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Overview of gut flora and probiotics

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Abstract

Scientific developments in recent years have opened new frontiers and enable a better understanding of the gastro-intestinal tract (GIT) as a complex and delicately balanced ecosystem. This paper focuses on more recent information related to the microbial population of the GIT and its functional role in human physiology and health. Special attention is also given to modern approaches for improving or stabilising the intestinal system and its functioning by the deliberate application of viable microbial cultures, so-called 'probiotics', selected for special functional properties. © 1998 Elsevier Science B.V.

Keywords: Bifidobacteria; Functional properties; Gastro-intestinal tract; Lactobacilli; Probiotics

1. Introduction

There is general agreement on the important role of the gastro-intestinal (GI) microflora in the health status of men and animals. The importance of lactobacilli for human health and longevity was first hypothesized by Metchnikoff at the beginning of this century. He, however, considered the gut microbes in total as detrimental rather than beneficial, and suggested that desirable effects might only be expected from their substitution by yogurt bacteria. Since then, attempts have been made, especially during the last two to three decades, to improve the health status by modulating the indigenous intestinal flora by live microbial adjuncts, now called 'probiotics'. Although a number of definitions have been proposed to describe probiotics, an appropriate one was suggested by Havenaar et al. (1992), according to which probiotics are defined as ''mono- or mixed cultures of live microorganisms which, when applied to animal or man, beneficially affect the host by improving the properties of the indigenous microflora''. This definition has certain advantages compared to others, e.g.:

- it does not restrict 'probiotic' activities to the

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intestinal microflora, but also to microbial communities at other sites of the body;
- the 'probiotic' might consist of more than one bacterial species; and
- it can be applied to both man and animals.

Several studies have focused on the pharmacokinetics of different probiotics in humans, and our knowledge, especially of the complex mechanisms behind these effects, is constantly increasing. Today we know that certain lactic acid bacteria (LAB) can induce specific immune regulators as a result of interaction with mononuclear phagocytes and endothelial cells of the host (Brassart and Schiffrin, 1997). Furthermore, it has been observed that particular strains of LAB showed adjuvant properties by stimulation of a specific antibody response after infection with (attenuated) pathogenic microorganisms (Pouwels et al., 1996). Another intriguing development is the observation that certain LAB can strengthen the gut mucosal barrier and thereby influence gut mucosal permeability and possible diarrhoea. This paper will evaluate the present status of probiotics and discuss recent developments in this area.

2. Development, composition and function of the microbial population in the intestinal system

2.1. The ecosystem

The bacterial flora of humans is the most intimate portion of their biological environment and mediates many interactions with the chemical environment. In particular, the gastro-intestinal microflora represents an ecosystem of the highest complexity and our understanding of this system and its interactions is still limited (Berg, 1996). This complex microbial population may be considered as ''an open ecosystem comprising a group of microbial populations coexisting in an equilibrium in a spatiotemporally defined region'' (Ducluzeau, 1989).

Compared to the ca. 2 m^2 skin surface of our body the GI tract (GIT) represents a much larger contact area with the environment (Van Dijk, 1997). As shown in Fig. 1, the mucosal surface of the small intestine is increased in several ways, e.g.: three-fold by forming circular folds, 7–10-fold by folding of the epithelium (intestinal villi) and 15–40-fold by the formation of microvilli in the enterocyte resorptive luminal membrane. The resulting surface of the

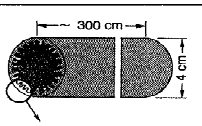
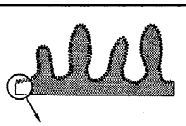
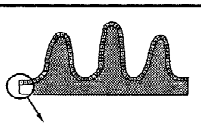
Structure	Schematic illustration	Increase of surface (Cylinder = 1)	Surface [m^2]
Intestinal wall (as cylinder)		1	0.33
Circular folds (Kerckring)		3	1
Intestinal villi		30	10
Microvilli		600	200

Fig. 1. Increase in the mucosal surface by folding (modified after Waldeck, 1990).

GI system is calculated to be 150–200 m^2 (Waldeck, 1990), thereby providing the necessary space for interactions during the digestive process and for adhesion to the mucosal wall and concomitant colonisation. The GIT of an adult human is estimated to harbour about 10^{14} viable bacteria (Luckey and Floch, 1972), i.e. 10 times the total number of eukaryotic cells in all tissues of man's body. The importance of these bacteria in the GIT was neglected for a long time, while the focus was merely placed on enteric pathogens and other factors leading to gastro-intestinal 'disorders'.

The intact intestinal epithelium with an optimal intestinal flora represents a barrier to the invasion or uptake of pathogenic microorganisms, antigens and harmful compounds from the gut lumen. In addition to the barrier function, the intestinal mucosa is efficient in assimilating antigens. Specialised antigen transport mechanisms present in the villus epithelium and Peyer's patches are essential for evoking specific immune responses (Heyman et al., 1982). In healthy individuals this barrier is stable, ensuring host protection and providing normal intestinal function and immunological resistance. Protection against infective agents is complemented intrinsically by the barriers of the gut-associated lymphoid tissues (GALT), considered to be the largest 'immune organ' in the human body. Per meter of small bowel, approximately 80% (10^{10}) of all immunoglobulin producing cells are found here (Shanahan, 1994). The gut flora itself is essential for mucosal immune stimulation (activation) and amplification of immunocompetent cells.

A number of terms are used in the literature to describe either the 'state of balance' within the microbial population or the status of particular microbial groups within the GIT. The balance, considered to be maintained by sensitive interactions between living and abiotic compounds in this 'enclave of external environment' (Ducluzeau, 1989), can be called 'eubiosis'. The opposite situation is termed 'dysbiosis' (Haenel and Bendig, 1975; Gedek, 1993). Although not precisely defined, this unstable state refers to qualitative and quantitative changes in the intestinal flora, their metabolic activity and their local distribution. Metabolic activity and certain turnover rates may be more important than actual numbers of particular bacterial species. The situations 'eubiosis' and 'dysbiosis' may not be explained only by numbers of leading species, and their definition therefore has limitations. Furthermore, studies on sampled material do not necessarily reflect intimate microbial interactions and in vivo growth kinetics.

