I. Introduction

The host and its indigenous (normal) microflora together form a well-functioning ecological system. Various exogenous and endogenous influences direct the balance/imbalance of the system. Antibiotics are the most common and effective drugs used for treatment of infectious diseases. However, there is increased awareness of the fact that the use of antibiotics often disturbs the indigenous protective microflora. Even sub-inhibitory concentrations of antibiotics lead to imbalance of the normal microflora and create a more pathogenic biofilm covering mucosal surfaces (Cuperus et al., 1995). Recovery of the indigenous flora may take weeks to months (Larson and Borriello, 1990). During this time the person is predisposed to diseases from pathogenic or opportunistic bacteria.

Antibiotic-associated diarrhea (AAD) is probably the most common manifestation of normal microflora alteration caused by antimicrobial treatment, with an incidence of up to 30 per 100 hospitalized patients.
AAD may be divided into two types: uncomplicated or nonspecific AAD, and *Clostridium difficile*–associated diarrheas (CDAD). To the first group usually belong mild and self-limited diseases, yet CDAD could be complicated with colitis, and it causes a major burden on the health care system (Wilkins and Lyerly, 2003). Since the main cause of AAD is alteration of intestinal microflora, the prophylaxis and therapy for restoring normal microflora seems to be the most natural approach with these groups of diseases. Unfortunately, it is hard to assess the specific contributions of different microorganisms of indigenous microflora in a well-balanced system with the host.

In this chapter we summarize the studies concerning the impact of different external factors, particularly antibiotics, on various groups of indigenous microflora and evaluate experimental animal models of CDAD. This approach could predict which groups of microbes are able to restore the normal symbiosis and help to develop further applications of microbial interference therapy and prophylaxis against *Clostridium difficile* infections. The chapter also summarizes some human clinical trials with non-pathogenic commensal lactobacilli that have been considered as probiotics with beneficial health effects, including prevention or amelioration of AAD.

II. Microbial Ecology of Intestinal Tract and Colonization Resistance

A. INDIGENOUS MICROFLORA

The gastrointestinal tract (GI) of a human is colonized with more than 400 different species of microorganisms in numbers of more than $10^{11} – 10^{12}$ per gram (reviews of Macfarlane and Cummings, 1999; Simon and Gorbach, 1984). The term *indigenous microflora* (IMF) signifies the groups of non-pathogenic or potentially pathogenic microbes that are permanently inhabiting a particular biotope (lumen or mucosa; ileum, jejunum, or colon) of a particular individual and are in symbiotic association with the host (Rusch, 1989; Savage, 1987).

There are great individual differences in the quantitative and qualitative composition of intestinal microflora, although the stability of microflora in particular individuals has been demonstrated (Meijer-Severs and Santen, 1986; Mikelsaar, 1992). Recently, with molecular methods (i.e., denaturing gradient gel electrophoresis [DGGE]), it was proved that the predominant mucosa-associated bacterial community along the colon was host specific and significantly different from the fecal community (Zoetendal et al., 2002). The individually different quantitative composition of fecal microflora depends on
the host genetics, as shown by investigating adult monozygotic twins (Mikelsaar et al., 1984). Monozygotic twins reveal the identity of many genetic markers (i.e., antigenic structure of somatic cells and secretions of the host, as well as the immune reaction, that are important for the selective colonization by the indigenous microflora (Warner et al., 1988). To date, the same relationship has been confirmed by using molecular methods by comparing the intestinal microflora of genetically identical monozygotic twins (Zoetendal et al., 1998).

In the large intestine, relying on cultivation assays, the predominant anaerobic bacteria are Gram-negative rods such as *Bacteroides* and *Fusobacterium* sp., Gram-positive rods such as *Bifidobacterium* sp., *Eubacterium* sp., *Clostridium* sp. and cocci such as *Peptostreptococcus* sp., *Veillonella* sp. (Hentges, 1983; Levy, 2000; Mikelsaar and Mändar 1993; Sepp et al., 1997).

More anaerobes can be detected by 16S rDNA probes than by cultivation techniques (Harmsen et al., 2000; Štšepetova et al., 2002). The molecular techniques have identified new groups of bacteria (*Ruminococcus* sp., *Phascolarctobacteria* etc.) colonizing the intestinal tract in high numbers. However, the anaerobes like bacteroids, clostridia, and eubacteria (accounting for 20% to 29%), and bifidobacteria (accounting for 3% of the total fecal population) are still among the most important predominant microbes (Franks et al., 1998). In the future, the applicability of new techniques (genomics, proteomics, metabolomics) can profoundly enhance our knowledge about the various components of the indigenous microflora.

Apart from indigenous microflora, each biotope consists of non-indigenous (transient, allochtonous) microbes originating from the environment or from IMF of the other biotopes (Rusch, 1989). These pathogenic or opportunistic pathogens (e.g., *C. difficile*) can inhabit a biotope either for a short time or in the case of more profound perturbation of the microbial ecosystem, even for prolonged periods.

**B. Lactic Acid Bacteria as Part of Normal Microflora**

Lactobacilli are the well-known component of intestinal microflora. Lactobacilli are Gram-positive, rod-shaped, facultatively anaerobic, non-sporulating, acid-tolerant, and catalase-negative bacteria with a DNA base composition of less than 53 mol% G + C. Lactobacilli can be divided into subgenera such as *Thermobacterium*, *Streptobacterium*, and *Betabacterium* according to their growth temperatures and hexose fermentation pathways (Kandler and Weiss, 1986). Modern molecular methods based on the comparison of highly conserved molecules of
16S ribosomal ribonucleic acid (16S rRNA) genes have shown that these subgroups are inconsistent with the phylogenetic relationship of the species within the genus (Song et al., 2000). The principal phylogenetic grouping of *Lactobacillus* spp. proposed is summarized as follows: (1) *L. delbrueckii* group; (2) the *L. casei*–*Pediococcus* group; and (3) the *Leuconostoc* group including the species from the genera *Lactobacillus*, *Oenococcus*, and *Weissella* (Stiles and Holzapfel, 1997).

