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The Intestinal Mucosa as a Habitat of the Gut Microbiota and a Rational Target for Probiotic Functionality and Safety

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INTRODUCTION

The human intestinal tract comprises a large variety of microorganisms, which create an ecosystem within the host that has a major effect upon host physiology and biology (1, 2). Recent years have shown an increase in studies regarding the influence of microorganisms on intestinal gene expression either *in vivo,* by colonizing germ-free mice with a defined microbiota (3), or *in vitro* by determining the interaction of bacteria with intestinal cell lines (4, 5). The impact of the gut microbiota on the host has been clearly demonstrated in model studies using *Bacteroides thetaiotaomicron*, a predominant gut commensal bacterium, the genome of which has been determined (6). When inoculated into germ-free mice, *B. thetaiotaomicron* could elicit the production of fucosylated glycans (Fucα1, 2Galβ-glycans) from the host via a molecular sensor FucR (7), and could also affect the expression of several mouse genes, and increase production of Ang4, an antimicrobial protein, which is involved in the host defence against pathogens (8). These examples all indicate that this commensal gut bacterium was able to interact with its host in a specific manner to affect formation of a particular ecosystem.

Fermented dairy products, in particular, have long been used to improve the composition and activity of the intestinal microbiota. Such products do not appear to have any health risks. Probiotics, although very similar to starter cultures used in the fermentation of dairy products, are usually of a different origin and are intended to survive gastrointestinal (GI) transit (9). Probiotics have an established safety record and several strains have been used for long periods of time in diverse populations; therefore the safety of these strains seems to be well established and agreed (10). However, in order to be able to continue to provide safe versions, particularly of novel strains, an assessment of the intrinsic properties of probiotics is necessary.

The intestine, and especially the colon, is heavily colonized by microbes. Translocation of these microorganisms to sites systemic to the gut poses a serious risk to the host. A number of diseases exist whereby this situation may occur. It would be of great benefit if probiotics could diminish this translocation and the severity of the ensuing disorder. The use of animal models is required to establish the safety and efficacy of new and existing probiotic strains for such applications (11, 12). Such models provide information on the effect of probiotics on translocation of members of the intestinal microbiota. Moreover, it has to be established that the used strain should not translocate either.

The intestinal mucosa forms the border between the heavy colonized intestine and more sterile areas. It is therefore of primary importance to both the microbiota and host, and is a major target for probiotic safety and functionality. In this article, the results from three studies will be shown, dealing with safety-related properties of members of the intestinal microbiota and of probiotic bifidobacteria, and with the efficacy and safety of established and new probiotics in relation to bacterial translocation.

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DIVERSITY OF MUCIN-DEGRADING GUT CONSORTIA AND CHARACTERIZATION OF A NOVEL MUCIN DEGRADER

Introduction

The GI tract is covered with a mucus layer composed of high molecular weight glycoproteins, mucins, that can serve as a barrier to protect the underlying epithelium from pathogen attachment. It also serves as a source of nutrients for commensal bacteria. The constant availability of these host glycans provides a major growth factor for colonization of intestinal microorganisms (13). However, on the other hand, excessive degradation of mucin may be considered a virulence factor, as loss of the protective mucus layer may expose gut cells more to pathogens (14, 15). Under normal circumstances, mucin-degrading bacteria live in mutual coexistence with host cells and the rate of degradation is balanced with the rate of synthesis by goblet cells. However, a disturbance of the mucus layer has been shown in cases of chronic inflammatory bowel disease (IBD) such as Crohn's disease and ulcerative colitis (16), where the origin and the activity of bacteria are still unclear. A link between IBD and intestinal sulphate-reducing bacteria is suspected but not fully confirmed (17). Improved knowledge of the microbiota, the GI tract and its mucus would help in understanding the role of these microorganisms in health and disease. Hence in the present study, by combining culture-based and culture-independent methods, we have described the microbiota able to utilize mucin, as measured by growth in a medium containing mucin as sole carbon source. The enrichments were analysed by denaturing gradient gel electrophoresis (DGGE) of PCR-amplified 16S ribosomal DNA (rDNA) sequences. Moreover, a novel intestinal organism was isolated and characterized, *Akkermansia muciniphila* strain Muc^T, that is able to use gastric mucin in pure culture.