2.2. Microbial numbers and the ecosystem

Both the variety and the overall sum of microbial numbers are determined by an array of complex factors, intrinsic to the respective GI sections, and 'extrinsic' related to, for example, diet, stress, drugs, etc. After the more or less neutral pH of the oral cavity, the low pH of the stomach (ranging from 2.5 to 3.5) is destructive to most microbes. The population averages 10^3 bacteria g^{-1}, and is dominated by Gram-positive bacteria such as streptococci and lactobacilli, and by yeasts (see Fig. 2). Due to the aggressive intestinal fluids (e.g., bile, pancreatic juices) and the short transit time, the duodenum also represents a hostile environment and contains relatively low numbers of merely transient microbes. In the early 1960s, Reuter and coworkers revealed new and carefully elaborated data on the microbial population of the different sections of the human GIT (Lerche and Reuter, 1962; Reuter, 1965a,b, 1969). Further comprehensive data of such an extent have not been added to our knowledge yet. Research in the following two decades concentrated on the easily available fecal flora and, so far, no comprehensive evaluation has been made using up-to-date mi-

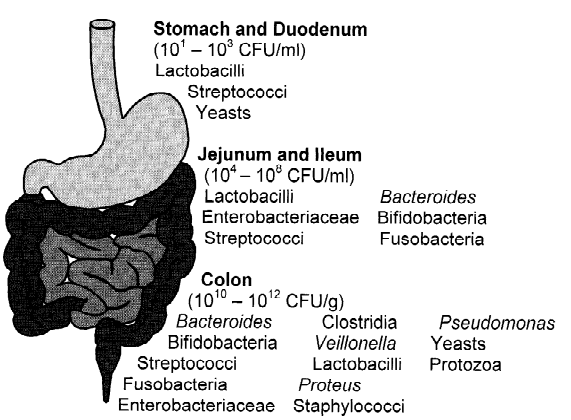


Fig. 2. Microbial colonisation of the human gastro-intestinal tract (modified after Simon and Gorbach, 1982).

crobiological means of sampling and identification. Still, the relatively sparse data available on the human jejunum and ileum suggest a continuous increase in the numbers (up to 10^8 g^{-1}) and variety of the flora towards the distal regions (Lerche and Reuter, 1962; Reuter, 1965b; Bhat et al., 1989; Bernhardt and Knoke, 1980; Nielsen et al., 1994). In addition to the LAB groups, with increasing numbers of bifidobacteria towards the more distal regions, Gram-negative facultative aerobic groups such as Enterobacteriaceae, and the obligatory anaerobic genera such as *Bacteroides* and *Fusobacterium*, also appear. Strict anaerobes are present in increasing numbers even above the ileo-caecal valve. Beyond the valve, however, the strict anaerobes outnumber the facultative anaerobes in the lumen by 100 to 1000 times, and bacterial numbers typically exceed 10^{11} g^{-1}.

It is estimated that the colon of healthy adults harbours about 300–400 different cultivable species belonging to more than 190 genera. A significant additional proportion of the colon microflora is, however, not cultivable by existing techniques (see Section 5.2). Among the known colonic microbial flora only a few major groups (‘main flora’, according to Gedek, 1993) dominate at levels around 10^{10}–10^{11} g^{-1}, all of which are strict anaerobes such as *Bacteroides*, *Eubacterium*, *Bifidobacterium* and *Peptostreptococcus* (Fig. 2). Facultative aerobes are considered to belong to the sub-dominant (intermediate) flora or ‘satellite flora’ (Gedek, 1993), constituting Enterobacteriaceae, streptococci and lactobacilli.

Compared to the colonic flora, the fecal flora undergoes distinct quantitative variations and seems to be a good qualitative indicator of the distal colonic microflora. It does not reflect, however, the intestinal flora and most definitely not those of the small intestine. Furthermore, our current knowledge on the stability of the strains, species and even genera relationships is still extremely limited. While stability in species composition may be a feature of the ‘normal’ microflora, stability of bacterial strains within the population may be less common (McCartney et al., 1996). Genetic fingerprinting techniques indicated the presence of a collection of *Bifidobacterium* and *Lactobacillus* strains ‘‘unique of each human’’ (Tannock, 1997). It was also suggested that the composition of these populations may remain relatively constant for some individuals and may fluctuate considerably for others.

Minor groups of pathogenic and opportunistic organisms, the so-called ‘residual flora’ according to Gedek (1993), are always present in low numbers. In a healthy state, the quantity even of toxic metabolites seems insufficient to act detrimentally on the host. Ducluzeau (1989) proposed the action on the host to be considerable only when the number of particular bacteria exceeds 5×10^7 g^{-1}. This may be the case for the colon, but lower numbers may be required to dominate in other regions and to promote significant pharmacokinetic interactions, e.g. in the jejunum. In these regions the concentration of metabolites is host mediated by active resorption pathways.

2.3. Colonisation, succession and influencing factors

During delivery, the new-born becomes contaminated with microorganisms from the birth canal of the mother and the environment. At first *Escherichia coli* and *Streptococcus* predominate, but in breast fed infants there is a sharp increase in the numbers of *Bifidobacterium* with a concomitant decrease of *E. coli* and *Streptococcus*, whilst *Clostridium* is low or absent. In formula fed babies this shift in composition is not observed, and their GI flora becomes rather complex with relatively high numbers of *Bacteroides*, *Clostridium* and *Streptococcus*. *Bifidobacterium* is present but not predominating. This is a strong indication that the diet can influence the ratio between the microbial species and strains of the intestinal flora. When breast fed infants are provided with food supplements, clostridia, streptococci and *E. coli* attain higher levels and the flora becomes similar to formula fed infants. Now also *Bacteroides* and Gram-positive cocci appear in progressively higher numbers. After weaning, a conversion to the normal adult flora occurs. Generally, streptococci and *E. coli* populations decrease and by the second year of life the intestinal microflora is similar to that of the adult (Mevissen-Verhage et al., 1985; Bullen et al., 1977).