In addition, based on the peptidoglycan type of the cell wall and their fermentation pathways for pentoses and hexoses, lactobacilli are divided into obligately homofermentative lactobacilli (OHOL), facultatively heterofermentative lactobacilli (FHEL), and obligately heterofermentative lactobacilli (OHEL) (Hammes and Vogel, 1995). The latter division is valuable to understand their physiology and impact on human health.

There are few studies on the prevalence of lactobacilli in the small intestine. Usually, the numbers of microorganisms in the proximal jejunum are approximately $10^4$/ml, while the oropharyngeal microflora predominates. However, in a recent study *Lactobacillus* sp. microorganisms were found only in two healthy subjects out of 20 (Sullivan et al., 2003). In contrast, in the fecal samples, the *Lactobacillus* strains were present in approximately 70% of adults who consume a Western-like diet (reviewed by Heilig et al., 2002). In elderly persons the prevalence of lactobacilli was even higher, reaching 90% (Mikelsaar et al., 1998; Speck, 1976).

Earlier studies have demonstrated that the *Lactobacillus* spp. counts reached $10^{10}$ CFU/g in the fecal microflora of adults and were outnumbered only by obligate anaerobes (Simon and Gorbach, 1984). More recent investigations have shown counts of $10^6$–$10^9$ CFU/g (Sepp et al., 1997). A good marker for characterisation of the intestinal microflora is the distribution of particular groups of microorganisms in the total count. The relative abundance of lactobacilli in the total count of fecal bacteria for particular persons is <2% in children up to 1 y and <0.1% in adults (Mikelsaar and Mändar, 1993; Sepp et al., 1997). These data have been confirmed by Sghir et al. (2000) showing that lactobacilli constitute less than 1% of the total bacterial community within human fecal microbiota.

Using a fluorescent *in situ* molecular hybridisation technique (FISH), Marteau et al. (2001) found that the counts of cecal lactobacilli were quite similar to those of fecal lactobacilli (8.4 vs. 8.8, log CFU/g, respectively). Conversely, their distribution in cecal and fecal samples was quite different (23% vs. 7%). This finding may stress the importance of lactic acid producing bacteria in mucosal flora of the caecum,
the particular biotope most frequently attacked by pathogenic bacteria like *C. difficile*.

The lactoflora of the human GI tract consists of various species, subspecies, and biotypes of homo- and heterofermentative lactic acid bacteria. The most frequently occurring lactobacilli belong to 6 species: *Lactobacillus acidophilus-group, L. salivarius, L. casei, L. plantarum, L. fermentum, L. brevis* in various combinations (Mikelsaar et al., 1998; 2002; Molin et al., 1993; Reuter, 1997; Song et al., 1999). The *L. acidophilus* group has now been divided into *L. acidophilus sensu strictu, L. gasseri, L. crispatus* and *L. johnsonii* (Holzapfel et al., 2001). In addition to these, the frequent occurrence of *L. reuteri* in the GI tract of humans and animals has also been shown (Axelsson, 1990; Kandler and Weiss, 1986; Reuter, 1997).

Rectal biopsies from 42 individuals showed that lactobacilli counts ranged from <2.0 to 7.0 log CFU/g mucosa, with a median of 4.0 log CFU/g (Ahrné et al., 1998), while the most frequently isolated species from rectal mucosa were *L. plantarum* and *L. rhamnosus*. This finding hints on some geographical differences, as in studies of Russian astronauts (Lencner et al., 1984) and Italian elderly people *L. rhamnosus* was very seldom found (up to 20%) in individuals (Silvi et al., 2003). Additionally, the geographic differences were apparent when comparing the fecal *Lactobacillus* species composition of Estonian and Swedish 1- to 2-year-old children (Mikelsaar et al., 2002).

Large individual differences complicate the picture of GI lactoflora even more. In a survey over a 15-year period of 10 healthy volunteers, the stable persistence of fecal *Lactobacillus* species were revealed for each person even though the persons aged during the study, thus having several health failures, and used some medicines (Mikelsaar and Mändar, 1993; Mikelsaar et al., 1998). Similarly, Kimura et al. (1997) showed by pulsed-field gel electrophoresis (PFGE) that eight of ten subjects tested harbored a unique collection of lactobacilli in the intestine.

**C. Colonization Resistance**

The term microbial colonization resistance (CR) has been defined as the limiting action of the IMF on colonization of the bowel by exogenous as well as endogenous potentially pathogenic microorganisms. According to van der Waaij and Berghuis (1974), the CR relies on the anaerobic microflora of the gut. Several studies indicate that anaerobic IMF really has the main role in microbial CR, and aerobic potentially pathogenic microorganisms do not contribute (Borriello,
The set of anaerobes involved in CR has been shown to be quite wide, including some 7 to 10 different species (Boureau et al., 1989). To date, some authors have postulated on the particular role of intestinal lactobacilli in the maintenance of CR (Lidbeck and Nord, 1993; Mikelsaar and Mändar 1993; Naaber, 1997; Salminen and Deighton, 1992). The above-mentioned large geographic and individual variations in the normal microflora of people may obscure the understanding of CR.

The dynamic mechanisms of CR are well reviewed by McFarland (2000), though a full understanding of the complex indigenous microbiota by which protection is offered has not been fully established. Several actions of indigenous bacteria against pathogens, defined as a barrier effect (Borriello, 1990), are possible: competition for nutrients, secretion of antimicrobial substances (bacteriocines, hydrogen peroxide, nitric oxide, short chain fatty acids, proteases etc.), blockage of adhesive receptor sites for bacterial cell wall components or secreted toxins, co-aggregation of bacteria to be removed as larger particles, attenuation of virulence by suppression of toxin production and immune stimulation.

Although microbes play the most important role in maintenance of CR, it could also be mediated by anatomical and physiological factors including salivation, swallowing, and normal gastrointestinal motility; production of gastric acid, lysozyme and mucus protecting the epithelial cells; intact mucosal lining; epithelial cell turnover; secretory IgA levels; and action of M cells, phagocytes, and lymphatic tissue (McFarland, 2000; Rolfe, 1997). Newly described elements of the innate immune system (e.g., antimicrobial peptides such as defensins formed by polymorphonuclear cells and enterocytes) are directed against bacteria (Mahida et al., 1997). It appears, however, that anatomical and physiological CR guaranteeing systems are not capable of keeping the concentration of potentially pathogenic microorganisms under control if the IMF is absent or disturbed.

