transient unwanted effects such as vertigo, GI upset, headache, nausea, and sleeplessness (Liu *et al.*, 2004). In view of these observations and its commercial promotion for a wide range of conditions, there is a need to appropriately assess its worth and safety. This is essential in relevance to its potential clinical value in the management of asthma (Hosseini *et al.*, 2001), childhood asthma (Lau *et al.*, 2004), mild hypertension (Liu *et al.*, 2004), and its use to prevent traveler's thrombosis (Belcaro *et al.*, 2004) alone or in combination with standardized ginger (*Zingiber officinalis*) root as the polyherbal, Zinopin for this condition, and in the treatment of motion sickness (Scurr and Gulatin, 2004). The potential also exists for interactions between insulin, oral hypoglycemics, and stimulant herbs such as Ma Huang (*Ephedra sinica*) and caffeine-containing beverages, although no cases have been reported (Miller and Murray, 1998). Flaxseed oil (*Liman usitatissimum*) can affect the absorption of glucose or drugs taken simultaneously (Blumenthal, 1998).

XVIII. USE OF PSYCHOACTIVES

Use of psychoactive plants and fungi for recreational, religious, or shamanistic purposes can affect individuals differently and often depends on a number of mitigating circumstances associated with individual responses to their use or abuse. It is not unusual for the consumer to experience various

unpleasant symptoms, especially nausea and vomiting, before experiencing enhanced auditory and visual effects. Users of the peyote cactus (*Lophophora williamsii*) during religious rituals commonly experience symptoms of nausea, chills, and vomiting, accompanied by dislocation of visual perspective and anxiety. Similarly, headache, dizziness, and nausea often accompany the excessive use of nutmeg or mace (*Myristica fragrans*) for recreational hallucinogenic purposes. In the Amazon, the shamanistic or recreational utilization of ayahuasca can cause nausea and vomiting, and these symptoms and limb numbness, facial twitching, and loss of muscular coordination can accompany employment of the South American snuff containing *Virola.* As sequelae to use, the least debatable and most troublesome adverse effect of smoking marijuana is impairment of short-term memory. Among chronic habituates, respiratory conditions such as chronic cough, bronchitis, bronchial irritation, shortness of breath, and chest tightness can also develop (Lewis and Elvin-Lewis, 2003). The consequences of long-term use of psychoactive plants on the CNS are understudied.

XIX. EFFECTS OF SLIMMING AGENTS

A number of studies have shown that use of natural slimming agents can be problematic (Lewis and Elvin-Lewis, 2003) and the overall risk/benefit ratios are not encouraging (Pittler and Ernst, 2004). Except for limited data on glucomannan and hydroxy-methylbutyrate, there is little evidence to support claims that any of these other products (e.g., chitosan, chromium picolinate, *Ephedra sinica, Garcinia cambogia,* Guar gum [*Cyamopsis tetragonoloba*], psyllium seed [*Plantago* species], pyruvate, and yohimbe [*Corynanthe yohimbe*]) are able to promote significant weight loss (Pittler and Ernst, 2004). Within this group, Glucomannan or Konjac flour, derived from tubers of *Amorhophallus konjac*, plantago psyllium, the water-soluble fiber from the husks of ripe seed of *Plantago ovata,* and Guar Gum, a dietary fiber from the Indian cluster bean (*Cyamopsis tetragonologus*), are bulking agents. These substances delay intestinal absorption of carbohydrates, so feelings of satiety are prolonged and glucose hemostasis is maintained for longer periods. These positive effects are often overridden by problems associated with absorption of a wide range of medications and fat-soluble vitamins. The bulking effect can cause bloating and flatulence, so taking them before retiring is not advised. Their use is also contraindicated in patients with intestinal, esophageal, or bowel obstructions (Lewis and Elvin-Lewis, 2003), which in the case of Guar gum resulted in a fatality (Opper *et al.*, 1990; Seidner *et al.*, 1990). In addition, allergic reactions have been associated with the use of glucomannan and psyllium (Lewis and Elvin-Lewis, 2003).

Stimulatory and anorexic effects are properties of caffeine and ephedrine, and these compounds or plants that contain them are often combined to promote optimal results in weight-loss formulations. Consumers respond differently to their excitatory effects, and serious reactions affecting the heart and CNS have resulted in the banning of *Ephedra sinica* and its alkaloids in these products in the United States and Canada. Caffeine is not regulated and can be found in several stimulating beverages such as coffee (*Coffea arabica*), black and green tea (*Camellia sinensis*), Yerba mate (*Ilex paraguariensis*), and guarana (*Paullinia cupana*).

Hydroxy citric acid extracted from *Garcinia cabogia* inhibits citrate cleavage enzyme and suppresses *de novo* fatty acid synthesis and food intake; however, clinical trials have been disappointing, and its use may elicit headaches, stomach pains, and upper respiratory and GI symptoms. Similar ineffective clinical effects have been seen with chitosan, a cationic polysaccharide derived from the exoskeleton of crustaceans. Promoted as a remedy to reduce fat absorption, it can evoke various GI symptoms, from constipation, diarrhea, flatulence, bloating, nausea, and heartburn. Comparable negative effects have been observed by using chromium picolinate, and reported effects in increasing lean body mass and increasing the basal metabolic rate have not translated into clinically meaningful data. On the other hand, hydroxy methylbutyrate use appears to be a promising way to modulate body mass toward a leaner appearance without causing any side effects (Lewis and Elvin-Lewis, 2003; Pittler and Ernst, 2004). *Aristolochia* in Chinese herbal formulations used for weight loss have been found to cause a rapidly progressive interstitial renal fibrotic syndrome and renal failure (Verherweghem *et al.*, 1993). Likewise in Japan, the development of Fanconi syndrome was related to the use of the slimming remedy Kanmokutsu containing *Aristolochia manshuriensis* (Tanaka *et al.*, 2000). The presence of sparteine in a number of herbal remedies used for slimming and diabetes has been reported to cause circulatory collapse and respiratory arrest (Galloway *et al.*, 1992) and classic anticholinergic effects (Tsiodras *et al.*, 1999). Following the use for several weeks of a slimming remedy consisting of the juices of *Sauropus androgynus* with pineapple or guava, an outbreak of rapidly progressive respiratory distress syndrome called *bronchiolitis obliterans* occurred in Taiwan (Lai *et al.*, 1996). Kelp (*Laminaria, Macrocystis, Nereocystis* species) contains high amounts of iodine, which alone or adjunct to supplemental thyroid therapy can enhance thyroid activity. Several cases of transient iodine-induced hyperthyroidism have been reported for healthy individuals taking kelp supplements for slimming; the symptoms disappeared when the supplements were no longer taken (De Smet, 1990; Eliason, 1998; Shilo and Hirsch, 1986). Adverse reactions to the use of kelp-containing skin patches have yet to be reported but would likely have the same potential.