Results and discussion

Fresh faecal samples were collected from six healthy volunteers and inoculated in a basal medium containing mucin as sole carbon source as described previously (18). V6–V8 regions of the 16S rDNA from the faeces and enrichment cultures were analysed by DGGE (Fig. 1) (19). Profiles of the enrichment cultures showed a large diversity of mucin-degrading bacteria consortium that differed between the volunteers. However, some dominant bands in the profiles seemed to be shared between individuals. A clone library of each enrichment culture was constructed from the 16S rDNA of each of the six individuals and the predominant bands were sequenced. Major bands from each enrichment were cloned and sequenced. This revealed that most of the clones were related to bacterial sequences with a homology $<97\%$, suggesting that they have not hitherto been cultivated (Table I). In healthy adults, it has been estimated that 1% of the cultivable colonic microbiota is able to degrade host mucin using specific enzymes (20) and the responsible bacteria were identified as strains of *Ruminococcus torques*, *Ruminococcus gnavus*, *Bifidobacterium bifidum* or *Clostridium* species. All of these are Gram-positive strict anaerobes (21). Similarly, the majority of the sequences obtained from the clone libraries in this study showed that they all originated from strict anaerobic Gram-positive bacteria. By use of serial dilution of a faecal sample, we isolated one Gram-negative anaerobic organism, *Akkermansia muciniphila*, which was highly specific for mucin utilization (18). Analysis of the 16S rDNA (1433 bp) gene revealed that the novel strain was related to, but phylogenetically distinct from, organisms belonging to the *Prosthecobacter* and *Verrucomicrobium* genera that are members of the division *Verrucomicrobia* (Fig. 2) (22). The most similar 16S rDNA sequences were 99% identical to strain Muc^T, but each of these was derived from studies of uncultured colonic bacteria: HuCA18, HuCC13 (23) and L10-6 (24). It is the second member of the *Verrucomicrobium* division to be isolated from the gut, following *Victivallis vadensis* (25). Bacteria related to *Verrucomicrobium* members have been detected in other environments (soil, fresh water) and have also been identified in low numbers in human faecal-derived 16S rRNA gene libraries (23, 26, 27). We conclude that many human intestinal bacteria participate in mucin degradation and have not been yet cultivated.

IN VITRO SAFETY ASSESSMENT OF PROBIOTIC BIFIDOBACTERIA

Introduction

Probiotics that are most commonly in use mainly belong to the genera *Lactobacillus* and *Bifidobacterium*. Species of these genera are generally regarded as safe, as indicated by their long history of use in fermented foods and their presence in the normal intestinal microbiota of humans (28). However, some lactobacilli and bifidobacteria have been associated with isolated cases of bacteraemia in patients with reduced immune function or severe underlying disease. It has also been suggested that the rate of *Lactobacillus* infection is increasing (29), although recent data do not support this view (30). It is uncertain if this perceived increase is real or due to a more active search for these organisms in clinical specimens. In most cases of *Lactobacillus* and *Bifidobacterium* infection, the host intestinal microbiota is the most likely source of infection (31). In this respect, it is important to note that bifidobacteria belong to the numerically dominant members of the intestinal microbiota (32). Despite this, they are extremely rarely involved in infections.

Although a large number of *Lactobacillus* and *Bifidobacterium* strains have GRAS status and many strains have a long history of safe use in foods (10), it is important