The intestinal physiology and host defense mechanisms play an important role in preventing overgrowth of the microflora and in determining the final

composition and distribution of the flora throughout the gastro-intestinal tract. The major factors influencing the composition of the microflora are summarised in Table 1. These factors may be related to changes in physiological conditions of the host (aging, stress, health status, ethnical environment), composition of the diet and environmental circumstances (e.g., contamination with pathogens, use of medicines). In this way the conditions underlying digestion (e.g., pH, substrate availability, redox potential, transit time, flow of enteric fluid, IgA secretion, etc.) may be modulated. This could result in a decline of the beneficial bacteria and in an increase in potentially harmful bacteria. Changes in diet or climate, aging, medication, illness, stress or infection generally lead to an increase in anaerobes and *E. coli* in the small intestine and to an increase of *Enterobacteriaceae* and streptococci in the colon concomitantly with a decrease of bifidobacteria (Mitsuoka, 1990, 1992). Implicit interactions of typical intestinal bacteria may also contribute to stabilisation or destabilisation, e.g. by the production of H_2O_2, acids and bacteriocins.

Table 1
Factors affecting the microflora of the gastro-intestinal tract

1. Host mediated factors
pH, secretions such as immunoglobulins, bile, salts, enzymes
Motility, e.g. speed, peristalsis
Physiology, e.g. compartmentalisation
Exfoliated cells, mucins, tissue exudate
2. Microbial factors
Adhesion
Motility
Nutritional flexibility
Spores, capsules, enzymes, antimicrobial components
Generation time
3. Microbial interactions
Synergy
Metabolic cooperation
Growth factors and vitamin excretion
Changes to E_h, pH, O_2 tension
Antagonism/stimulation
Short-chain fatty acids, amines
Changes to E_h, pH, O_2 tension
Antimicrobial components, siderophores
Nutritional requirements, etc.
4. Diet
Composition, non-digestible fibres, drugs, etc.

2.4. Functions of the intestinal flora

By a number of physiological functions the intestinal flora contribute to overall health. Disturbance of the ecological balance in the gastro-intestinal system may therefore be detrimental to health. Bacteria typical of the 'normal' intestinal flora may possess a range of beneficial features, and are (e.g.) able to degrade certain food components, produce certain B vitamins, stimulate the immune system and produce digestive and protective enzymes. The normal flora is also involved in the metabolism of some potentially carcinogenic substances and may play a role in drug efficacy. These effects can be either beneficial or detrimental to health. Furthermore, the colon mucosa is dependent on short chain fatty acids (SCFA) produced by the colonic microflora. Products of polysaccharide metabolism SCFA are passively absorbed by the enterocytes (Hoverstad, 1989). Roediger (1982) estimated that 40–50% of the required energy has to be provided by the colonic microflora, suggesting one form of established mutualism in a region where SCFA are also partially responsible for bowel motility and circulation (Kvietys and Granger, 1981).

The role of the gut flora as a barrier against pathogenic and opportunistic microorganisms is surprisingly effective, considering the large amounts of allochthonous ('non-resident') bacteria entering the GIT. Most of them have no chance to establish within the system. One approach to understanding aspects of microbial interactions related to in situ barrier effects was to study restoration effects in simplified two-strain models, using a continuous flow system (Du Toit et al., 1998a). It could be shown that the probiotic *Enterocccus faecium* SF68 promoted restoration of sublethally damaged *Lactobacillus reuteri*, a representative of the autochthonous gut flora. More complex in vitro models and in vivo experiments need to be conducted for further elucidating the complex microbial interactions and host dependent factors involved in restoration. These aspects will be addressed briefly in Section 5 (Future developments).

Increased interest exists in possibilities of manipulating the composition of the gut microflora by foods or food ingredients. The aim is to increase the numbers and activities of those microorganisms

suggested to possess health promoting properties such as *Bifidobacterium* and *Lactobacillus* species. The question now arises what scientific evidence is available to support the postulated mechanisms behind these beneficial effects of probiotics.

3. Present status of probiotics

Different product types or supplements containing viable microorganisms with probiotic properties are commercially available either in lyophilised form or as fermented food commodities. Strains of *L. acidophilus* and *L. casei* strain Shirota probably have the longest history among known bacterial strains for application on account of their health benefits. In present-day commercial probiotic products, *Lactobacillus* spp. are well represented, followed by *Bifidobacterium* spp., some other LAB genera and even a few non-lactics (see Table 2). These non-lactics, however, are rarely used in dairy or other food commodities, but find application rather as lyophilised or encapsulated 'pharmaceutical' preparations.

With the emphasis mainly on 'novel-type' fermented dairy products, a steadily increasing range of yogurt-like products is available on the European market (see Table 3). *Streptococcus thermophilus*, although generally associated with these 'mild' yogurt types, is applied for technical reasons and sustains the low acid fermentation typical of these novel yogurt types. (In some cases, only one strain, e.g. *L. casei* strain Shirota, is responsible for the fermentation.) Probiotic strains for these products are generally derived from the GIT of the adult human host. This is exemplified, for example, by the high frequency in which strains of autochthonous *Lactobacillus* spp., associated with the human gut, are applied in these products, *Lactobacillus salivarius* being the only exception (see Table 4). Especially *L. casei* and the so-called 'acidophilus' group appear to have special value as probiotic agents. Formerly considered as one species, *L. acidophilus* was shown to be heterogeneous, first by Lerche and Reuter (1962), and later, on the basis of DNA homology groups, by Lauer et al. (1980) and Johnson et al. (1980). This information is summarised in Table 5, in which the present species status of these homology groups, their typical hosts, and some phenotypic features are given.

4. How do probiotics work?

Although probiotic microorganisms are considered to promote health, the actual mechanisms involved have not yet been fully elucidated. In addition to desirable technical features, factors related to health promotion or health sustaining, serve as important criteria for strain selection. Three categories of key criteria have been defined as desirable for probiotic bacteria (Havenaar et al., 1992) and are briefly discussed below.