XX. EFFECTS OF IMMUNE STIMULANTS

After the advent of the AIDS pandemic, the prophylactic use of herbal supplements with immunostimulating properties have become widespread with the notion that these confer enhanced protective effects for a normal healthy individual. Although this concept has limited value, many still subscribe to the practice. *Echinacea* (*E. purpurea* or *E. angustifolia*) is possibly the most popular drug in Western herbal practices today and is widely promoted for its value in preventing or obtunding symptoms of the common cold and lower urinary tract infections (Blumenthal, 2000). Its effect for these purposes has yet to be conclusively proven, perhaps because appropriately supervised clinical trials are only now beginning to be conducted (Sperber *et al.*, 2004), the etiology of the common cold is so complex, and optimal doses have yet to be ascertained. Numerous *in vitro* and *in vivo* studies have proven its immunostimulating, bactericidal, virostatic, and wound healing properties and have further demonstrated that its alkamides, glycoproteins, caffeic acid derivatives (cichoric and echnosides), and polysaccharides are related to these phenomena (Lewis and Elvin-Lewis, 2003). Animal studies have shown it can increase the number of circulating leukocytes, promote phagocytosis, and stimulate the production of cytokines. By inhibiting tissue and bacterial hyaluronidases, it improves wound healing (Kemp and Franco, 2002). However, individuals taking immunostimulating herbs who are genetically predisposed to autoimmune disease are at risk of precipitating or exacerbating their symptoms. Several immunostimulating herbs have been implicated in these adverse events. For example, reports of flares of pemphigus vulgaris have been linked to use of *Echinacea* and the alga *Spirulina platensis,* and the development of dermatomyositis following use of a polyherbal containing the algae *S. platensis* and *Aphanizomenon flos-aquae* has occurred. Tumor necrosis factor-α (TNF-α) may play a role in some cases (Lee and Werth, 2004). Recurrent erythema nodosum has also been linked to *Echinacea* use (Soon and Crawford, 2001). Furthermore, atopic individuals using *Echinacea* can develop sensitivity to its allergens or respond by cross-sensitivity to other Asteraceous plants with the consequence of developing allergic episodes of rhinitis, dermatitis, and anaphylaxis (Beilory, 2002; Mullins and Heddle, 2002). *Echinacea* is likely to cause leukopenia if used for long periods, and recommendations to use formulations of Echinacea/goldenseal formulations as a prophylactic measure to ward off colds are, therefore, inappropriate (Kemp and Franco, 2002). Echinacea has also been implicated in a case of hepatotoxicity when used in combination with amiodarone, ketoconazole, and methotrexate (Miller, 1998).

XXI. PERIOPERATIVE USE OF HERBS AND SURGERY

The medical community is becoming increasingly aware and apprehensive of how herbal use may affect the patient's response to surgery or the outcome of healing. Currently, the American Society of Anesthesiologists (ASA) recommends that all herbal medications should be discontinued 2–3 weeks before an elective surgical procedure. Their primary concerns are related to increased risks of bleeding of such popular herbs as *Ginkgo biloba,* ginseng (*Panax ginseng*), and supplement (not culinary) uses of ginger (*Zingiber officinalis*) and garlic (*Allium sativum*), and their additive effects with other anticoagulants. Salicylate-containing plants might also be considered in this category particularly if they are used internally (e.g., black cohosh [*Cimicifuga racemosa*]). Ginseng also has the ability to evoke hyperglycemia. Numerous interactions are associated with SJW, which by its induction of CYP 3A4 increases the requirements for lidocaine, calcium channel blockers, midazolam (versed), and cyclosporin, as well as the interactions with anesthetics and sedative-hypnotic drugs of kava (*Piper methysticum*) and valerian (*V. officinalis*), which can prolong sedation when used with anesthetics or benzodiazepines and barbiturates. Also, with anesthesia, both *Echinacea* and kava may cause liver toxicity in those with concomitant liver disease. *Echinacea,* as an immune stimulant, may result in rejection of an organ transplant or if used on a prolonged basis may result in immunosuppression and otherwise affect the healing process. Though banned in the United States, *Ephedra*-containing products are still available over the Internet and through Chinese herbal practitioners, and any patient taking these is at risk of increasing blood pressure or developing life-threatening cardiac arrhythmias or CNS disturbances. Should either valerian or *Ephedra* be discontinued abruptly, perioperative hemodynamic instability or delirium can develop in a few hours. Ephedra use may evoke life-threatening hyperpyrexia if used with monoamine oxidase inhibitors (MAOIs) and significant arrhythmias with halothane. In addition, hypersensitivity myocarditis, leading to cardiomyopathy, has been observed. Plastic surgeons are particularly concerned about those herbs that can elicit photosensitivity or photoallergic reactions, noteworthy among these are kava and SJW. Although the potential exists for a number of foods such as mustard (*Brassica*), figs (*Ficus carica*), *Citrus* species, and numerous taxa within the Apiaceae including celery (*Apium graveolens*), carrot (*Daucus carota*), dill (*Anethum graveolens*), fennel (*Foeniclum vulgare*), parsnip (*Pastinaca sativa*), and so on, to elicit these reactions, they are likely of little clinical relevance (Ciocon *et al.*, 2004). For a complete list in this category, refer to Lewis and Elvin-Lewis (2003).