III. Antibiotic-Associated Diarrhea (AAD)

A. AAD AND Clostridium difficile-ASSOCIATED DIARRHEA (CDAD)

Gastrointestinal symptoms, particularly diarrhea, are relatively common side effects of antibiotic usage. Antibiotic associated diarrhea designates the diarrheas manifesting during or after recent antimicrobial therapy. AAD not caused by C. difficile is usually clinically mild watery diarrhea without complications, and it is resolved after drug
withdrawal. In contrast, CDAD can cause constitutional symptoms (fever and leukocytosis) and severe complications (colitis, bloody diarrhea, toxic megacolon). Symptoms of CDAD often persist after drug withdrawal and relapses are common (Bartlett, 1992).

The reported incidence of AAD ranges from 0.44 to 26/100, depending on host factors and hospitalization status (McFarland, 1998). Although in the majority of cases the exact mechanisms of the side effects of antibiotic consumption are not well understood, the proposed mechanisms include (1) direct action of antibiotics on intestinal function; (2) inducing predisposition to infection with enteric pathogens; and (3) factors secondary to the disturbance of normal intestinal flora that do not involve infection with a known pathogen (Borriello, 1992; Högenauer et al., 1998; McFarland, 1998; Midtvedt, 1989).

*C. difficile* is the most important causal agent of AAD. From 0.5% to 56% of AAD are caused by *C. difficile*, depending on the patient group investigated. The lowest frequency is observed in non-hospitalized patients. However, probably all nosocomial outbreaks of AAD are caused by *C. difficile* (Bartlett, 1992; De-Barbeyrac et al., 1989; Drapkin, 1992; McFarland, 1998).

1. **Antibiotics**

It is known that nearly all CDADs are related to previous antibiotic treatment and nearly all antimicrobial drugs can induce CDAD. However, several investigations support the idea that the ability of a particular antimicrobial to induce CDAD depends on its spectrum of activity and its pharmacokinetic properties. Antibiotics active against anaerobic bacteria (clindamycin, erythromycin) have been found most frequently associated with CDAD (McFarland, 1993). Administration of third-generation cephalosporins, broad-spectrum penicillins, and clindamycin is associated with the highest risk for CDAD (Bartlett, 1992; Hirschhorn et al., 1994; McFarland, 1993; Stoddart and Wilcox, 2002; Zimmermann, 1991). The challenge with these data is that there exists geographically and individually different compositions of indigenous microflora. The antibiotic causing CDAD in one person may not cause it in the other because of a wide variety of susceptibility to antibacterials of individual predominant microbes of the gut.

Moreover, it seems that the frequency and severity of CDAD do not appear to be antibiotic dose-related, in contrast to AAD that is due to other causes. The assumption is that modification of fecal flora is an essential feature of the drugs, but a confounding interrelated variable is their antibacterial activity against *C. difficile* (Bartlett, 1992). Unexpectedly, according to studies of the hamster model and
of patients, drugs with good activity against \textit{C. difficile} (including ampicillin and vancomycin) may also induce CDAD. Seemingly, it depends on the selective concentration of the drug in the lumen and mucosa of the gut.

B. \textit{Clostridium difficile} and CDAD

1. Etiopathogenesis

\textit{Clostridium difficile} is a Gram-positive spore-forming obligate anaerobe that was first isolated by Hall and O'Toole in 1935 from the feces of an infant and designated as \textit{Bacillus difficile} (Knoop et al., 1993). Although pseudomembranous colitis (PMC) was described more than a century ago, it started emerging in the 1960s because of increasing use of new antibiotics, particularly after the introduction of clindamycin. The link between PMC and \textit{C. difficile} was made at the end of the 1970s when Larson showed the cytotoxic activity of PMC patients' fecal filtrate on cell culture (Larson et al., 1977). Afterwards, the isolation of \textit{C. difficile} from the intestinal tract of PMC patients and clindamycin treated animals confirmed its etiological role (Bartlett and Gorbach, 1977; Bartlett et al., 1977; George et al., 1978; Larson et al., 1978)

The virulence of \textit{C. difficile} is mainly associated with two toxins, A and B, commonly referred as enterotoxin and cytotoxin. Until recently, production of toxin A was thought to be the most important factor in the pathogenesis of CDAD. Surprisingly, several outbreaks of toxin A-negative and toxin B-positive strains have been reported during the last decade (Wilkins and Lyerly, 2003). However, in these isolates, toxin B has a broader substrate specificity than both toxins producing isolates. The role of other virulence factors is more obscure. Adhesins have been proposed as being important, but their relevance in the colon is not very clear. The outer cell coat, called the \textit{S-layer}, has also been proposed as an important virulence factor of \textit{C. difficile}. Currently, suppression of indigenous intestinal microflora due to administration of antibiotics, subsequent colonization of the intestinal tract, and production of toxins (A and B or only B) by \textit{C. difficile} appear to be the most important factors for the development of intestinal infection (Bartlett, 1994; Poxton et al., 2001; Wilkins and Lyerly, 2003).

2. Prevalence

\textit{C. difficile} readily colonizes neonates and infants at the time when the microflora succession has not been finished and there is only scarce flora established. In children with mature microbial ecology, the \textit{C. difficile} is cleared from the intestines (Wilson, 1993). Once more the large
geographical differences can be noted as from the Swedish 1- to 2-year-old children with some 34%, while only 4% of Estonians of the same age were colonized with *C. difficile* (Sepp et al., 1997). In healthy adults the carriage rate of *C. difficile* also varies in the intestine of persons from different geographical areas, from 2% in Sweden to 15% colonized in Japan (Knoop et al., 1993) Unfortuately, it is not known if these numbers represent a transient colonization or if *C. difficile* is a permanent component of the stable flora of these subjects (Bartlett, 1994).