Table 2
Microorganisms applied in probiotic products

Lactobacillus species	*Bifidobacterium* species	Other LAB	Non-lactics
L. acidophilus	*B. adolescentis*	*Ent. faecalis*[a]	*Bacillus cereus* ('toyoi')[a,d]
L. casei	*B. animalis*	*Ent. faecium*	*Escherichia coli* ('Nissle 1917')[d]
L. crispatus	*B. bifidum*	*Lactoc. lactis*[c]	
L. gallinarum[a]	*B. breve*	*Leuc. mesenteroides*[c]	*Propionibacterium freudenreichii*[a,d]
L. gasseri	*B. infantis*	*Ped. acidilactici*[c]	
L. johnsonii	*B. lactis*[b]	*Sporolactobacillus inulinus*[a]	*Saccharomyces cerevisiae* ('boulardii')[d]
(*L. paracasei*)	*B. longum*	*Strep. thermophilus*	
L. plantarum			
L. reuteri			
L. rhamnosus			

[a]Mainly used for animals.
[b]Probably synonymous with *B. animalis*.
[c]Little known about probiotic properties.
[d]Mainly as pharmaceutical preparations.

Table 3
'Probiotic' lactobacilli detected in novel-type yoghurts (data combined from own results and from Reuter, 1997a)

Manufacturer	Origin	*Lactobacillus* viable counts (log CFU g^{-1})	Lactobacilli indicated by the manufacturer	Lactobacilli identified by DNA–DNA hybridization
A	Germany	7.9–8.9	*L. casei* Shirota	*L. paracasei (casei)*[b]
B	Germany	6.4–8.1	*L. acidophilus* LA-1	*L. johnsonii*
C	Germany	8.0–8.4	*Lactobacillus casei* GG	*L. rhamnosus*
		6.3–7.8		*L. acidophilus*
D	Germany	5.4–6.4	BactoLab cultures	*L. acidophilus*
E	Germany	7.4–8.1	*L. casei* Actimell	*L. paracasei (casei)*
F	Germany	6.8–8.2	*L. acidophilus*	*L. acidophilus*
		6.2–7.8	*L. casei*	*L. paracasei (casei)*
G	Germany	3.9–5.7	*L. acidophilus* LA-7	*L. acidophilus*
H	Germany	4.7–6.2	*L. acidophilus* LA7	*L. acidophilus*
I	Germany	5.2–5.9	Not indicated	*L. acidophilus*
J	Germany	5.5–6.8	*L. acidophilus*	ND
K	Germany	7.1–7.8	BIOGARDE cultures	*L. johnsonii*
L	Germany	8.6–8.7	*L. casei*	*L. paracasei (casei)*
M	Germany	8.1–8.4	*L. acidophilus* LA-H3	ND
		4.7–5.3	*L. casei* LC-H2	
C	Netherlands	6.8[a]	*L. acidophilus* Gilliland	*L. crispatus*
		6.4[a]	*L. casei*	*L. paracasei (casei)*[a]
N	Sweden	9.2	*L. acidophilus*	*L. acidophilus*
O	Germany	7.3		*L. acidophilus*[a]
P	Switzerland	7.6[a]	*L. acidophilus*	*L. acidophilus*[a]
		9.0[a]	*L. casei*	*L. paracasei (casei)*[a]
		5.2[a]	*L. reuteri*	*L. reuteri*[a]
Q	Switzerland	8.3[a]	*L. casei*	*L. rhamnosus*[a]
R	France	8.3[a]	*L. casei*	*L. paracasei (casei)*[a]

[a]According to Reuter (1997a).
[b]*L. casei* suggested for *L. paracasei* by Dicks et al. (1997).

Table 4
Autochthonous lactobacilli associated with the human host (according to Reuter, 1997b)

I. Homofermentative[a]
'*L. acidophilus*'
L. acidophilus sensu stricto
L. gasseri
L. crispatus
L. johnsonii
'*L. salivarius*'
L. salivarius
subsp. *salivarius*
subsp. *salicinius*
'*L. casei*'
L. casei
subsp. *casei*
subsp. *tolerans*
L. rhamnosus

II. Heterofermentative[b]
L. reuteri (formerly *L. fermentum II*) including *L. oris* and *L. vaginalis*

[a]Predominantly lactic acid and no production of gas from glucose.
[b]Production of both lactic acid and other organic acids (acetic and formic acid) and of CO_2 from glucose.

Table 5
Typical features of the species of the 'acidophilus-group' (modified after Mitsuoka, 1992; Reuter, personal communication)

Species	Habitat[a]	mol% G + C in the DNA	'Biotypes' according to:	DNA homology groups according to:	
			Lerche and Reuter (1962)	Lauer et al. (1980)	Johnson et al. (1980)
L. acidophilus	All?	32–37	I,II	Ia	A-1
L. amylovorus	P/C	40	IV (III)	Ib	A-3
L. crispatus	H/W	35–38	III	Ic	A-2
L. gallinarum	W	33–36		Id	A-4
L. gasseri	H/C	33–35	I	IIa	B-1
L. johnsonii	H/P/W	32–38	I,II	IIb	B-2

[a]H, humans; P, pig; C, cattle; W, poultry.

4.1. General microbiological criteria

These aspects refer to safety (nonpathogenicity), survival of the defense system located in the upper regions of the human GIT (saliva, gastric and bile juice), presumable human origin, and genetic stability. Most difficult appears to be the safety assessment of a probiotic strain. Whilst this issue seems unproblematic for commercial strains with some 'safety record', future approval of new strains may require back-up by sound scientific data. Guidelines are presently in discussion and will probably take account of intrinsic properties of a strain, its interactions in vivo with the host, and its pharmacokinetics (Marteau et al., 1993; Pelletier et al., 1996; Saxelin, 1996). Pronounced differences have been observed in the survival rate of probiotic strains passing through the stomach and upper intestinal tract (Marteau et al., 1993). In vitro model studies, taking account of the low pH of 2.5 in the stomach, and the toxic effects of bile salts, show marked differences in the sensitivity of commercial strains, as is shown in Fig. 3. Safety assessment of new strains may also take into account their acute toxicity, which has been shown to exceed an LD_{50} of 6 g kg^{-1} body weight, for strains of *L. fermentum*, *Enterococcus faecium*, *L. helveticus* and *Lactobacillus* GG (Donohue and Salminen, 1996) (see also Section 6).

