Functionality of probiotics and intestinal lactobacilli: light in the intestinal tract tunnel

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The commercial interest in functional foods that contain live microorganisms, also termed probiotics, is paralleled by increasing scientific attention to their functionality in the digestive tract. Most studies are focused on intestinal *Lactobacillus* species, which are part of the natural gastro-intestinal microbiota, and include analysis of colonisation factors and other interactions with the host, the design of novel or improved strains with specific health benefits, and the application of sophisticated molecular tools to determine their fate and activity *in situ*.

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Abbreviations

ABC	ATP-binding cassette
AggH	aggregation helicase
CnBP	collagen-binding protein
LAB	lactic acid bacteria
LTA	lipoteichoic acid
TGGE	temperature gradient gel electrophoresis

Introduction

The increasing consumer awareness that diet and health are linked is stimulating innovative development of novel products by the food industry. In particular, scientific evidence is accumulating to support the claims of functional foods containing probiotics, which can be defined as "living microorganisms, which upon ingestion in certain numbers, exert health benefits beyond inherent basic nutrition" [1]. The probiotic products traditionally incorporate intestinal species of *Lactobacillus* because of their long history of safe use in the dairy industry and their natural presence in the human intestinal tract, which is known to contain a myriad of microbes, collectively called the microbiota [2,3]. Members of the *Bifidobacterium* have more recently been added to foods for probiotic purposes, probably encouraged by the discovery of their consistent presence as part of the normal microbiota of the human intestine [4]. The primary claim of probiotics is their beneficial influence on the intestinal ecosystem, which in turn may provide protection against gastro-intestinal infections and inflammatory bowel diseases. The desirable effects on human health include antagonistic activity against pathogens, anti-allergic effects and other effects on the immune system (Table 1). Whereas several of these health claims remain controversial, well-planned clinical trials increasingly support the claims for carefully selected probiotic strains [5]. Some probiotic strains for which a substantial amount of literature has been generated include *Lactobacillus rhamnosus* GG, *Lactobacillus johnsonii* La1 and *Bifidobacterium lactis* Bb12, and screening for new commercial isolates continues [6,7]. Nevertheless, there is a reasonable amount of frustration and scepticism among the scientific community because the mechanistic details underlying probiotic effects are simply not known. In fact, although it has been recognised for decades that the gut commensal microbiota is necessary for development and maintenance of normal health, the effects of individual members on our health are still not understood.

Table 1

Beneficial effects of probiotics strains: demonstrated and proposed.

Alleviation of lactose intolerance
Positive influence on intestinal flora
Prevention of intestinal tract infections
Reduction of growth of *H. pylori*
Improvement of immune system
Reduction of inflammatory reactions
Anti-carcinogenic
Anti-allergic
Regulation of gut motility
Reduction of serum cholesterol
Feeling of well-being

The application of novel molecular methods in combination with bacteriological and biochemical disciplines now provides opportunities to analyse the complex intestinal ecosystem [3]. These technologies are being increasingly used for analysis of probiotic and intestinal microorganisms [8,9]. As a result, unravelling of the mechanisms behind the activities and interactions in the digestive tract between both the indigenous and probiotic bacteria and their host, as well as following the fate of probiotic bacteria, has essentially begun. This review focuses on the recent molecular advances in properties of intestinal and probiotic lactobacilli that may influence their role in the intestine. Significant advances in genetic modification of lactobacilli for creation of novel strains with beneficial properties, for use in functional foods with advantages for the consumer or oral vaccination, are described. The application of novel molecular techniques in tackling the research challenges ahead will also be addressed.

Colonisation factors

Colonisation within the intestinal tract, while essential for the survival of our indigenous lactobacilli, is also considered to be a valuable property for probiotic strains. Established

Table 2

***Lactobacillus* genes identified with potential functionality in colonisation.**

Species	Gene(s)	Function (proposed)	Reference
L. reuteri	*cnb*	Collagen binding adhesion	[15]
L. reuteri	*mub*	Mucus binding adhesion	[22••]
L. reuteri	*aggH*	Auto-aggregation	[13••]
L. fermentum	*mapA*	Mucus adhesion promoting	[P1]
L. acidophilus	*slpA/B*	S-layer, antigenic variation	[23]
L. brevis	*slpA*	S-layer	[61]
L. johnsonii	*dlt*	Lipoteichoic acid synthesis, adhesion	[21]
L. casei	*dlt*	Lipoteichoic acid synthesis	[62]
L. johnsonii	*rml*	dTPD-L-rhamnose synthesis	[21]
L. gasseri	*rml*	dTPD-L-rhamnose synthesis	[63]
L. plantarum	*bsh*	Bile salt hydrolyase	[64]
L. johnsonii	*bshβ*	β-subunit of bile salt hydrolase	[25]
	ORF1, ORF2	Conjugated bile salt uptake	
L. johnsonii	*bshα*	α-subunit of bile salt hydrolase	[21]
L. plantarum	*pln*	Peptide-induced bacteriocin production	[65]

strains should have a competitive advantage and presumably have a lengthier probiotic effect. This is supported by a report describing a spontaneous aggregation-deficient mutant of a *Lactobacillus crispatus* strain that could no longer colonise the human gastro-intestinal tract like the wild-type strain [10]. In addition, recent indications are that at least direct contact with intestinal epithelial cells is a prerequisite for some probiotic effects on the immune system, such as the enhanced leucocyte phagocytic activity against enterobacteria that was detected following administration of probiotic strains to healthy individuals [11]. Different mechanisms, such as adherence, aggregation or high growth rate to avoid 'washing-out' or in the case of probiotic strains continued consumption, might be involved in achieving colonisation or contact. Advances in understanding of the molecular and genetic basis of biofilm development in bacterial infection also indicate the importance of bacterial communication by signalling molecules and defence mechanisms within these microbial communities [12••]. These factors are also likely to play a major role in colonisation of intestinal microbes. Although their ultimate role in animal or human colonisation awaits further *in vivo* studies, there has been significant progress in the genetics of extracellular or cell-wall associated binding molecules and other potential colonisation factors (Table 2).