Higher age has been reported as a risk factor for colonization by *C. difficile* and CDAD (Bennett and Greenough, 1993). Some 19% of elderly residents have been found colonized by *C. difficile* in long-term-care facilities. Also, the colonization of healthy elderly people in the population is higher than the average for the general population (Nakamura et al., 1981; Simor et al., 1993). The reasons are probably altered CR caused by changes in the gut IMF in older people (Hébuterne, 2003; Hopkins and Macfarlane, 2002). In elderly persons, bifidobacteria decrease or disappear, while lactobacilli, enterococci, enterobacteria, and clostridia increase (Kleessen et al., 1997; Mitsuoka et al., 1990). Hopkins et al. (2001), using viable counts and estimations of 16S rRNA abundance, have reported the skewed bifidobacterial results, and a healthy 67-year-old male had, for instance, very high counts of these organisms, belonging to several species. In elderly persons the loss of some properties of the intestinal mucus necessary for adhesion of endogenous bifidobacteria have been assessed (Fang He et al., 2001). Individually different *Lactobacillus* flora was described in the feces of healthy elderly Italian people. However, *L. fermentum* and *B. longum* were the most represented species and suggested the design of functional foods to fortify the intestinal microflora of the elderly (Silvi et al., 2003).

According to different studies, the carriage rate in hospitalized patients varies from 7% to 21%. During an outbreak, even more than 50% of patients in the ward may become colonized by *C. difficile*. Interestingly, from 50% to 85% of these patients may remain asymptomatic, while in others, CDAD develops (Cartmill et al., 1994; Clabots et al., 1992; Johnson and Gerdin, 1998; Johnson et al., 1990; McFarland et al., 1990; Simor et al., 1993). It is not fully understood which factors control the population level of *C. difficile* in the gut and the expression of the disease.

3. *CR and C. difficile*

The importance of IMF in maintaining CR to *C. difficile* can most impressively be demonstrated in hamsters: *C. difficile* cannot colonize and cause disease in normal hamsters, but in antibiotic-treated
animals, even small doses of \( C. \) difficile cause lethal infection, and further, previous administration of fecal microflora of normal hamsters to antibiotic-treated animals prevents CDAD (Borriello, 1990). Despite clear evidence of the protective role of IMF, the exact mechanisms and bacteria that guarantee CR against \( C. \) difficile remain unknown.

We have found that children colonized with \( C. \) difficile and patients with CDAD usually have lower counts of lactobacilli in their intestine. Marked individual differences in counts of anaerobes, lactobacilli, and other members of IMF in healthy, as well as in \( C. \) difficile colonized/infected patients, have been assessed (Naaber et al., 1997). Another study has shown quite an opposite trend: in a small number of CDAD patients studied, high prevalence and viable counts of lactobacilli and enterobacteria have been found. However, the decreased total bacterial counts and these of bifidobacteria show simply the influence of previous metronidazole therapy sustaining lactobacilli and coliforms in these four patients (Hopkins and Macfarlane, 2002). It can be speculated that not only the total counts of lactobacilli (or some other bacterial group), but also the species composition of intestinal lactoflora may be important in the maintenance of CR against \( C. \) difficile.

The antibiotic susceptibility pattern of lactobacilli is not uniform (Table I). Keeping in mind individually different compositions of lactoflora, it can be understood why some people are more prone to CDAD by the same antibiotic prophylaxis than the others. Among antibiotics shown to be associated with the highest risk for CDAD the penicillins, erythromycin, and tetracycline affect most species of human lactobacilli. However, several species of \( Lactobacillus \) are resistant to cephalosporins of different generations. Cefoxitin and cefuroxime used for prophylaxis in surgery affect the \( L. \) acidophilus group, \( L. \) brevis and \( L. \) buchneri quite differently from the other lactobacilli. Interestingly, the combination of ampicillin and gentamicin often used in control of infections in intensive care seriously affects gastrointestinal lactobacilli. In contrast, treatment of CDAD by metronidazole is most safe for lactobacilli responsible for the maintenance of CR, similarly to vancomycin that suppress only the \( L. \) acidophilus- group of lactobacilli.

Moreover, the inconsistency of abundant clinical and experimental data indicates that there is no single microbial group that controls the establishment of \( C. \) difficile in the intestinal tract by a single mechanism. It is more likely that several diverse microbes may be involved by different mechanisms. As IMF varies from person to person, different microbes may have a leading role in CR against \( C. \) difficile in different individuals.
### TABLE I
Susceptibility of Human Lactobacillus sp. (LB) to Most Common Groups of Antimicrobials