XXII. DRUG AND HERBAL INTERACTIONS

There is a wide range of potentially adverse interactions that can occur when conventional drugs are used concomitantly with certain foods and/or herbal medications (Blumenthal, 1998). Once rare, reports of problematical conditions are becoming commonplace (Williamson, 2003). For example, these associations can serve to potentiate or antagonize drug absorption or metabolism, affect the patient's metabolism, or cause unwanted side reactions such as hypersensitivity (Blumenthal, 2000; Brinker, 2001; Cupp, 1999; Izzo and Ernst, 2001). According to Brazier and Levine (2003), most interactions are pharmacokinetic and associated with metabolic affects on CYP enzymes. The most common examples are interactions with grapefruit, warfarin (and other cardiovascular drugs), and SJW (*H. perforatum*). In addition, a wide range of herb–drug interactions are associated with treatment of CNS conditions.

A. GRAPEFRUIT

Although grapefruit (*Citrus paradisi*) would not be generally categorized as an herbal remedy, uses of *Citrus* in folk medicine are widely known (Lewis and Elvin-Lewis, 2003). It is considered a functional food in that it is regularly eaten or its juice drunk because of its potassium, β-carotene, and high vitamin C content. It is also favored in many weight-loss diets. Although it is believed that grapefruit aides in weight loss by impacting on insulin levels and speeding up metabolism, a 2003 study indicates that its naringenin, by impairing glucose uptake in adipose tissue, may exacerbate insulin resistance in susceptible individuals (Harmen and Patel, 2003). The accidental discovery of its ability to interact with a wide range of drugs occurred when grapefruit juice was used to mask the flavor of alcohol in a clinical evaluation of an analogue of the calcium blocker nifedipine, namely felodipine (Bailey *et al.*, 1989). When plasma concentrations proved higher than expected, it was discovered that plasma felodipine concentrations were more than fivefold greater with grapefruit juice than with the alcohol mixture or water (Bailey *et al.*, 1989). This finding serves as an example of how foods that are purposely taken for their healthy benefits may also present serious hazards to health. The elderly are particularly at risk since they frequently consume grapefruit juice, and in spite of packaging inserts that caution of its use with certain drugs, they still may be unaware of the seriousness of the adverse reactions that can be evoked (Bailey and Dresser *et al.*, 2000; Bailey *et al.*, 1998; Dresser, 2004). Moreover, the clinical relevance of these drug interactions with grapefruit juice is not predictable,

TABLE VI
DRUG AND HERBAL INTERACTIONS: GRAPEFRUIT

Drug type	Adverse effects
Cardiovascular: dyslipidemia	Rhabdomyolysis
Cardiovascular: hypotension with dihydropyridines	Excessive vasodilation
Cardiovascular: antiarrhythmic agents	Enhances drug toxicity
Cardiovascular: congestive heart failure	Enhances drug toxicity
Cardiovascular: angina pectoris	Atrioventricular conduction disorders
Cardiovascular: antiplatelet	Attenuates activity
Allergies: fexofenadine	Lowers plasma levels
Ergotamine	Stroke, gangrene
Erectile dysfunction	Serious systemic vasodilation
Appetite suppression: sibutramine	Increases heart rate and blood pressure
Antidiabetic: repaglinide	Hypoglycemia

because oral clearance of drugs can vary, as does the activity of intestinal CYP 3A4 in individual patients. Toxicity is more likely to occur if a patient is given higher than usual doses of a potentially interactive drug and then begins drinking grapefruit juice for the first time (Huang *et al.*, 2004). Table VI summarizes the following data in tabular form.

For example, ingestion of grapefruit juice (e.g., 200–300 ml) or grapefruit segments can cause an irreversible inactivation of CYP 3A4 so that presystemic metabolism is reduced, and after 24 hours, oral drug bioavailability of medications is increased by reduction in intestinal or hepatic efflux transport. At least 20 drugs fit this category (Bailey *et al.*, 1998). Of importance are its effects on a number of cardiovascular drugs (Bailey and Dressler, 2004). Interactions with these drugs may increase the risk of rhabdomyolysis when dyslipidemia is treated with the HMG-CoA reductase inhibitors atorvastatin, lovastatin, or simvastatin or cause excessive vasodilation when hypertension is managed with the dihydropyridines such as felodipine, nicardipine, nifedipine, nisoldipine, or nitrendipin. Studies with elderly patients taking the calcium channel blocker felodipine indicate that a normal dietary amount of grapefruit juice can reduce its presystemic metabolism and elicit a pronounced, unpredictable, and sustained pharmacokinetic interaction. Also, a marked interaction similar to that for grapefruit juice can be found in individuals drinking red wine that have a preexisting high intestinal CYP 3A4 content. Felodipine concentration–blood pressure response relationships affected different blood pressure values seen in these studies (Offman *et al.*, 2001).

Enhancement of drug toxicity for antiarrhythmic agents such as amiodarone, quinidine, disopyramide, or propafenone, and for the congestive heart failure drug, carvediol has been demonstrated. Grapefruit juice may also elicit atrioventricular conduction disorders, if taken with verapamil used to treat angina pectoris as well as attenuate the antiplatelet activity of clopidogrel. Other drugs used in the treatment of peripheral and central vascular disease also have the potential to interact with grapefruit juice. Also, grapefruit juice may markedly lower the plasma concentration of fexofenadine, which is used to treat allergies. In contrast, the therapeutic effect of the angiotensin II type 1 receptor, losartan may be lowered. Interaction between ergotamine for migraine may cause gangrene or stroke. Intervention with nimodipine with stroke may cause systemic hypotension.