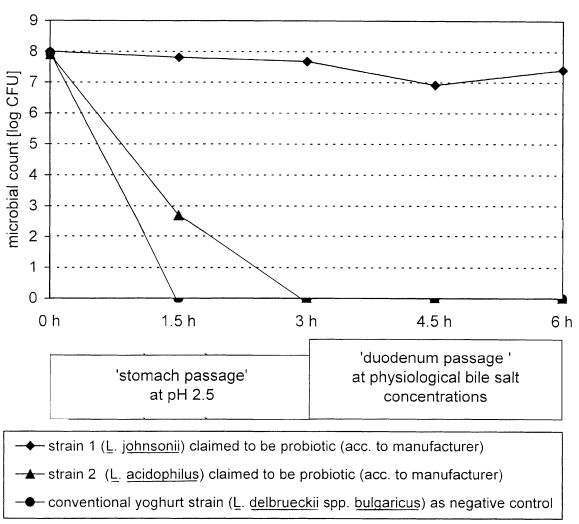


Fig. 3. Inactivation of three different commercial LAB strains associated with probiotic products, using a simulated stomach–duodenum passage. The values represent the average of duplicates for each of three separate experiments conducted over time. Strain 1 (*Lactobacillus johnsonii*) exhibited good acid and bile tolerance and survived these conditions at a high rate. Strain 2 (*Lactobacillus acidophilus*), also labelled as 'probiotic', survived acid stress at levels presumably insufficient to exert significant probiotic effects. Strain 3 (*Lactobacillus delbrueckii* spp. *bulgaricus*) represents a classical yoghurt strain and served as a 'non-probiotic' negative control.

4.2. Technological aspects

The possibility of cultivating the organism on an industrial scale has most important practical consequences for the manufacturer. Strains should be adapted to a suitable carrier or fermentable substrate (e.g., milk), and the final product should have an acceptable shelf-life and sensory attributes such as colour, taste, aroma and texture. Probiotic strains claimed to be present in a product should remain viable in sufficiently high numbers and retain metabolic activity even beyond the 'use-before' date.

4.3. Functional effects and underlying mechanisms

Numerous beneficial effects have been suggested to result from probiotic activities in the gut. Sound

scientific evidence in support of health claims is based either on a limited number of in vivo studies or on deductions from well-founded in vitro 'model' studies. Some of the most important functional effects, backed up by scientific evidence, have been summarised by Salminen et al. (1996), and include aspects such as immune modulation and strengthening the gut mucosal barrier, due to: (1) gut microflora modification, (2) adherence to the intestinal mucosa with capacity to prevent pathogen adherence or pathogen activation, (3) modification of dietary proteins by the intestinal microflora, (4) modification of bacterial enzyme capacity especially of those suggested to be related to tumour induction, and (5) influence on gut mucosal permeability. Examples referring to well-characterised probiotic strains are shown in Table 6 (see also Salminen et al., 1996). These observations are predominantly based on studies either with lyophilised bacterial strains or fermented milks. In contrast to probiotic foods which are aimed at the 'normal' (healthy) population, most studies have been conducted on adults and children with intestinal disorders (Salminen et al., 1996). Probiotic products applied as therapeutics and aimed at curing a disorder may therefore be considered as pharmaceuticals ('probiotic drugs'). The issue of health claims connected with probiotic foods is presently heavily debated. Authorities will probably rely on judicial considerations for solving present discrepancies.

Referring to early hypotheses, numerous studies have convincingly shown the stabilising influence on the gut ecosystem of either probiotic cultures or fermented milks containing such strains. A diet supplemented with yoghurt containing live LAB was found to enhance host resistance against *Salmonella typhimurium* (DeSimone et al., 1988; Paubert-Braquet et al., 1995) or persistent diarrhoea (Boudraa et al., 1990). Fermented milk containing *L. acidophilus* La1 and bifidobacteria was shown to induce changes in the intestinal flora and to modulate the immune response in humans (Link-Amster et al., 1994; Majamaa and Isolauri, 1997). Several probiotic strains have been proven effective in the treatment of different types of intestinal disorders. Significant reduction in either the duration or severeness of gastroenteritis was achieved when different LAB strains (Kaila et al., 1992; Saavedra et al., 1994), *Lactobacillus* GG (Isolauri et al., 1994; Raza et al., 1995), *L. casei* Shirota (Sugita and Tagawa, 1994) or *Bifidobacterium bifidum* were administered. These effects were suggested to result from immune response promoted by particular LAB strains (Kaila et al., 1992; Majamaa et al., 1995). Acute enteritis was successfully treated in double-blind controlled trials with *Enterococcus faecium* (Camarri et al., 1981) and *Lactobacillus* GG (Isolauri et al., 1991). The latter strain was also found effective in the treatment of recurrent *Clostridium difficile* colitis (Biller et al., 1995). In addition, live *L. acidophilus* cultures were found to support the intestinal integrity during radiotherapy (Salminen et al., 1988).

Although application of probiotics shows promising results on immunomodulation, the underlying mechanisms are not yet well understood. There are indications that specific cell wall components or surface layers may be involved. Apparently some similarity exists with Gram-negative bacteria, e.g.