<table>
<thead>
<tr>
<th>Antibiotic groups</th>
<th>Species of LB*</th>
<th>Susceptible</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Penicillins</td>
<td>LB</td>
<td>100%</td>
<td>Hamilton et al., 1994</td>
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<tr>
<td></td>
<td></td>
<td></td>
<td>Felten et al., 1999</td>
</tr>
<tr>
<td>Ampicillin</td>
<td>LB</td>
<td>96%</td>
<td>Mändar et al., 2001</td>
</tr>
<tr>
<td>Methicillin</td>
<td><em>L. paracasei</em></td>
<td>55%</td>
<td>Testore et al., 2002</td>
</tr>
<tr>
<td>Cephalosporins</td>
<td>LB</td>
<td>52–100%</td>
<td>Testore et al., 2002</td>
</tr>
<tr>
<td>1st generation</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2nd generation</td>
<td>LB</td>
<td>62%</td>
<td>Charteris et al., 1998</td>
</tr>
<tr>
<td>Cefoxitin</td>
<td><em>L. acidophilus-group</em></td>
<td>19–53%</td>
<td>Mändar et al., 2001</td>
</tr>
<tr>
<td></td>
<td><em>L. brevis</em></td>
<td>14%</td>
<td>Mändar et al., 2001</td>
</tr>
<tr>
<td></td>
<td><em>L. buchneri</em></td>
<td>50%</td>
<td>Mändar et al., 2001</td>
</tr>
<tr>
<td>Cefuroxime</td>
<td>LB</td>
<td>43–100%</td>
<td>Testore et al., 2002</td>
</tr>
<tr>
<td></td>
<td><em>L. brevis</em></td>
<td>43%</td>
<td>Mändar et al., 2001</td>
</tr>
<tr>
<td>3rd generation</td>
<td>LB</td>
<td>50–76%</td>
<td>Testore et al., 2002</td>
</tr>
<tr>
<td>Vancomycin</td>
<td>LB</td>
<td>26%</td>
<td>Zarazaga et al., 1999</td>
</tr>
<tr>
<td></td>
<td><em>L. acidophilus group</em></td>
<td>93%</td>
<td>Mändar et al., 2001</td>
</tr>
<tr>
<td>Macrolides</td>
<td>LB</td>
<td>100%</td>
<td>Muli and Struthers, 1998</td>
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<tr>
<td>Erythromycin</td>
<td></td>
<td></td>
<td>Felten et al., 1999</td>
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<td></td>
<td>62%</td>
<td>Mändar et al., 2001</td>
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<td></td>
<td></td>
<td></td>
<td>Testore et al., 2002</td>
</tr>
<tr>
<td>Tetracycline</td>
<td>LB</td>
<td>90–100%</td>
<td>Charteris et al., 1998</td>
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<td></td>
<td></td>
<td>Mändar et al., 2001</td>
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<tr>
<td></td>
<td></td>
<td></td>
<td>Testore et al., 2002</td>
</tr>
<tr>
<td>Aminoglycosides</td>
<td>LB</td>
<td>36–79%</td>
<td>Testore et al., 2002</td>
</tr>
<tr>
<td>Gentamycin</td>
<td>microaerobic milieu</td>
<td>93–100%</td>
<td>Mändar et al., 2001</td>
</tr>
<tr>
<td></td>
<td>anaerobic milieu</td>
<td>0%</td>
<td>Charteris et al., 1998</td>
</tr>
<tr>
<td>Fluorokinolones</td>
<td>LB</td>
<td>40–100%</td>
<td>Hamilton et al., 1994</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Zarazaga et al., 1999</td>
</tr>
<tr>
<td>Ciprofloxacin</td>
<td><em>L. acidophilus</em>, <em>L. paracasei</em>, <em>L. buchneri</em></td>
<td>13%, 94%, 50%</td>
<td>Mändar et al., 2001</td>
</tr>
</tbody>
</table>

(continued)
C. OTHER CAUSES OF AAD

Although *C. difficile* is the most important pathogen that overgrows after antibiotic-induced alteration of intestinal IMF, some other microorganisms are also proposed to cause AAD by this mechanism. These pathogens include *Clostridium perfringens*, *Staphylococcus aureus*, *Klebsiella oxytoca*, and *Candida* spp. However, their role, pathogenetic mechanisms involved, and incidence of AAD are not yet fully understood (Borriello, 1992; Högenauer et al., 1998). The overgrowth of a particular species or genus could be an indicator of an imbalance of microflora rather than the real cause of diarrhea.

In most cases of AAD a known specific enteric pathogen cannot be isolated. Since metabolic activity of gut IMF is important for the normal functioning of the intestine, the alteration of intestinal microflora can change the motility of the colon, as well as absorption and secretion. The main mechanisms proposed, carbohydrate malabsorption and decreased metabolism of bile acids, have been described in a recent review by Högenauer et al. (1998). These disorders have been mainly associated with a decrease of obligate anaerobes. Some antibiotics such as erythromycin and amoxicillin/clavulanate have been described to affect intestinal motility directly. Since antibiotics always alter intestinal microflora and thus metabolic functions of IMF, it is not clear which mechanisms are predominant in clinical cases of AAD.

IV. *In Vitro* Studies and Animal Experiments

A. INHIBITION OF *IN VITRO* GROWTH OF *C. DIFFICILE* BY LACTOBACILLI

Several bacterial species isolated from feces have been found to be antagonistic against *C. difficile* on agar plates. These microorganisms include anaerobes (*Clostridium bifermentas*, *Clostridium beijerinckii,*
Peptostreptococcus productus, Bifidobacterium adolescentis, Bifidobacterium infantis, Bifidobacterium longum) and lactobacilli (L. acidophilus, L. salivarius) as well as facultative aerobes such as Enterococcus spp., Streptococcus spp. (Barclay and Borriello, 1982; Bogovič-Matijašić et al., 1998; Forestier et al., 2001; Lee et al., 2003; Malamou-Ladas and Tabaqchali, 1982; Rolfe et al., 1981; Tvede and Rask-Madsen, 1989). However, in most of these experiments a few IMF strains have been tested against only one indicator C. difficile strain.

Reports about in vitro activity of lactobacilli against C. difficile are controversial. Strus et al. (2001) found all tested lactobacilli to be equally antagonistic to C. difficile as well as against the other enteric pathogens. Therefore they postulated a similar mechanism of inhibition of lactobacilli against all anaerobic bacteria. Lee et al. (2003) found that only 12 strains of 109 lactic acid bacteria tested were antagonistic against one C. difficile test strain. Only three of these C. difficile suppressing strains were lactobacilli (L. salivarius).

To solve this discrepancy, we screened the antagonistic activity of 51 intestinal Lactobacillus strains against 23 clinical C. difficile isolates and found that five strains (L. paracasei and L. plantarum) were antagonistic against all and 18 strains were antagonistic against 9 C. difficile isolates. Twenty-seven Lactobacillus strains had no antagonistic activity against any tested C. difficile isolate (Naaber et al., 1998b, 2002). Thus, since the antagonistic activity of lactobacilli as well as the sensitivity of C. difficile to this antagonism is strain specific, the results of in vitro studies seemingly depend on the selection of indicator strains.