By reducing the activity of the P-glycoprotein transporter, GFJ is apparently involved in altering the systemic bioavailability of the immunosuppressive agent cyclosporine and the protease inhibitor, saquinavir. *In vitro* studies indicate that this phenomenon may be due to inhibition of organic anion transporting polypeptides (OATP), which decreases intestinal transport uptake as oral bioavailability is reduced. *In vitro* studies indicate that naringin at 3000 μmol/L and 6′,7′-dihydroxybergamottin at 33 μmol/L can reduce P-glycoprotein activity in half; naragin content in grapefruit segments is sufficiently high to evoke some P-glycoprotein inhibition activity in humans. OATP activity may also be reduced by both orange juice and apple juice, but apple juice differs in the spectrum of uptake transporters, which are affected (Dresser *et al.*, 2002).

The use of grapefruit juice with the appetite suppressant, sibutramine can increase heart rate and blood pressure, and with the antidiabetic agent, repaglinide, hypoglycemia has been observed. Serious systemic vasodilation can occur with the use of sildenafil, tadalafil, or vardenafil for erectile dysfunction, especially if it is combined with nitrate use. Interaction between ergotamine for migraine and grapefruit juice may cause gangrene or stroke. In stroke, interaction with nimodipine may cause systemic hypotension. Although it is recognized that drug responses are variable, and interactive events, leading to serious overdose toxicity, are difficult to prevent, the mandatory avoidance of grapefruit juice during pharmacotherapy is recommended. This is particularly true if the drug to be used is low in inherent oral bioavailability from presystemic metabolism by CYP 3A4 or efflux transport by P-glycoprotein. Clinically relevant interactions seem likely for most dihydropyridines, terfenadine, saquinavir, cyclosporin, midazolam, triazolam, and verapamil and may occur with lovastatin, cisapride, and astemizole (Bailey *et al.*, 1998). The elderly should be carefully advised regarding this risk, because they are likely to consume grapefruit juice unless cautioned to do otherwise (Bailey and Dressler, 2004; Dressler *et al.*, 2000).

B. WARFARIN AND OTHER DRUGS USED TO TREAT CARDIOVASCULAR CONDITIONS

Use of certain herbs with warfarin can affect its therapeutic value and cause life-threatening events (Table VII). For example, the Chinese herb Danshen (*Salvia miltiorrhiza*) may alter the pharmacokinetics of warfarin by increasing its plasma concentrations through enhancing absorption rates and decreasing its clearance (Samuels, 2005), whereas feverfew (*Tanacetum parthenium*) can retard it absorption by significantly suppressing prostaglandin production without inhibiting cyclooxygenase (COX) activity (Heptinstall *et al.*, 1987; Makheja and Bailey, 1981, 1982). Also, the flavones baicalin and oroxylin in skullcap (*Scutellaria baicalensis*) can hinder coagulation (Samuels, 2004), and tannins in Daikon-So (*Geum japonicum*) can inhibit key serine proteinases of thrombin and factor Xa and fibrinogen hydrolysis (Dong *et al.*, 1998). Lessening effects with warfarin have been associated with concomitant use of Passionflower (*Passiflora* species), Juniper (*Juniperus* species), and Vervain (*Verbena officinalis*) and increased with the use of the Reishi mushroom, (*Ganoderma japonicum*), papaw (*Asimina triloba*), ginseng (*Panax* spp), Devil's claw (*Harpagophytum procumbens*), *Ginkgo biloba,* ginger (*Zingiber officinalis*), Red Clover (*Trifolium pratense*),

TABLE VII
HERBAL INTERACTIONS WITH WARFARIN

Common name	Adverse effects
Daikon-So	Inhibits both serine proteinases of thrombin, factor Xa of fibrin hydrolysis
Danshen	Increases plasma concentrations
Devil's claw	Increases effects
Feverfew	Retards absorption
Ginger	Increases effects
Ginkgo	Increases effects, spontaneous bleeding, subarachnoid hemorrhage
Ginseng	Increases effects
Horse-Chestnut	Increases effects
Juniper	Lessens effects
Papaw	Increases effects
Passionflower	Lessens effects
Red Clover	Increases effects
Reishi mushroom	Increases effects
Skullcap	Hinders coagulation
Vervain	Lessens effects

and Horse-Chestnut (*Aesculus hippocastanum*) (Argento *et al.*, 2000; Miller *et al.*, 2004). For example, it has been hypothesized that the pharmacodynamic effects of *Ginkgo biloba,* used to treat generative and vascular conditions associated with memory loss, is related to a combination of platelet-activating factor antagonistic effects, free radical scavenging activity, and modulation of cholinergic function (Nathan, 2000). Its ginkgolides, which are unique terpene lactones, possess specific platelet-activating factor antagonist activities (Williamson, 2003). Unfortunately, in certain patients, use of this herbal remedy can elicit episodes of spontaneous bleeding (Skogh, 1998) such as subarachnoid hemorrhage (Vale, 1998) and, when warfarin, aspirin (acetylsalicylic acid), or ibuprofen are taken concomitantly, may elicit events such as intracerebral hemorrhage (Matthews, 1998; Mehta, 2003), bleeding of the iris into the anterior chamber of the eye (Rosenblatt and Mindel, 1997) due to increases in ocular blood flow velocity (Chung *et al.*, 1999) or fatal intracerebral mass bleeding (Meisel *et al.*, 2003) have been reported. Concomitant use of ginkgo with calcium channel antagonists such as nicardipine, nifedipine, and diltiazem may be unwise (Williamson, 2003) because studies in rats suggest that Ginkgo extract can attenuate the hypotensive response of nicardipine through induction of CYP 3A2 and other liver metabolizing enzymes (Shinozuka *et al.*, 2003). Table VII summarizes these data in tabular form.

