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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