There is little known about the mechanisms involved in this antagonistic activity. Bogovič-Matijašić et al. (1998) isolated and characterized two bacteriocins of L. acidophilus that showed activity against C. difficile strains as well as several other obligatory and facultative anaerobic bacteria. Forestier et al. (2001) studied the activity of L. casei subsp. rhamnosus culture supernatant against C. difficile and found that the inhibitory substance was resistant to treatment with protease and heat and had a molecular mass below 3 kDa. They proposed that this could be a bacteriocin-like substance or an organic acid. Several studies support the opinion that acidification of the colonic content with short chain fatty acids produced by lactobacilli and other members of IMF can suppress C. difficile growth (Borriello and Barclay, 1986; Ito et al., 1997; May, 1994; Rolfe, 1984; Yamamoto-Osaki et al., 1994). We have also found a close correlation between antagonistic activity of different lactobacilli against C. difficile and their H₂O₂ and lactic acid production (Naaber et al., 2002). However, these relationships were not absolute: some highly antagonistic strains were
both H₂O₂ negative and low lactic acid producers. According to these studies several antagonistic compounds of lactobacilli could be involved in the inhibition of *C. difficile*. Whether some of these are important in the maintenance of CR *in vivo* is not completely evident.

**B. In Vitro Studies of Other Mechanisms Involved in CR**

A study carried out by Borriello and Barclay (1986) with a batch culture model showed that the inhibition of *C. difficile* depends on the presence of complete viable intestinal IMF rather than inhibitory substances in culture filtrates. This indicates the importance of some other mechanism in the maintenance of CR against *C. difficile* besides the effect of antimicrobial substances. Studies with continuous flow cultures suggest that competition for nutrients, especially amino acids, may be an important mechanism in CR (Wilson and Perini, 1988; Yamamoto-Osaki *et al.*, 1994). However, continuous flow cultures are extremely dependent on culturing and incubation parameters, and their applicability to the gut environment is unclear (McFarland, 2000).

Since adhesion of a pathogen is essential for colonization and expression of virulence, the competition for mucosal receptors and inhibition of adhesion of *C. difficile* by IMF could be one mechanism of CR. Some experiments have shown that lactobacilli can inhibit adhesion of several other enteric pathogens (Coconnier *et al.*, 1993; Forestier *et al.*, 2001; Mack *et al.*, 1999). Specific carbohydrates decorating the cell wall of different species of lactobacilli, revealed by lectin typing (Annuk *et al.*, 2001), suggests the possibility of blocking the adhesive sites of pathogens by co-adhesion with lactobacilli. Unfortunately, there are no data on how lactobacilli or other members of IMF influence *C. difficile* adhesion. Since lactobacilli and other probiotic bacteria are frequently administrated in dairy products and sometimes combined with prebiotics, we have investigated the possible effects of these additives to *C. difficile* adhesion. We found that xylitol (as a likely prebiotic), bovine colostrums and milk whey can inhibit *C. difficile* adhesion to Caco-2 cells (Naaber *et al.*, 1996), showing the potential for designing and applying appropriate functional foods for the prevention of *C. difficile* colonization.

**C. Animal Models**

Several attempts have been made to reconstitute resistance to *C. difficile* infection in animals by using fecal homogenates from healthy normal animals of the same or different species or from
humans. In most of these studies with different germ-free animals, the administered complete fecal flora provided resistance to *C. difficile* (Itoh *et al.*, 1987; Wilson *et al.*, 1981, 1986). In contrast to these studies, some attempts to use a particular anaerobe or combinations for reconstituting CR against *C. difficile* have been unsuccessful (Borriello, 1990; Wilson *et al.*, 1986). However, there has been described a trixenic mouse model colonized with *Clostridium indolis*, *Clostridium cocleatum* and a fusiform flagellate *Eubacterium* sp. capable of inhibiting the implantation of *C. difficile* (Boureau *et al.*, 1989). In a recent experiment with this model (Thomas *et al.*, 2002) the barrier mechanism of the protective flora was assessed, focusing on the interactions taking place in the cecal mucus layer and inside of crypts. Modern molecular methods (FISH) combined with scanning electron microscopy showed that the three barrier species with mucus degrading ability, and not *C. difficile*, were embedded in the mucus layer of caecum. The tissue association of the *C. difficile* strain was 10-fold lower than that of the flagellate, showing that adhesion, deep mucus colonization and crypt association are the mechanisms responsible for the barrier effect. The challenge of animal studies is the different microbial ecology of the gut from humans and the possibility to draw only indirect conclusions.

A few animal experiments have been performed to study the role of lactobacilli in the maintenance of stability of intestinal IMF after exposure to *C. difficile*. Itoh *et al.* (1987) found that *C. difficile* overgrowth was associated with a decrease of intestinal lactobacilli in an ampicillin compromised mouse model. However, a mixture of three strains of lactobacilli together with other intestinal bacteria did not eliminate *C. difficile* in gnotobiotic mice in their experiment. Moreover, Wong *et al.* (1996) showed that human strains of *Lactobacillus* sp. fed to mice usually did not survive well, and the metabolism of introduced strains was significantly lowered. We have used a cefoxitin compromised mouse model to study changes in intestinal microflora and CR against *C. difficile* (Naaber *et al.*, 1995). Although administration of cefoxitin did not change the total counts of intestinal lactobacilli, mice became more susceptible for colonization by *C. difficile*. This colonization was short-term and no real infection developed. However, in these experiments detection of just total counts of lactobacilli could miss changes in the species composition of lactoflora and was not able to track the highly antagonistic strains.

Since in most experiments probiotic bacteria alone fail to protect against *C. difficile* infection (Borriello, 1990), we have tried the combination of prebiotic together with probiotic. Administration of

Considering the results of *in vitro* experiments and animal models, we can conclude: (1) antimicrobial activity of lactobacilli and susceptibility of *C. difficile* to this antagonistic activity varies in different strains; (2) there is some association between the counts of intestinal lactobacilli and CR against *C. difficile*, but the exact role of lactobacilli and the mechanism of action are not clear; and (3) according to animal models, however, it seems that lactobacilli are not the only group of bacteria responsible for the protection against AAD and CDAD. The dramatically different intestinal flora of rodents and guinea pigs may also be the reason for some failures.