The Chinese formula Kangen Karyu contains peony root (*Paeonia veitchii, P. lactiflora, P. ovata*), safflower (*Carthamus tinctorius*), saussurea/costus root (*Saussurea lappa*), cnidium/Schezuan lovage root (*Ligusticum chuanxiong*), cyperus/nut-grass rhizome (*Cyperus rotundus*), and Danshen (*Salvia miltiorrhiza*) (Bensky and Gamble, 1993). This polyherbal is used to reduce blood viscosity and improve microcirculation and can significantly enhance bleeding time (Makino *et al.*, 2002), as well as suppress the metabolism and excretion of warfarin, without affecting its serum binding and absorption (Makino *et al.*, 2003). In isolated cases, elevated INR values have also been associated with a patient taking warfarin, digoxin, atenolol, and fluvastatin and a TCM remedy of Chinese wolfberry (*Lycium barbarum*) (Lam *et al.*, 2001), and another taking warfarin and boldo *(Perumus boldo)* together with fenugreek (*Trigonella goenum-graecum*) (Lambert and Cormier, 2001). Similar INR increases have been noted with two patients taking pumpkin seed (*Curcubita pepo*) and saw palmetto (*Serenoa repens*) for prostate enlargement; in these cases, only one of these individuals was also taking warfarin (Yue and Jansson, 2001). Apparently there is little clinical significance to the observation that a few individuals on either warfarin or procoumon can experience enhanced INR levels when drinking large quantities of tonic water containing quinine (Stockley, 2002). Eating large amounts of mango fruit while on warfarin can also increase INR levels

(Monterrey-Rodrigues *et al.*, 2002). Because bromelian in papaya and pineapple modulates the arachidonate cascade, INR rates are also increased with concomitant use of warfarin (Shaw *et al.*, 1997). In contrast, the vitamin K in a variety of green vegetables, particularly broccoli, in large amounts can act as an antagonist to the effects of anti-coagulant therapy (D'Arcy, 1993); in warfarin overdose, vitamin K is used to normalize the INR.

A ginseng component 2A-1-1 has also been found to inhibit platelet aggregation by blocking the receptor-dependent Ca^{2+} channels (ROC) and inhibiting Ca^{2+} influx of human platelets (Zeng *et al.*, 2004). Prolongation of thrombin, prothrombin, and thromboplastin times is also characteristic of several of the 26 herbs found in Bak Goong Pill, also known as *Bai Geng Wan* (Gou *et al.*, 2003). Gugulipid (*Commiphora mukul*) can diminish the effects of other cardiovascular drugs including diltiazem and propranolol (Dalvi *et al.*, 1994; Singh *et al.*, 1994), and *Plantago psyllium* and *P. ovata* bulking agents can enhance the cholesterol-lowering action of cholestyramine and possibly decrease the absorption of digoxin and warfarin. Glucomannan thickening flour made from *Amorphophallus konjac* may act similarly (Elvin-Lewis, 2001; Lewis and Elvin-Lewis, 2003; Williamson, 2003). Numerous adverse events have been associated with the use of aconite (*Aconitum*), *Ephedra,* and licorice (*Glycyrrhiza glabra*) (Ernst, 2003; Mashour *et al.*, 1998). Together with digitalis, additive effects with thiazide diuretics have been seen with aconite inducing K loss, by increasing absorption and retarding absorption. Fatal hypertension can occur when use of digitalis is combined with *Ephedra* and scotch broom (*Cystisus scoparius*). In addition, a tyramine-induced hypertensive crisis has occurred when rhubarb root (*Rheum officinale*) is combined with digitalis use.

C. ST. JOHN'S WORT

It is believed that SJW, used to treat mild to moderate depression, has the potential to reduce the systemic bioavailability of many conventional drugs and to diminish blood levels of cyclosporine, amitriptyline, digoxin, indinavir, warfarin, human immunodeficiency virus (HIV) protease inhibitors, phenprocoumon, and theophylline. This is based on the concept that enhancement of blood clearance of warfarin, digoxin, theophylline, or ethinylestradiol/desogestrel is mitigated by the presence of SJW. An additive effect has been proposed, because all, including SJW, can cause the induction of the CYP isoenzymes CYP 3A4, CYP 2C9, and CYP 1A2, as well as the transport protein P-glycoprotein, needed to metabolize and remove these drugs from circulation (Henderson *et al.*, 2002; Miller *et al.*, 2004).

In addition, possible pharmacodynamic interactions with selective serotonin reuptake inhibitors and serotonin (5-HT_{1d}) receptor agonists such as triptans used to treat migraine have been identified (Henderson *et al.*, 2002). Taken together with loperamide or selective serotonin reuptake inhibitors (sertraline, paroxetine, nefazodone), delirium or mild serotonin syndromes have occurred (Izzo and Ernst, 2001). One reported incident of a patient taking testosterone after an orchidectomy, in addition to sertraline and SJW, describes a major manic episode accompanied by grandiose delusions resulting in hospitalization (Barbenel *et al.*, 2000). Another case of acute mania and psychosis was related to use by a 23-year-old woman of a polyherbal remedy called "Valdispert balans," which contained high doses of SJW and valerian (*V. officinalis*). As antidepressants, both are used to treat anxiety, but usually not together. She was diagnosed as having substance-induced mood disorder, with manic features (American Psychiatric Association, 2000) and completely recovered after discontinuing the herbal remedy and taking a short course of olanzapine. The authors further caution about the hazards of taking these herbal remedies by patients with bipolar disorders, in the event that its use could trigger a manic attack (Guzelcan *et al.*, 2001). Although numerous case reports accumulate, many clinical trials cited in support of these interactions have been based on evidence collected from inappropriately designed studies, which are lacking in scope and methodological quality, especially regarding the inclusion of controls and randomization (Mills *et al.*, 2005). Moreover, it is now recognized that many non-responders exist, which only tends to confound the issues of treatment efficacy or adversity (Gelenberg *et al.*, 2004).

When SJW is used with the oral contraceptive ethinylestradiol/desogestrel, intermenstrual bleeding has been observed (Izzo and Ernst, 2001). A British survey among female college students taking contraceptive pills indicates that although most (64%) are aware that concurrent use of antibiotics can decrease contraceptive effectiveness, only 14% are aware that SJW can act in a similar manner. This information is disconcerting, because SJW use as a mild antidepressant in these populations can increase the risk of unwanted pregnancies (Hindmarsh and Oakeshott, 2002). Currently, the concomitant use of SJW and warfarin is not recommended by the United Kingdom Medicines and Healthcare Products Regulatory Agency (MHRS), and it further advises that those doing so should stop and adjust their dose of warfarin accordingly (Williamson, 2003). A paper by Huang *et al.* (2004) illustrates a number of studies on drug pharmacokinetics and responses to SJW. Table VIII summarizes these data.