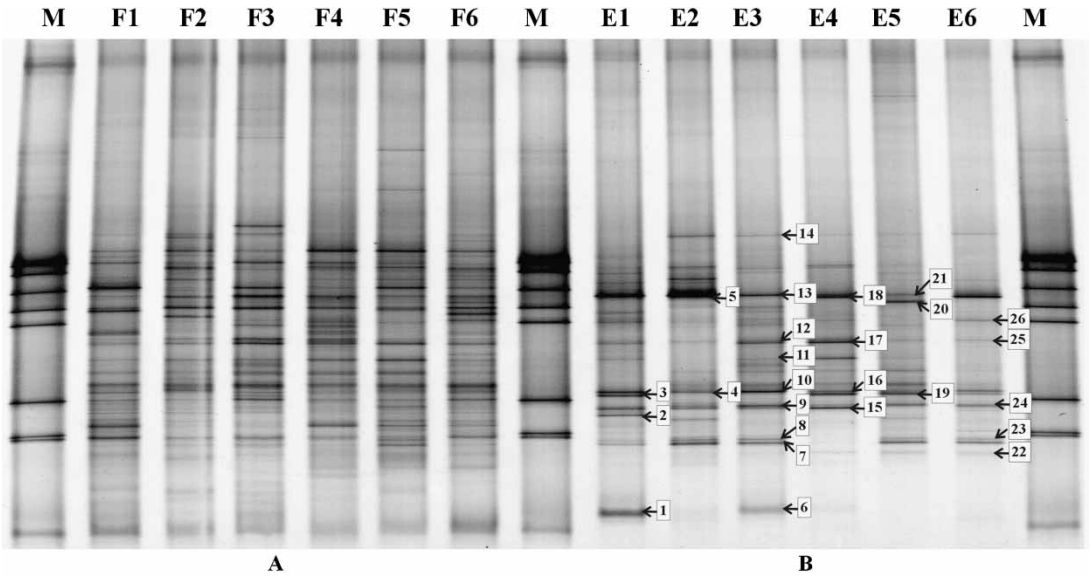

Fig. 1. DGGE analysis of amplified V6–V8 regions of the 16S rDNA gene of faecal (F) samples (gel A) and enrichment (E) cultures on mucin medium (gel B). M represents the DGGE marker. The bands identified from the 16S rDNA clone libraries are numbered. The origin of the bands is presented in Table I. DNA isolation, PCR, DGGE analysis of the V6–V8 regions of 16S rDNA and sequencing analysis were performed as described previously. PCR and DGGE of the enrichment cultures were performed as described previously (19).

Table I

Closest relatives as determined by comparative sequence analysis, level of identity with this relative, clone designation and accession no. of each band identified in Fig. 1

n	Species	%	bp	Clones	GenBank accession no.
1	*Akkermansia muciniphila*	100	1433	NA	AY271254
2	*Ruminococcus obeum*	99	522	1–21	AY451996
3	*Ruminococcus obeum*	95	551	1–11	AY451995
4	*Ruminococcus torques*	96	438	2–3	AY451997
5	*Ruminococcus obeum*	96	534	2–18	AY451998
6	*Clostridium clostriiformes*	95	978	3–1	AY451999
7	*Eubacterium ramulus*	99	1414	3–3	AY452000
8	*Ruminococcus obeum*	96	1457	3–5	AY452001
9	*Ruminococcus obeum*	92	1457	3–9	AY452002
10	*Ruminococcus obeum*	95	1457	3–10	AY452003
11	*Clostridium* sp.	93	1004	3–13	AY452004
12	*Ruminococcus obeum*	94	1457	3–16	AY452005
13	*Desulfomonas pigra*	99	1505	3–17	AY452006
14	*Ruminococcus torques*	99	1418	3–18	AY452007
15	Bacterium mpn-isolate group18	99	410	4–2	AY452008
16	*Fusobacterium prausnitzii*	96	543	4–9	AY452009
17	*Ruminococcus productus*	93	472	4–13	AY452010
18	*Escherichia coli*	98	502	4–17	AY452011
19	*Clostridium ramosum*	98	534	5–10	AY452012
20	*Escherichia coli*	99	533	5–12	AY452013
21	*Clostridium ramosum*	97	546	5–13	AY452014
22	*Ruminococcus obeum*	98	538	6–4	AY452015
23	*Ruminococcus obeum*	97	520	6–10	AY452016
24	*Ruminococcus obeum*	97	512	6–13	AY452017
25	*Ruminococcus obeum*	95	524	6–14	AY452018
26	*Ruminococcus* sp. CO27	96	506	6–16	AY452019

The 26 sequences of the 16S rDNA determined in this study were deposited in the GenBank database.
NA: Not applicable (Pure Culture).