V. Use of Biotherapeutic Agents in Clinical Studies

Standard treatment of CDAD includes oral administration of vancomycin or metronidazole for 7 to 10 days (Pothoulakis and LaMont, 1993; Tabaqchali and Jumaa, 1995). Although definite improvement is usually noted just 3–4 days after such treatment, one or multiple serial relapses of CDAD can occur in 10 to 20% of patients (Fekety and Shah, 1993). For other AAD that are not caused by *C. difficile*, no specific antimicrobial treatment is recommended. Therefore, new strategies for treatment and prophylaxis of CDAD and other AAD that restore intestinal IMF and improve CR to *C. difficile* have been explored extensively. The most natural, and in animal experiments, successful approach is administration of the whole intestinal microflora of healthy persons. There are several reports about treatment of patients with relapsing CDAD by using rectal infusion of normal feces or a mixture of intestinal bacteria (Schwan *et al.*, 1984; Tvede and Rask-Madsen, 1989). Although some of these attempts were successful, there are serious ethical and practical problems with this kind of treatment. Therefore, biotherapeutic strains with known safety and properties are preferred.

A. PROBIOTICS, PREBIOTICS, AND SYNBIO蒂CS

Preparations that have beneficial effects on human health by modulation or reparation of IMF include probiotics, prebiotics and synbiotics. Oral probiotics can be defined as living microorganisms,
which upon ingestion in certain numbers exert health benefits beyond inherent basic nutrition by improving the intestinal microbial balance (Fuller, 1989; Salminen, 2001). A prebiotic is a non-digestible food ingredient that can improve the host’s health by selectively stimulating the growth and/or activity of bacteria in the colon (Gibson and Roberfroid, 1995) Synergistic combinations of prebiotics and probiotics are called *synbiotics*.

Probiotic products currently on the market may be presented in the form of powders, tablets or capsules, liquid suspensions, or sprays. The main branch of the probiotics industry entails preparations specifically designed for carriage of particular probiotic strains such as yogurts and different fermented foods, including cheeses. The European probiotic yogurt market is very fast growing, grossing nearly 800 million EURO in 1998 (Fooks et al., 1999).

More commonly used probiotics contain lactic acid bacteria (lactobacilli, streptococci) and bifidobacteria. The main function of probiotics is to restore the impaired colonization resistance, which can be achieved by following the important requirements including the ability to survive transport to the active site; adhesion to the gut mucosa in the active site; ability of reproduction; ability to exist in symbiosis with mucosal biofilm; ability to exist in the presence of factors that disrupt CR.

Exact mechanisms of the protective effect of probiotic lactobacilli have not yet been fully elucidated, but several putative mechanisms have been postulated. These include direct or indirect suppression of pathogenic microorganisms in the gut, support for reestablishment of IMF and increase the defense of the host: (1) several products of lactobacilli such as short-chain fatty acids, hydrogen peroxide and bacteriocins have substantial antagonistic activity against several pathogens at least in vitro; (2) lactobacilli can prevent colonization of pathogens by blocking of adhesion sites on mucosa; (3) lactobacilli can block toxin receptor sites; (4) lactobacilli can suppress the overgrowth of pathogens by competition for essential nutrients; (5) lactobacilli may affect nonhumoral immunity (e.g., increase macrophage activity); and (6) lactobacilli can attenuate the virulence of pathogens (Fooks et al., 1999).

In our laboratory, some additive putative mechanisms of action by lactobacilli for providing CR have been proposed. It was shown that different lactobacilli serve as antagonists to pathogens more effectively if tested either in microaerobic or anaerobic environments (Annuk et al., 2003). This could drive searching for probiotics against *C. difficile* specific strains, particularly *L. acidophilus* or *L. casei* group.
members predicted to be active in the cecum. In a human clinical trial we have shown that administration of probiotic lactobacilli, particularly *Lactobacillus rhamnosus* GG, increases simultaneously the population of the other IMF members (such as anaerobes) that may have an important role in the maintenance of CR (Sepp *et al.*, 1993). Recently we succeeded in showing that some probiotic strains, particularly *L. fermentum* ME-3 (DSM 14241) express substantial antioxidative activity (Kullisaar *et al.*, 2002), which can diminish the deleterious effect of excessive oxidative stress on epithelial cells during intestinal salmonellosis (Tamm *et al.*, 2002).

In animal models we have assessed the ability of lactobacilli for translocation (Mikelsaar and Türi, 1990; Naaber *et al.*, 2000) without causing infection. The same was shown by Berg (1995); in fact, the phenomenon recently has been suggested as a basis for action of lactobacilli with leukocytes subsequently entering circulation (Cross *et al.*, 2002). The components of the Gram-positive bacterial cell wall or intact bacterial cells can actively communicate with immune cells transducing the nuclear factor κB and STAT-mediated signals. The host responds to such stimuli by the release of pro- or anti-inflammatory cytokines, depending on the properties of the *Lactobacillus* strains (Maassen *et al.*, 2000; Miettinen *et al.*, 2000; Wallace *et al.*, 2003). Moreover, the beneficial effects of probiotics (LGG) on intestinal epithelial cells have been attributed to either preventing cytokine induced apoptosis (Fan Yan and Polk, 2002) or increased enterocyte production (Banasaz *et al.*, 2002). This may drive our attention to search for more specific immune-enhancing and mucosa-restoring probiotic strains against *C. difficile*.