TABLE VIII
DRUG AND HERBAL REACTIONS: ST JOHN'S WORT

Drug	Adverse effects
Amitriptyline	Reduces systemic bioavailability
Cyclosporin	Reduces systemic bioavailability
Digoxin	Reduces systemic bioavailability, mitigates clearance
Ethinylestradiol/ desogestrel	Mitigates clearance, intramenstrual bleeding, decrease contraceptive effectiveness
HIV protease inhibitors	Reduces systemic bioavailability
Indinavir	Reduces systemic bioavailability
Loperamide	Delirium, mild serotonin syndromes
Phenprocoumon	Reduces systemic bioavailability
Serotonin reuptake inhibitors: sertraline, paroxetine, nefazodone	Delirium, mild serotonin syndromes
Theophylline	Reduces systemic bioavailability, mitigates clearance
Warfarin	Reduces systemic bioavailability, mitigates clearance

D. HERB–DRUG INTERACTIONS AFFECTING THE CENTRAL NERVOUS SYSTEM

When a patient is undergoing conventional treatment for disorders of the CNS, use of certain herbs, including the smoking of tobacco or cannabis, is not advisable. Heavy tobacco smoking is a common practice among psychotic patients, and this habit can seriously affect the metabolic clearance and pharmacokinetic and pharmacodynamic properties of the psychotropic drugs they are taking. A similar action may also exist for cannabis smoking. This is because the polycyclic aromatic hydrocarbons in tobacco smoke can induce hepatic aryl hydrocarbon hydroxylases that facilitate blood clearance of drugs such as imipramine, clomipramine, fovoxamine, traxodone, tiotixene, fluphenazine, haloperidol, olanzapine, chlorpromazine, and clozapine. Similarly, increased clearance of the benzodiazepines alprazolam, lorazepam, oxazepam, diazepam, and demethyl-diazepam can occur. Plasma concentrations of amitriptyline and nortriptyline may be variably affected by tobacco smoking, whereas carbamazepine is minimally affected, probably because the hepatic enzymes are already stimulated by its own autoinductive properties (Desai *et al.*, 2001). Cannibis smoking may also increase chlorpromazine clearance (Chetty *et al.*, 1994). Cigarette smoking does not appear to affect the use of amfebutamone (bupropion). Smokers receiving

chlorpromazine and benzodiazepines may also experience reduced drowsiness (Desai *et al.*, 2001). Cannibis-induced manias have also been reported for patients concurrently using either fluoxetine (Stoll *et al.*, 1991) or disulfiram (Lacoursiere and Swatek, 1983).

Seizures have occurred in schizophrenic patients taking evening primrose oil alone or with fluphenazine. Its experimental use within schizophrenic patient populations has exacerbated negative symptoms of the disease and can produce electroencephalographic evidence of temporal lobe epilepsy (Vaddadi, 1981). The oil is derived from the seeds of *Oenothera biennis* L. and as ω-6 polyunsaturated fatty acids consists of is linoleic acid (LA) and γ-linolenic acid (GLA). Reduction of plasma levels of phenytoin and loss of seizure control has been associated with concurrent use of the Ayurvedic drug Shankhapushpi (Dandekar *et al.*, 1992). This polyherbal contains *Centella asiatica, Convolvulus pluricaulis, Nardostacys jaatamansi, Nepteta elliptica, N. hindostana,* and *Ansosma bracteatum.* Similar effects have been seen with the traditional Chinese herbal ingredient Paeoniae Radix (*Paeonia veitchii, Paeonia lactiflora, Paeonia ovata*) (Chen *et al.*, 2001). Ginseng may act as an antidepressant antagonist with phenelzine, triazolam, and lorazepam by causing manic episodes associated with headaches, tremulousness, insomnia, irritability, and visual hallucinations (Gonzalez-Seijo *et al.*, 1995). Caffeine in tea, coffee, and other beverages may also increase clozapine serum levels because they are both metabolized mainly by CYP 1A2 and caffeine inhibits clozapine metabolism. In clinical evaluations, possibly because of genetic differences among the participants, ingestion of instant coffee was found to elicit variable responses on the plasma levels of clozapine (Raaska *et al.*, 2004). This may also be due to the ability of other components in the coffee to precipitate out the neuroleptic chlorpromazine and, thus, affect its bioavailability; similar effects are also known for tea (Cheeseman and Neal, 1981). In an earlier clinical trial, tea or coffee drinking had no effect on either blood levels of antipsychotic drugs or their behavior (Bowen *et al.*, 1981). The use of oral contraceptives (e.g., haloperidol) with chasteberry fruit (*Vitex agnus-castus*) is contraindicated; this combination also has a reciprocal weakening effect on dopamine receptor antagonists (Blumenthal *et al.*, 1998).

The combination of herbal sedatives such as valerian (*V. officinalis*), passion flower (*Passiflora* species), and anticholinergic Solanaceae (*Atropa belladonna, Datura stromonium, Hyocyamus niger,* and *Mandragora officinarum)* with alcohol or antihistamines can potentiate the effects of antidepressants, antihistaminics, and antispasmodics, causing drowsiness and obtunding the ability to use machinery (D'Arcy, 1993; De Smet *et al.*, 1996).

Psychoactive activity is potentiated when tetracycline, propanolol, or alcohol is used with either cinnamon (*Cinnamomum zeylanicum*) (in excess

of culinary amounts) or "magic mushrooms" (*Psilocybe semilanceata*) (D'Arcy, 1993; Ernst, 1998).