Table 6
Successful probiotic bacteria and their reported effects (as reported by Salminen et al., 1996)

Strain	Reported effects in clinical studies
Lactobacillus acidophilus LA1	Immune enhancer, adjuvant, adherent to human intestinal cells, balances intestinal microflora
Lactobacillus acidophilus NCFB 1748	Lowering faecal enzyme activity, decreased faecal mutagenicity, prevention of radiotherapy related diarrhoea, treatment of constipation
Lactobacillus GG (ATCC 53013)	Prevention of antibiotic associated diarrhoea, treatment and prevention of rotavirus diarrhoea, treatment of relapsing *Clostridium difficile* diarrhoea, prevention of acute diarrhoea, Crohn's disease, antagonistic against cariogenic bacteria, vaccine adjuvant
Lactobacillus casei Shirota	Prevention of intestinal disturbances, treatment of rotavirus diarrhoea, balancing intestinal bacteria, lowering faecal enzyme activities, positive effects in the treatment of superficial bladder cancer, immune enhancer in early colon cancer, immune enhancement
Streptococcus thermophilus; *Lactobacillus bulgaricus*	No effect on rotavirus diarrhoea, no immune enhancing effect during rotavirus diarrhoea, no effect on faecal enzymes
Bifidobacterium bifidum	Treatment of rotavirus diarrhoea, balancing intestinal microflora, treatment of viral diarrhoea
Lactobacillus gasseri (ADH)	Faecal enzyme reduction, survival in the intestinal tract
Lactobacillus reuteri	Colonising the intestinal tract, mainly animal studies so far, possibly an emerging human probiotic

Bacteroides spp. or *Enterobacteriaceae*, where it has been shown that peptidoglycan and lipopolysaccharide components of the cell wall not only express detrimental effects but, under certain conditions, also induce a large spectrum of activities which may be considered advantageous for the host (Hamann et al., 1998, this issue). These activities include induction of the non-specific resistance to microbial infections and irradiation, as well as necrosis of certain tumour types. It has been clearly shown that the effects of peptidoglycan–lipopolysaccharide complexes, independent of whether they are toxic or beneficial, represent mediated phenomena. They are the result of interaction with mononuclear phagocytes and endothelial cells of the host, the production of endogenous mediators such as tumour necrosis factor or other interleukines, and the action of such mediators on the host. It is very intriguing to realise that both Gram-positive and Gram-negative resident bacteria of the intestinal tract may exert similar effects, provided that the subtle equilibrium in the microbial composition of the intestinal flora is maintained. Interactions between host epithelial cells, the gut associated lymphoid tissue and the intestinal microflora with or without probiotic bacteria are presently under study in detail (Farstad et al., 1997). The overall challenge still remains to link effects of probiotics with health related responses of the host. Validated disease models might be valuable for this purpose since several animal studies have shown higher survival rates after gut flora modification with probiotic bacteria.

Of special significance are observations on protective effects of particular LAB against carcinogenesis. Reported as antimutagenic (Pool-Zobel et al., 1993a) or antigenotoxic (Pool-Zobel et al., 1993b) effects, these phenomena are probably related to the ability of such strains to prevent or reduce DNA damage as the early event in the process of carcinogenesis. The incidence of colon tumours in rats was significantly reduced, for example, when *L. acidophilus* was administered (Goldin et al., 1980), whilst *B. longum* was shown to prevent the induction of colon, liver and mammary tumours by the cooked food carcinogen IQ (Reddy and Rivenson, 1993). However, only limited convincing evidence, both scientific and epidemiologic (De Vrese, 1996), for anticarcinogenic effects of LAB in humans is available (Aso and Akazan, 1992; Aso et al., 1995). Some observations even appear to be contradictory (Moore and Moore, 1995), although consumption of LAB with the diet seems to reduce the excretion of mutagenic activity in faeces and urine (Lidbeck et al., 1992; Hayatsu and Hayatsu, 1993).

Adhesion is considered an important property of probiotic LAB able to colonise the GIT. This is mediated either non-specifically by physico-chemical factors or, specifically, by adhesive bacterial surface molecules and epithelial receptor molecules. Human cell lines such as Caco-2 or HT 29 cells may serve as in vitro models for assessment of adherence ability. These and faecal recovery studies have shown (e.g.) that traditional ‘dairy’ strains do not possess these properties (Elo et al., 1991; Saxelin et al., 1995; Salminen et al., 1996).

The postulated ability of some gut microflora to reduce the serum cholesterol level is still a matter of dispute. It is however an established fact that cholesterol and bile salt metabolism are closely linked. Bile salts may be deconjugated by the enzyme bile salt hydrolase (BSH) (E.C. 3.5.1.24), typical of some gut bacteria. According to the ‘BSH hypothesis’ the free bile salts are excreted more readily and may thus contribute to reducing serum cholesterol levels (Chikai et al., 1987). This hypothesis is, however, not undisputed and is not supported by current knowledge on the passive absorption kinetics of free bile acids in the GIT (Aldini et al., 1996; Marteau et al., 1995). Enhanced faecal loss of bile acids, however, may result in an increased requirement for cholesterol as precursor for the de novo synthesis of bile salts, thereby reducing cholesterol levels. Recent observations based on pig and minipig feeding experiments with highly BSH active lactobacilli, strongly suggest a cholesterol lowering effect by such strains (De Smet et al., 1995; Du Toit et al., 1998b). The fact that a number of commercial probiotic strains exhibit high BSH activities should be taken into consideration, also in view of the formation of potentially toxic levels of secondary bile acids in the gut, but also with regard to the question of the desirability or not of this property for probiotic strains.

5. Future developments

In spite of considerable progress in ‘probiotic’ research over the last five years, not all probiotic bacteria available on the market have a solid sci-

entific record. If nutritional and health benefits are to be derived from products containing probiotic bacteria, it is imperative that we understand the mechanisms underlying these benefits. The 'probiotic concept' will only be accepted by regulatory bodies and authorities if these mechanisms are elucidated and appropriate selection criteria for probiotic microorganisms are defined. It is clear that the selection of strains for probiotic use must be based on criteria which are coherent with the claim the probiotic is used for.

Rational selection and validation of promising microbial strains should be based on evidence obtained in in vitro models with a reliable predicted value or function, and followed by studies in humans. Acceptance of the probiotic concept by both the scientific world and regulatory bodies must be based on evidence obtained from fundamental research with respect to the three M's: Mechanisms to verify, Models to certify and Methods to quantify specially controlled studies in humans. The first M, possible mechanisms, has been discussed above and some scientific evidence has been presented. The question is, how to proceed and how to select models that would enable reliable elucidation of the underlying mechanisms.