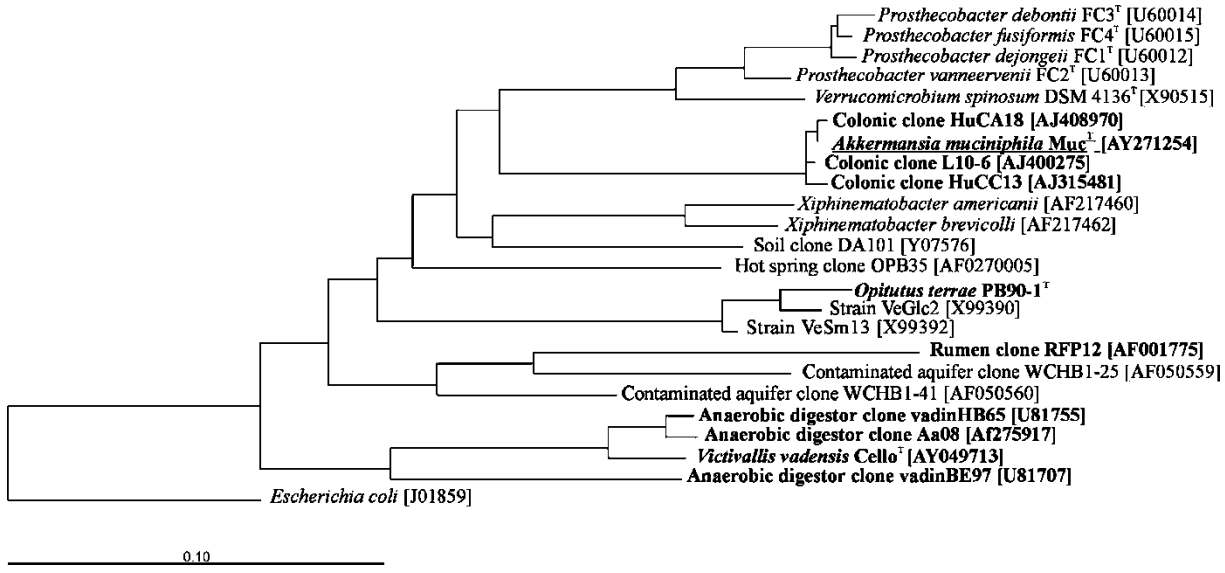

Fig. 2. Phylogenetic tree showing the position of *Akkermansia muciniphila* (underlined) among selected clones or strains belonging to the *Verrucomicrobia* division. The tree, which was rooted by using *Escherichia coli* as the outgroup, was generated by the neighbour-joining method. Bold type indicates the clones or isolates that originated from intestinal or similar anaerobic ecosystems. Phylogenetic analyses were performed with the ARB software package (22). Bar represents 10% sequence divergence.

that the safety of novel and existing starter and probiotic cultures is confirmed. As most bifidobacteria are non-pathogenic, it is difficult to identify inherent strain properties that may be related to health risks. We therefore chose to compare faecal, blood and probiotic *Bifidobacterium* isolates for adhesion to immobilized human collagen type IV, fibrinogen or intestinal mucus. Adhesion to the intestinal mucosa is one of the principal selection criteria for probiotics (9). However, adhesion is also one of the first steps in microbial pathogenesis (33). It is therefore important to determine whether adhesion of probiotic bifidobacteria to these substrata is different for faecal and clinical blood culture isolates. These three groups of bacteria were chosen since probiotics are usually of faecal/intestinal origin and also the blood isolates are generally thought to be of intestinal origin (34). Differences in adhesion between these groups may indicate whether these properties relate to health risks in specific populations. On the other hand, lack of such differences would suggest that adhesion is not a risk factor and therefore would support the safety of these bacteria for food use.

Furthermore, resistance to serum mediated killing, α- or β-haemolytic activity, induction of respiratory burst in peripheral blood mononucleocytes and phosphatidylinositol-specific phospholipase C (PI-PLC) activity were determined. These properties are considered virulence factors for a number of pathogens. Their absence or general presence would generate information on the importance of such properties for the safety of probiotic bifidobacteria (34).