Blood levels of lithium, used to treat bipolar disorder, must be checked regularly to avoid toxicity. However, in patients taking bulk fiber preparations of ispaghula or psyllium, which could affect its absorption, blood levels may be lower (Toutongi *et al.*, 1990). Alternately, lithium toxicity was elicited in a patient taking a polyherbal diuretic for purposes of slimming (Pyevich and Bogenschutz, 2001), an association that has been well known with other diuretic agents for some time (Jefferson, 1980). Because reserpine (an alkaloid from *Rauwolfia serpentina*) depletes neurotransmitter release, it can reduce the effects of levodopa (Stockley, 2002). Exacerbations of parkinsonian tremor can occur following the chewing of betel nut when patients are taking flupentixol or procyclidine (Deahl, 1989).

E. MISCELLANEOUS HERB–DRUG INTERACTIONS

Herbal effects on absorption can affect the bioavailability of antibiotics and has been demonstrated for Khat (*Catha endulis*) users (Attef *et al.*, 1997). Absorption and decreased metabolism of the asthma drug theophylline are also affected by piperine from black pepper (*Piper nigrum, Piper longum*).

XXIII. SUMMARY AND CONCLUSION

It has been generally considered that when compounded and prescribed appropriately, the safety of traditional herbal medications is high. Most are moderately bioreactive, and thus, within the context of empirical selective methods, their benefits are likely to far outweigh their risks. However, because these have become adapted to current widespread use, are subject to changes and adulterations by entrepreneurs or inexperienced practitioners, and are used without supervision, the possibility of unexpected reactions increases, particularly if they are taken with conventional medications. It is likely that most minor adverse reactions are simply underreported, particularly among those who tend to self-medicate. Although initiatives are underway for global oversight to ensure herbal products are appropriately formulated and administered, the realization of this goal may take many years to achieve. In the interim, there is little option but to rely on the integrity and skill of those that formulate, promote, or prescribe them with the anticipation that one's health is not compromised in the process.

Anyone selecting these methods, particularly in the context of neo-Western herbalism should realize that there is a wide variability in the herbal products that are available and using reliable sources is essential. Prospective

herbal users must be conscious that these formulations are composed of raw materials that can vary in quality or quantity, that they may contain any number of undocumented substances, and their pharmacokinetic and pharmacognostic capabilities can thus be unreliable. Their use can be of benefit, interact with other herbs or drugs, or be potentially harmful. Certain foods, or functional foods, may also potentiate or antagonize pharmaceutical treatments, and under certain conditions, clinical-use restrictions may apply. Also, new data are constantly being generated by research designed to delineate their clinical value and identify any potential dangers that might arise. It may mean that certain well-known products have expanded uses or may no longer be considered safe to use. If one lacks appropriate interpretive skills to understand this information, it is conventional wisdom to seek advice from those with this ability before embarking on a regimen of self-medication. Keeping one's allopathic physician informed of what one is doing is also recommended.

In light of current policies that are unevenly evoked to ensure safety and efficacy, reliance on promotional information, no matter how skillfully executed, this is folly unless authoritative scientific evaluations are well known to back up any claims of worth and parameters of use. There is little meaning in the belief that ''natural'' is safe, any more than one can expect the same for a pharmaceutically derived product. The following guidelines for rational herb use follow many recommendations already outlined by Murphy (1999) and Drew and Myers (1997) (Table IX).

It is also the responsibility of conventional practitioners to apprise themselves of herbal and other alternative practices that are popular within their community of patients. This will allow them to better formulate strategies regarding integrative use and to understand their patients and the implications that it has to their health. This recommendation is made because surveys have indicated that at least one-third of patients are likely to use unconventional therapy; the majority of these do so for chronic conditions but are unlikely to inform their allopathic physician (Barret *et al.*, 1999; Eisenberg *et al.*, 1998). Disconcerting also are the number of children with chronic conditions that are being provided with dietary supplements without their physician's knowledge (Ball *et al.*, 2005). Therefore, care must be taken in interviewing each patient to be open-minded and nonjudgmental during the initial process so that they are willing to reveal the totality of their self-medication or alternative medicinal practices. Formal discussions should help understand their preferences and expectations, and by encouraging patients to keep a symptom diary, potentially harmful situations will become evident on follow-up visits (Eisenberg, 1997). Armed with appropriate knowledge of any potential interactions or adverse effects that might occur, the physician may then be able to guide them as to whether it is

TABLE IX
GUIDELINES FOR RATIONAL HERB USE[a]

- Be informed, seek out unbiased, scientific sources
- For guidance, rely on professionally trained and registered practitioners of herbal medicine
- Be aware that your physician's or pharmacist's knowledge of herbal remedies may be limited
- Inform your physician of self-medication regimens
- Remember that drug interactions can be problematical
- Do not depend on product claims alone
- Read labels carefully, do not exceed recommended dose ranges
- Know benefits and risks of potential side effects
- Know potential drug interactions
- Never use if pregnant or nursing
- Avoid giving to children
- Avoid giving to the elderly
- Do not use for serious illnesses
- Do not use for prolonged periods
- Know your source, formulator, or manufacturer
- Select standardized formulations
- Understand that batch-to-batch variations of the formula may occur
- To avoid misidentification, do not collect plants yourself
- Make sure packaging is appropriately labeled with its contents
- Make sure the labeling includes scientific names
- Store appropriately to prevent loss of potency
- Report any adverse reactions you believe are linked to its use

[a]Amended from Table VIII in Elvin-Lewis, 2001.

TABLE X
EVALUATION OF ADVERSE EffECTS[a]

- Temporal association between exposure and effect
- Disappearance of effect after product discontinued
- Reappearance of effect when product reintroduced
- Association of product use and interactions with medicines
- Occupational chemicals and recreational drugs
- Association of underlying disease states considered
- Association of exposure and effects known in scientific literature

[a]Amended from Table VII in Elvin-Lewis, 2001.

advisable to continue with any particular practice. It is appreciated that practitioners may recognize acute symptoms of toxicity but are unlikely to link effects associated with hepatotoxicity, teratogenicity, or carcinogenicity to use. The guidelines in Table X are related to temporal associations to herbal use and may be valuable when adverse effects are suspected

(Elvin-Lewis, 2001). Many recommendations follow those cited by Murphy (1999) and Drew and Myers (1997).