5.1. In vitro models

It is generally believed that adherence to mucosal surfaces contributes to the efficacy of a probiotic strain since adherent strains could confer a competitive advantage, important for bacterial maintenance in the gastro-intestinal tract. Furthermore, by blocking the attachment sites, probiotics might contribute to the prevention of infection by pathogenic microorganisms. The non-mucus secreting enterocyte-like Caco-2 cell line displays typical features of enterocytic intestinal cells. By using this cell line as in vitro model, it was shown that *L. acidophilus* and *L. rhamnosus* strains adhere in relatively high numbers and are able to prevent attachment of pathogenic microorganisms such as *Salmonella typhimurium*, *Yersinia enterocolitica* and enteropathogenic *E. coli* (Coconnier et al., 1993; Bernet et al., 1994; Hudault et al., 1997). The value of these observations can, however, be questioned. First of all, only a three-fold difference between 'good' and 'poor' adherent strains was detected (Lehto and Salminen, 1996), which microbiologically is not highly significant. In addition, the growth conditions may vitally influence the expression of bacterial surface structures. The cell surface, for example of an overnight culture, is quite different from that of bacteria which just passed the stomach and small intestine and have been stressed by gastric acid, bile and pancreatic juice. The use of microorganisms surviving these conditions and the use of mucus secreting cell lines, such as HT-29MTX (Lesuffleur et al., 1991), may represent the in vivo situation more closely. Finally, although there are differences in in vitro adherence between different probiotic strains, no evidence has thus far been found for any significant differences in colonisation under in vivo conditions. As yet, none of the well known probiotic strains has been shown to colonise the GI tract permanently.

In vitro methods are suitable for assessment of some of the above-mentioned criteria. However, it is important to note that results from such studies may not be predictive of the actual in vivo situation. For example, the survival of microorganisms at low pH or in the presence of bile in test tubes is not a reflection of interactions in the stomach and the intestine, where more complex physiological conditions exist. Therefore, a dynamic model that mimics the successive in vivo gastro-intestinal conditions as closely as possible, would present a major advantage to conventional in vitro methods. Such a dynamic model of the stomach and small intestine has been developed at TNO Nutrition and Food Research at Zeist, The Netherlands, and was described in detail by Minekus et al. (1995). Recently, a new type of large intestinal model has been developed (Minekus et al., personal communication). It is designed to be complementary to the dynamic multi-compartmental model of the stomach and small intestine. This colon model combines removal of metabolites and water, necessary to obtain physiological concentrations of microorganisms, dry matter and microbial metabolites. High densities of microorganisms, comparable to that found in the colon in vivo, were achieved by absorption of water and dialysis of metabolites through hollow fibres inside the compartments. Incubation with faecal flora resulted in total anaerobic bacterial counts of $> 10^{10}$ CFU ml^{-1} with stable physiological levels of *Bifidobacterium*, *Lactobacillus*, Enterobacteriaceae and *Clostridium*. The dry matter content was approximately 10%, while the total short chain fatty acids concentration was maintained at physiological levels

with similar molar ratios of acetic acid, propionic acid and butyric acid as measured in vivo. The validation studies, so far, have shown that this model accurately reproduces the in vivo conditions and that the results can be extrapolated to the human situation. Validation and application of this in vitro gastrointestinal model in relation to the survival of ingested lactic acid bacteria (Marteau et al., 1997) and other research topics have been published (Havenaar and Minekus, 1996). Obviously, disadvantages of such models should also be considered when planning an experiment, for example with regard to epithelium and immune response. However, combined with other in vitro models the TNO model offers a range of additional possibilities for probiotic research. Furthermore, such combined models can be used for studying bioconversions by the intestinal flora of drugs, toxic components or (pro)carcinogens.

5.2. Methods for studying gut flora composition

A drawback in studying the role of probiotics in the modification of the intestinal microflora is the lack of appropriate techniques to sample or identify changes in the endogenous microflora. Practically all results are based on faecal samples, which obviously are a poor description of the situation in the terminal ileum or the ascending colon. Furthermore, our microbiological techniques are hardly sufficient to even isolate or identify the predominant microbial species in the GIT.

In order to study the effect of probiotics on the composition of the microflora, monitoring at certain intervals before, during and after administration of the probiotic is essential. The use of selective media for growth of different groups of bacteria is widely used. However, in several cases, bacteria cannot correctly be enumerated by plate counting since these viable counts do not correlate with microscopical counts (Langendijk et al., 1995). In addition, many species cannot be cultured in vitro at all. It is speculated that at present less than 20% of all microorganisms can be cultured (Ward et al., 1990), whereas only a fraction of the total culturable and a few unculturable species are known. Examples of known unculturable intestinal bacteria are segmented filamentous bacteria in mice (Snel et al., 1994), or *Epulopiscium fishelsoni*, an intestinal inhabitant of the surgeon fish and considered as the largest bacterium (Angert et al., 1993). Difficulties in culturing techniques have led to the development of novel molecular methods based on DNA technology by which bacteria can be identified without cultivation (Amann et al., 1995).

Presently, ribosomal RNA (rRNA) is commonly used as a tool to study microbial populations. During the last two decades the gene encoding for 16S rRNA of *Escherichia coli* was sequenced, and many more 16S rRNA sequences of other bacteria have been determined. The polymerase chain reaction (PCR) and improved sequencing technology have greatly facilitated retrieval of new sequences. At present, several thousands of 16S rRNA sequences of different bacteria are available in genetic databases, even from intestinal bacterial species that cannot be cultured in vitro (Angert et al., 1993; Snel et al., 1994). The 16S rRNA gene is approximately 1540 bases long, and includes several variable regions while the overall structure is highly conserved. This makes it possible to use this gene to study phylogenetic relationships between microorganisms (Woese, 1987).

An approach to analyse the genetic diversity of complex microbial populations is denaturing gradient gel electrophoresis (DGGE) or temperature gradient gel electrophoresis (TGGE). This technique is based on the separation of PCR amplified fragments of genes coding for 16S rRNA, all having the same length (Muyzer et al., 1993). This results in unique separation patterns for different microbial populations, and will contribute to the description of changes or differences in the microflora composition of (uncharacterised) microbial populations.