Methods and materials

Faecal bifidobacteria (nine isolates) were isolated from healthy adult volunteers, two isolates from clinical blood cultures were obtained from patients with severe underlying diseases (30). The bacteria were minimally subcultured to avoid adaptation to laboratory conditions. Three probiotic strains were isolated from products or obtained from the producers of such products.

Adhesion to immobilized human collagen IV, fibrinogen and intestinal mucus was performed with radiolabelled bacteria essentially as described earlier (35). Adhesion was expressed as the percentage of radioactivity recovered following adhesion, relative to the radioactivity of the bacterial suspensions added to the substrata.

Haemolysis was determined as described by Baumgartner and co-workers (36), using human instead of sheep blood. Serum resistance was determined as described by Burns and Hull (37). The induction of respiratory burst was basically performed as described by Lilius and Marnila (38) and expressed as mV/100 000 peripheral blood mononucleocytes (PMN). Phosphatidylinositol-specific phospholipase C (PI-PLC) activity was determined as described by Rodriguez and co-workers (39). Appropriate positive and negative controls were included in the assays, which were all performed in triplicate.

Results

Adhesion to human mucus, collagen and fibrinogen varied substantially for all three groups of bifidobacteria, ranging from $< 1\%$ to almost 12%. Because of a large variation

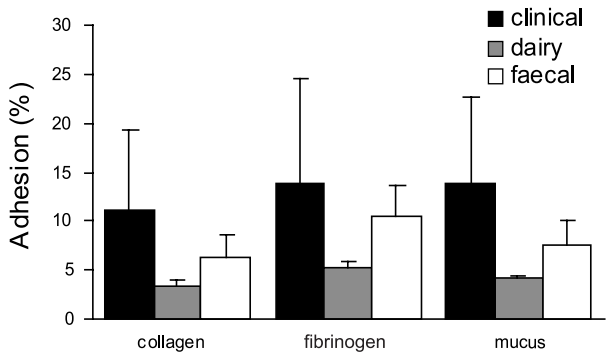

Fig. 3. Adhesion of bifidobacteria of probiotic, faecal and clinical (blood) origin to immobilized human intestinal mucus, human collagen IV and human fibrinogen. Results are expressed as the average of three independent experiments, error bars indicate the standard deviation (modified after (34)).

within the three groups, statistical analyses (Kruskal-Wallis, $p > 0.05$) did not reveal significant differences (Fig. 3) (34). For the bifidobacteria tested a positive correlation was observed for the adhesion to collagen and fibrinogen (Spearman rank correlation coefficient, $p < 0.0005$).

None of the *Bifidobacterium* strains tested exhibited α- or β-haemolysis of human blood group O erythrocytes.

With the exception of one faecal *B. longum* isolate, all strains tested were found to be resistant to serum-mediated killing. Of the 14 *Bifidobacterium* strains tested, 12 were found to grow in the presence of 80% human serum. All strains survived and grew in heat-inactivated serum.

The respiratory burst induced in PMN varied from 287 to 20 000 mv/100 000 PMN. There was a trend ($p = 0.083$) for dairy strains to induce a stronger respiratory burst, although this did not reach statistical significance (Fig. 4). None of the tested strains exhibited PI-PLC activity.

Discussion

In the current study, we investigated the adhesion of bifidobacteria isolated from probiotic products, blood and faeces, to human intestinal mucus, collagen and fibrinogen.

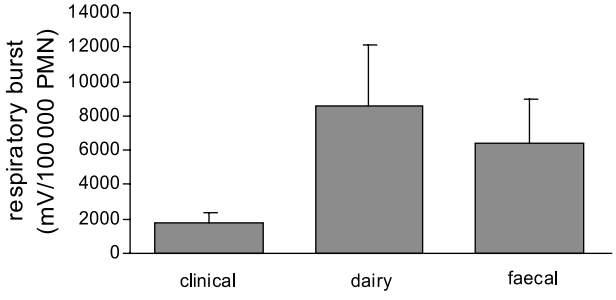

Fig. 4. Induction of respiratory burst by bifidobacteria of probiotic, faecal and clinical (blood) origin in peripheral blood mononucleocytes. Results are expressed as the average of three independent experiments, error bars indicate the standard deviation (modified after (34)).