APPENDIX

COMMON AND SCIENTIFIC NAMES OF MEDICINAL PLANTS

African cherry	*Prunus africana*
Aloe	*Aloe vera*
Angelica	*Angelica dahurica*
Arnica	*Arnica montana*
Balsam of Peru	*Myroxylon pereirae*
Bajialoam	*Dysoma pleianthum*
Belladona	*Atropa belladona*
Birch (sweet)	*Betula lenta*
Black cohosh	*Cimicifuga racemosa*
Blood root	*Sanguinaria Canadensis*
Blue cohosh	*Caulophyllum thalictroides*
Boldo	*Perumus boldo*
Cats claw	*Uncaria tomentosa/guianensis*
Caowu	*Aconitum kusnezoffii*
Cascara	*Rhamnus purshiana*
Celandine (greater)	*Chelidonium majus*
Celandine (lesser)	*Ranunculus ficaria*
Cinnamon	*Cinnamomum zeylanicum*
Chamomile	*Chamaemelum nobile*
Chanwu	*Aconitum carmichaeli*
Chaparral	*Larrea tridentata*
Chasteberry	*Vitex agnus-castus*
Chinese foxglove	*Rehmannia glutinosa*
Chinese wolfberry	*Lycium barbarum*
Cloves	*Syzygium aromaticum*
Coffee	*Coffea arabica*
Creosote bush	*Larrea tridentata*
Cyperus/nut grass	*Cyperus rotundus*
Daikon-So	*Geum japonicum*
Danshen	*Salvia miltiorrhiza*
Devil's claw	*Harpagophytum procumbens*
Echinacea	*Echinacia pallida*
European mistletoe	*Viscum album*
Evening primrose oil	*Oenothera biennis*
Fenugreek	*Trigonella goenum-gracecum*
Fever few	*Tanacetum parthenium*
Fig	*Ficus carica*
Flax	*Liman usitatissimum*
Fructus Aristolochiae	*Aristolochia debilis, A. fangchi*

(*continued*)

APPENDIX (*continued*)

Fo ti	*Polygonum multiflorum*
Gancao	*Glycyrrhiza uralensis*
Gas plant/Burning Bush	*Dictamnus albus subspecies dasycarpus*
Garlic	*Allium sativum*
Germander	*Teucrium chamedrys, T. polium*
Ginger	*Zingiber officinalis*
Ginkgo	*Ginkgo biloba*
Ginseng (Chinese)	*Panax ginseng*
Ginsengs (others cultivated)	*P. notoginseng, P. zingerberensis*
Ginseng (American)	*Panax quinquefolius*
Ginseng (Siberian)	*Eleuthrococcus senticosus*
Glucomannin/Konjac	*Amorhophallus konjac*
Goldenseal	*Hydrastis canadensis*
Golden thread (Huanglian)	*Coptis chinensis*
Grapefruit	*Citrus paradisi*
Guar	*Cyamopsis tetra-gonoloba*
Guarana	*Paullinia cupana*
Guggul tree	*Commiphora mukul*
Hawthorn	*Crategus monobyna*
Himalayan Mayapple	*Podophyllum emodi*
Hindu datura	*Datura metel*
Horse-Chestnut	*Aesculus hippocastanum*
Hyayruro, lucky bean	*Ormosia coccinea*
Impila	*Callilepsis laureola*
Juniper	*Juniperus* spp.
Kava	*Piper methysticum*
Karela	*Momordica charantia*
Khat	*Catha endulis*
Kelp	*Laminaria, Macrocystis, Nereocystis* spp.
Licorice	*Glycyrrhiza glabra*
Magic mushrooms	*Psilocybe semilanceata*
Ma Huang/Ephedra	*Ephedra sinica*
Mandrake	*Mandragora officinarum*
May apple	*Podophyllum peltatum*
Meadow-sweet	*Filipendula ulmaria*
Milk thistle	*Silymarum marianum*
North African thistle	*Atractylis gummifera*
Nutmeg/mace	*Myristica fragrans*
Papaya	*Carica papaya*
Passionflower	*Passiflora* spp.
Papaw	*Asimina triloba*
Peony (Paeoniae Rubrae Radix)	*Paeonia veitchii, obovata*
Pennyroyal	*Hedeoma pulegoides, Mentha pulegium*
Peyote cactus	*Lophophora williamsii*
Pineapple	*Ananus comosus*
Pumpkin	*Curcubita pepo*
Prickly pear	*Opuntia streptocantha*
Psyllium	*Plantago ovata*

APPENDIX (*continued*)

Radix petasidis	*Petasties officinalis*
Rauwolfia	*Rauwolfia serpentina*
Red Clover	*Trifolium pratense*
Reishi Mushroom	*Ganoderma japonicum*
Sangre de grado	*Croton lechleri*
Sassafras	*Sassafras albidum*
Saw palmetto	*Serenoa repens*
Senna (*Folia sennae*)	*Senna alexandrina*
Shandigyeb	*Sophora tonkinensis*
Skullcap	*Scutellaria lateriflora*
Spirulina	*Spirulina platensis*
St. John's wort	*Hypericum perforatum*
Sweet melilot	*Melilotus officinalis*
Sweet woodruff	*Galium odoratum*
Tansy	*Tanacetum vulgare*
Tea (green/black)	*Camellia sinensis*
Tea Tree	*Melaleuca alternifolia*
Tonka beans	*Dipteryx odorata*
Tussilaginis Farfare, Flos	*Tussilago farfara*
Valerian	*Valeriana officinalis*
Vervain	*Verbena officinalis*
Willow	*Salix alba*
Wintergreen	*Gaultheria procumbens*
Yerba mate	*Ilex paraguariensis*
Yohimbe	*Corynanthe yohimbe*

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