From sequence data, rRNA-targeted oligonucleotide probes can be developed that are species- or group-specific. For analysis of intestinal microflora, such probes labeled with a radio-active marker have been used for the first time to study ruminal microbial ecology (Stahl et al., 1988). In this study, hybridisation was quantified in a dot-blot procedure. Later, for similar studies, oligonucleotide probes were labeled with radio-active or fluorescent markers and used for in situ hybridisation, which enables specific identification of individual cells (DeLong et al., 1989; Amann et al., 1990, 1995). Techniques based on 16S rRNA detection have recently been used to detect probiotic *Bifidobacterium* species in infant faeces (Kok et al., 1996).

Restriction enzyme analysis has been applied for probiotic lactobacilli (Charteris et al., 1997) and bifidobacteria (McCartney et al., 1996; Roy et al., 1996). This method also served to characterise strains within the *L. acidophilus* group (Roussel et al., 1993), of *L. reuteri* (Stahl and Molin, 1994) and *L. casei* (Ferrero et al., 1996). Likewise, oligonucleotide probes (Langendijk et al., 1995; Charteris et al., 1997), RAPD-PCR (Du Plessis and Dicks, 1995) and ribotyping (McCartney et al., 1996) have shown promising results for species and even strain-level differentiation of intestinal LAB.

Further new techniques for studying bacterial colonisation of bacteria are described by Contag et al. (1995). They marked strains of *Salmonella typhimurium* with bioluminescence through transformation with a plasmid conferring constitutive expression of bacterial luciferase. They were able to detect photons transmitted through tissues of animals infected with these bioluminescent strains. This enables in vivo detection of intestinal bacteria without invasive procedures.

5.3. Interactions between the intestinal microflora and epithelial cells

Although attachment is thought to be an important factor, mechanisms that allow certain species to establish at specific parts of the intestinal tract are largely unknown. Highly significant, however, is the recent observation that the intestinal microflora can influence the expression of epithelial glycoconjugates which may serve as receptor for attachment of (pathogenic) microorganisms. As discussed before, the interface between a mammalian host and the microflora in the lumen is the mucous gel layer and the underlying cell coat (glycocalix) which consist of glycoconjugates on the apical surface of the epithelium. It is hypothesized that integration of the ecosystem into the host may be achieved, at least in part, through dynamic host–microbe interactions that allow microbes to modify cellular differentiation programs and thus create favorable niches (Bry et al., 1996). This hypothesis was supported by experiments in which one signalling bacterial genetic locus from indigenous *Bacteroides thetaiotaomicron* and one associated responding mammalian gene were studied.

Recent papers by Bry et al. (1996), Matsumoto et al. (1992) and Umesaki et al. (1993), (1995), (1997) report that host epithelial cells in the small intestine express fucosylated glycoconjugates in response to the presence of specific strictly anaerobic bacteria (*Bacteroides thetaiomicron* and a Segmented Filamentous Bacterium ('SFB'), respectively). The observation that one species can induce epithelial surface structures such as fucosylated glycoconjugates, by which the attachment of other bacteria is influenced, has significance for the use of (e.g.) Caco cell lines or gnotobiotic animals as model system, to study either adherence or infectious diseases as well as for strategies to prevent and to treat gastrointestinal diseases. To induce and sustain fucosylated glycoconjugate production, it is thought that bacteria should reach a critical population density. This may be an indication for a soluble bacterial factor that produces a concentration-dependent response in the epithelium. Another (theoretical) possibility might be a density-dependent change in the metabolic properties of the bacteria that affects production of a signalling molecule, a process known as 'quorum sensing' (Kaiser, 1996).

It is not fully known whether these changes in fucosylation observed in the small intestine are also present in the large bowel or the stomach and, if so, which microorganism might bring about such changes. Especially for the stomach this information would be important, because certain glycoconjugates have been identified as receptors for the attachment of *Helicobacter pylori*.

6. Safety

The use of LAB in food products has a long safety record. Strains used in probiotic dairy products are also considered to be non-pathogenic for humans. In several clinical and epidemiological studies LAB and bifidobacteria have been examined for their role in health (Donohue and Salminen, 1996). In addition, while it has been suggested that probiotic strains have been implicated in some cases of patients with bacteraemia, it is important to note that none of the pathogenic strains isolated in such cases was related to lactobacilli used in probiotic food products (Donohue and Salminen, 1996; Saxelin et al., 1996).

There is general consensus that the consumption of probiotics, even in dosages as high as 10^{12} cfu d^{-1}, failed to exhibit any toxicity. Nevertheless, future approval of 'new' probiotic strains should also take into account safety aspects. A scheme proposed by Donohue and Salminen (1996) for safety assessment also takes intrinsic properties of probiotic strains into consideration, in addition to metabolic products, toxicity, mucosal effects, dose–response effects, clinical assessment and epidemiological studies. In view of their frequent association with human infection and possible easy acquisition of (glycopeptide) antibiotic resistance, the use of enterococci as probiotics is still a matter of some concern (Adams and Marteau, 1995; Farrag et al., 1996; Bonten et al., 1996).

7. Conclusions

Increasing knowledge underlines the important role of the intestinal flora for maintaining health and in the prevention of disease. Evidence is emerging that the intestinal flora does not exist as an entity by itself, but is constantly interacting ('communicating') with the environment, the central nervous system, the endocrine system and the immune system (Bry et al., 1996; Shanahan, 1997; Umesaki et al., 1997; Wang et al., 1997). Disturbance of this delicate balance may lead to other disorders and thus facilitate establishing a state of disease.

Probiotics offer dietary means to support the balance of intestinal flora. They may be used to counteract local immunological dysfunctions, to stabilise the gut mucosal barrier function, to prevent infectious succession of pathogenic microorganisms or to influence intestinal metabolism. Many of the proposed mechanisms have to be validated in controlled clinical trials in humans. Novel methods are urgently needed to monitor changes in the composition of the intestinal flora and their mutual interaction with the host's immunological functions and metabolism.

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