No statistically significant differences could be observed in the adhesive properties between the three groups to any of the three substrata tested. Therefore, it seems unlikely that a specific selection of probiotic bifidobacteria with high adhesive capacities to the intestinal mucosa poses any risk to the consumer. The observed positive correlation in adhesion to the tested extracellular matrix proteins suggests that similar adhesins may be used for adhesion to these substrata. This would, however, require further investigation.

None of the bifidobacteria tested exhibited haemolytic activity, this may be due to the fact that bifidobacteria do not require iron for their growth (40). Therefore, haemolysis does not appear to be a potential risk factor for bifidobacteria. Although all except one of the tested bifidobacteria were resistant to serum-mediated killing, this does not seem to be a risk but rather an intrinsic property of bifidobacteria. In general, Gram-positive bacteria are not sensitive to the complement system present in serum. An inability to induce a respiratory burst in PMN may enhance the survival, after translocation, of a bacterium in the blood. Although the clinical isolates exhibited a lower induction of respiratory burst, this was not significant. Based on these observations, this point does warrant further investigation. PI-PLC activity has been suggested to be associated with translocation of lactobacilli, although this was tested for only two strains of *L. rhamnosus* (39). However, this activity was absent from all strains tested, also the clinical isolates, and is therefore not likely to be a relevant risk factor for probiotic bifidobacteria.

Thus, no properties have so far been identified that may generate a concern for the consumption of probiotic lactobacilli and bifidobacteria. As no general risk factors were found to be associated with the clinical lactobacilli and bifidobacteria, this may indicate that the condition of the patient contributed more to translocation than did the properties of the bacteria. However, it cannot be excluded that other properties not yet tested could be involved.

Further *in vitro* studies to assess the importance of more potential risk factors need to be carried out. From such studies, strains that possess more risk factors will be used in animal trials to correlate *in vitro* findings to the *in vivo* situation (41). Studies as described above are also being performed with lactobacilli, for which larger numbers of strains are available.

The ultimate safety test is always a human feeding trial. Existing probiotic bifidobacteria have a long history of safe use and some of the emerging strains have successfully been assessed for their *in vivo* safety in humans (42).

In conclusion, the findings of the studies described here support the general view that probiotic bifidobacteria are wholly safe for human consumption.

BACTERIAL TRANSLOCATION

Introduction

The 'gut origin of sepsis' hypothesis proposes that bacteria, which are normally resident within the lumen of the intestinal tract, translocate across a damaged intestinal epithelial barrier and act as a source of sepsis at distant sites (43, 44). Animal studies support this concept (11, 12). Most bacterial infections in critically ill or immunocompromised patients are caused by the patients' own microbiota, and many persons dying from sepsis or multiple system organ failure have enteric bacteraemia for which no septic focus can be identified (44, 45). A number of factors have been shown to predispose towards bacterial translocation. These include shock with reduced splanchnic blood flow, parenteral nutrition, intestinal epithelial damage and antibiotic therapy (12). Intestinal bacterial overgrowth (46), intestinal atrophy and increased gut permeability (47) are all believed to result in an escape of bacteria (48) and endotoxin (49) from the intestinal lumen to the mesenteric lymph nodes and portal circulation, where they stimulate peritoneal, intestinal and hepatic macrophages to release inflammatory mediators. Hepatic macrophages (Kupffer cells) appear to play a role in clearance of translocated bacteria or endotoxin from the portal circulation. Impairment of the activity may potentiate the systemic effects of gut barrier failure (44).

Experimental rat models

A set of bacterial strains has been tested in two experimental rat models, i.e. a colitis model and a liver failure model. The test strains were *Lactobacillus plantarum* 299v (a probiotic strain originating from healthy human colonic mucosa; Probi AB, Lund), *Lactobacillus paracasei* 8700:2 (a probiotic strain with ability to grow in ripening cheese; originating from healthy human intestinal mucosa; Probi AB), *Lactobacillus gasseri* 5B3 (originating from healthy human vagina), *Bifidobacterium* 3B1 (originating from healthy human vagina) and *Bifidobacterium infantis* CURE19 (a probiotic strain originating from infant faeces; Probi AB).