**B. Lactobacilli in Prophylaxis and Treatment of CDAD and AAD**

One of the most widely used and investigated *Lactobacillus* strains with probiotic properties is the *Lactobacillus rhamnosus* strain GG (LGG). LGG is resistant to bile and low pH; it can adhere to intestinal mucosa and produce antimicrobial substances (Goldin and Gorbach, 1996; Saxelin, 1995; Silva *et al.*, 1987). This strain has been used successfully for the prevention of relapses of *C. difficile* colitis (Table II) in some earlier studies (Biller *et al.*, 1995; Gorbach *et al.*, 1987). However, the patient number in these studies was small (5 and 4). A double-blind placebo controlled trial was performed in Sweden with *Lactobacillus plantarum* 299v to prevent further recurrent episodes of CDAD (Wullt *et al.*, 2003). The recurrence of clinical symptoms (main outcome) was seen in only 4 patients of 11 who
<table>
<thead>
<tr>
<th>Probiotic strain</th>
<th>Indication/Antibiotic</th>
<th>Number of patients</th>
<th>Therapeutic effect</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>LGG</td>
<td>Prevention of relapses of CDAD</td>
<td>5</td>
<td>Decreased frequency of relapses</td>
<td>Gorbach et al., 1987</td>
</tr>
<tr>
<td>LGG</td>
<td>Prevention of relapses of CDAD</td>
<td>4</td>
<td>Decreased frequency of relapses</td>
<td>Biller et al., 1995</td>
</tr>
<tr>
<td>L. plantarum</td>
<td>Prevention of recurrent CDAD</td>
<td>20</td>
<td>Decreased frequency of relapses</td>
<td>Wullt et al., 2003</td>
</tr>
<tr>
<td>LGG</td>
<td>Treatment of AAD/Erythromycin</td>
<td>16</td>
<td>Shortened duration of AAD</td>
<td>Siitonen et al., 1990</td>
</tr>
<tr>
<td>LGG</td>
<td>Prevention of AAD/Various</td>
<td>188</td>
<td>Decreased frequency of AAD (17% vs. 48%)</td>
<td>Vanderhoof et al., 1999</td>
</tr>
<tr>
<td>LGG</td>
<td>Prevention of AAD/Various</td>
<td>267</td>
<td>No effect</td>
<td>Thomas et al., 2001</td>
</tr>
<tr>
<td>L. acidophilus +</td>
<td>Prevention of AAD/ampicillin</td>
<td>98</td>
<td>Decreased frequency of AAD (8.3% vs. 21%)</td>
<td>Gotz et al., 1979</td>
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<tr>
<td>L. bulgaricus</td>
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<td>L. acidophilus +</td>
<td>Prevention of AAD/neomycin</td>
<td>39</td>
<td>Decreased frequency of AAD (20% vs. 42%)</td>
<td>Clemens et al., 1983</td>
</tr>
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<tr>
<td>L. acidophilus +</td>
<td>Prevention of AAD/amoxicillin-clavulanate</td>
<td>27</td>
<td>Decreased frequency of AAD</td>
<td>Witsell et al., 1995</td>
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<tr>
<td>L. bulgaricus</td>
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<tr>
<td>L. acidophilus +</td>
<td>Prevention of AAD/amoxicillin</td>
<td>38</td>
<td>No effect</td>
<td>Tankanow et al., 1990</td>
</tr>
</tbody>
</table>
received metronidazole in combination with *L. plantarum* 299v and in 6 out of 9 treated only with metronidazole in combination with a placebo. There were a relatively small number of patients as a high rate of underlying diseases obstructed inclusion and reduced compliance, yet the results encourage the performance of larger multi-centre studies for the benefits of probiotics in patients with CDAD.

Concerning AAD, administration of LGG containing yogurt shortened the duration of erythromycin induced diarrhea (2 vs. 8 days) in 16 patients compared to placebo (Siitonen *et al.*, 1990). A similar beneficial result after erythromycin-induced GI effects were obtained with *Bifidobacterium longum* (Colombel *et al.*, 1987). In another study administration of LGG resulted in a significant decrease of antibiotic associated diarrhea in 188 children (Vanderhoof *et al.*, 1999). However, a more recent randomized placebo-controlled trial did not detect any changes in the rate of antibiotic associated diarrhea in LGG treated patients (Thomas *et al.*, 2001).

In several studies a commercial probiotic containing *L. acidophilus* and *L. bulgaricus* has been used for the prevention of AAD. In most of these, administration of probiotic caused some reduction of AAD (Clemens *et al.*, 1983; Gotz *et al.*, 1979; Witsell *et al.*, 1995). However, one double-blind placebo-controlled study did not show any benefit of *L. acidophilus + L. bulgaricus* for the prevention of amoxicillin induced diarrhea (Tankanow *et al.*, 1990).

An interesting biotherapeutic agent is a non-pathogenic yeast, *Saccharomyces boulardii*, that was isolated in Indochina and which grows at the unusually high temperature of 37°C (reviewed by Marchand and Vanderplas, 2000). This agent is intrinsically resistant to antibiotics except for nystatin. The mode of action seemingly relies on antisecretory mechanisms by two proteins of *S. boulardii*. One protein, 120 kD, reduces the formation of cyclic AMP in the intestinal cells driving the enterocytes for secretory diarrhea. The second protein, 54 kD, is a protease that acts on toxin A of *C. difficile*. Several successful clinical trials against *C. difficile* relapsing colitis have been described: the mortality was decreased and the effects of toxin A and B on mucosa were inhibited (Capano *et al.*, 1998; Castagliuolo *et al.*, 1999; Castex *et al.*, 1990).

The reasons for conflicting data may be due to different samples of patients (adults vs. children), differing gastrointestinal motility (adults vs. elderly with constipation), different individual gastrointestinal microflora with different susceptibility to antibiotics, and last but not least, different types/species of probiotics used (Table III). As shown above, not all clinical strains of *C. difficile* were equally susceptible
against the tested wide set of lactobacilli. Use of certain antibiotics (vancomycin) for treatment *C. difficile* infection may also suppress the probiotic strain if it belongs to the *L. acidophilus*-group. Moreover, it can be considered from clinical trials that the efficacy of one probiotic may not be the same in all patients, explainable with the above-mentioned geographical vs. ethnic vs. individual differences of GI microflora, or caused by the suitable vs. non-suitable gut environment for expression of probiotic properties (anaerobic; presence of non-absorbed prebiotic substances in colon).

### VI. Conclusions and Further Perspectives

The complexity of intestinal microecosystems makes it extremely difficult to evaluate the role of some particular microorganisms in maintenance of stability of IMF and CR against pathogens. This complexity also puts limits to in vitro studies of CR and its mechanisms.

However, studies of AAD and particularly CDAD have shown that damaging of intestinal lactobacilli may play an important role in the pathogenesis of these diseases, and some lactobacilli can be successfully used for protection against CDAD and other AAD.

Until now, elaboration of effective probiotics is mainly based on empirical success or failure. Investigation of probable protective properties of lactobacilli, such as antagonistic activity against pathogens
and antioxidative capacity, can lead to the development of new combinations of individually selected pro- and prebiotics for different patients with CDAD.

References