Colitis model

Mucosal barrier dysfunction is a feature of colitis irrespective of aetiology or species. Such dysfunction may be responsible for the systemic inflammatory response and complications seen in patients with inflammatory bowel disease (IBD) (50). The indigenous intestinal microbiota and an intact mucosa are vital components of the body defence against luminal pathogenic bacteria. Disruption of these defences in IBD may permit bacterial translocation and contribute to disease severity (51). The pathogenesis of IBD remains unknown. Genetic and environmental factor contributions are evident, and the luminal microbiota plays a major role in the initiation and perpetuation of chronic IBD (52). The effect of colonic inflammation on intestinal microbiota, specifically lactobacilli and bifidobacteria, is not clear.

We investigated the effect of different *Lactobacillus* and *Bifidobacterium* strains administered orally for 7 days before induction of colitis and continued for 7 days with dextran sulphate sodium (DSS) (5% in drinking water). The colitis lesions induced by DSS resemble those of human ulcerative colitis both symptomatically and histologically. We found that bacterial translocation to mesenteric lymph nodes decreased significantly in all treatment groups compared with a colitis control. Moreover, translocation of Enterobacteriaceae to the liver decreased in all treatment groups. Thus, administration of certain strains of *Lactobacillus* and *Bifidobacterium* significantly improves the disease activity index (DAI) and reduces bacterial translocation in a rat model. *L. plantarum* 299v, *Bifidobacterium* 3B1 and *Bifidobacterium infantis* CURE-19 seemed to have the best effect.

Liver injury model

Liver function, the intestine and the immune system not only influence each another, but are also affected by nutrients and their route of delivery (53). The gut is a major reservoir for bacteria and under normal conditions a series of local and systemic protective mechanisms prevents passage of pathogenic bacteria beyond the intestinal lumen, and these defence mechanisms are severely impaired in the acute liver injury induced by D-galactosamine (54). Intestinal microbiota composition is important in physiological and pathophysiological processes in the human gastrointestinal tract. Septic complications represent frequent causes of morbidity in liver diseases and following hepatic operations.

We therefore studied the effect of different *Lactobacillus* and *Bifidobacterium* strains on bacterial translocation, extent of liver injury and intestinal microbiota in an acute liver injury model. Sprague-Dawley rats were used and *Lactobacillus* and *Bifidobacterium* strains were administered orally twice daily for 8 days. Liver injury was induced on the eighth day by intraperitoneal injection of D-galactosamine (1.1 g/kg body weight). Samples were collected 24 h after the induction of liver injury. Liver enzymes and bilirubin serum levels, bacterial translocation and intestinal microbiota were evaluated.

We found that administration of different *Lactobacillus* and *Bifidobacterium* strains in an acute liver injury model has different effects on bacterial translocation and hepatocellular damage. *L. plantarum* 299v and *B. infantis* CURE-19 reduced bacterial translocation and hepatocellular damage. *L. gasseri* 5B3 reduced bacterial translocation but did not show significant effects on hepatocellular damage. *L. paracasei* did not reduce bacterial translocation

and hepatocellular damage, but translocated to extraintestinal sites. This clearly indicates the strain specificity of the health effects of lactobacilli and bifidobacteria.

CONCLUSIONS

Within the intestinal microbiota new members with new functions continue to be identified. Their role in the health and disease of the host remains to be fully elucidated. The studies described above have shown that the intestinal mucosa is an important habitat and provides energy for members of the intestinal microbiota. The barrier function provided by the intestinal mucosa can be safely strengthened by selected probiotics. Through investigating the interaction between members of the intestinal microbiota and probiotics, new insights may be obtained on the mechanisms by which the latter exert their health benefits. This, together with studies on the intrinsic properties of probiotics, is likely to lead to the development of new safe applications for probiotics.

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