Lactobacillus reuteri 1063 is an auto-aggregating strain isolated from the small intestine of pig. The aggregation phenotype was linked to a cell-surface protein, designated AggH (aggregation helicase), by inactivation of the *aggH* gene and the observation of increased aggregation behaviour when *aggH* was introduced into a related aggregating *L. reuteri* strain [13••]. Also, cleavage of the purified maltose-binding protein fusion protein (MBP–AggH–LacZ α) released an AggH–LacZ α peptide product that exhibited strong aggregation activity. Remarkably, AggH had extensive similarity to ATP-dependent DEAD-box RNA helicases, which are a family of proteins identified in a wide range of organisms performing fundamental biological reactions, such as DNA replication [14]. This surprising similarity prompts the speculation that AggH may have a dual role in DNA uptake during genetic transfer as well as aggregation.

The gene coding for a collagen-binding protein (CnBP) from the human intestinal *L. reuteri* strain NCIB 11951 was characterised and the deduced amino acid sequence was similar to the Class III solute transporters that belong to the ATP-binding cassette (ABC) family [15]. Recently, it was found that genes for a streptococcal adhesin and a co-aggregation factor, which were also part of ABC transporter operons, were in fact involved in manganese uptake [16,17]. It was suggested that the binding proteins in contact with Mn^{2+} or other components complexed with serum proteins may promote adhesion *in vivo* [17]. Similarly, the CnBP amino acid sequence is very similar to MAPP, a mucus adhesion promoting protein from another intestinal *Lactobacillus* spp. [P1], and the basic surface protein A (BspA) of *Lactobacillus fermentum*, which is the binding protein component of an ABC uptake system for L-cysteine [18]. Thus, this class of adhesins may also have a dual function in being involved in binding to host cells or their components, as well as transport of specific compounds. A further interesting suggestion is the possibility that these proteins, in analogy with other ABC transporters, may be involved in signalling to the bacterium that it has colonised a surface [19].

Lipoteichoic acid (LTA) was shown to be involved in the mechanism of adhesion of cells of the industrial probiotic *L. johnsonii* La1 to intestinal Caco-2 cells [20]. Both LTA purified from the La1 strain and the La1-spent culture medium containing LTA released during growth inhibited La1 adhesion to the Caco-2 cells in a dose-dependent manner, demonstrating its role. Genes involved in LTA synthesis (*dlt* genes) and other genes coding for potentially cell-wall located molecules have been identified during the genome sequencing project of *L. johnsonii* La1 [21].

Recently, the first proteinaceous adhesin, Mub, which mediates adhesion of *L. reuteri* 1063 to mucus, has been identified [22••]. The gene, 9807 basepairs in length, encodes one of the largest bacterial cell-surface proteins characterised and possesses motifs typical of secreted and cell-wall-anchored proteins from Gram-positive bacteria. The protein contains two types of long repeat sequences that share some similarity with an antigen from a hepatitis virus and a human ocular epithelial protein, two proteins possibly associated with mucus-containing environments. In contrast to the CnBP that is present in all *L. reuteri* strains, the Mub gene was present in less than 20% of strains tested.

Cell-surface crystalline or S-layers may also aid colonisation for some *Lactobacillus* strains. S-layers are constituted

of a single protein that is assembled into a monomolecular array that covers the cells. Various functions have been ascribed to S-layers, such as protective coats, molecular sieves, and adhesion [23]. Among the intestinal lactobacilli, study of the S-layer genetics of *Lactobacillus acidophilus* revealed that the two S-layer encoding gene homologues, which contain conserved and variable regions, were interchangeable by inversion of the chromosomal *slp* segment [24]. Although only the *slpA* gene was found to be expressed, it is probable that certain intestinal conditions may induce switching to the other phase and offer competitive advantages of this phase-variation during colonisation of a host.

The advantages of conjugated bile salt hydrolysis by bacteria within the intestinal tract to either colonisation of the microorganism or to host health are still somewhat enigmatic. Controlled studies with carefully constructed and isogenic strains that are deficient in the genes for bile salt hydrolase may provide some answers. In addition to the genes encoding the β- and α-subunits of bile salt hydrolyase, the genetic characterisation of a conjugated bile salt uptake system was also reported recently in *L. johnsonii* strain 100-100, which could lead to such functional studies [21,25]. It is noteworthy that a substantial number of genes encoding narrow-host-range bacteriocins in various lactobacilli have been characterised, but again their significance for colonisation awaits *in vivo* studies with isogenic mutants.

Signalling between intestinal microbes and host

The intestinal host defence mechanisms comprise complex systems involving the innate and adaptive immune responses, and protective effects of the indigenous microbiota. The commensal microorganisms colonising the intestinal mucosa provide a barrier effect against pathogens by using a variety of mechanisms, such as occupation of niches, competition for nutrients and production of antimicrobials. Furthermore, evidence is accumulating that these symbiotic microorganisms, including certain lactic acid bacteria (LAB) can modulate the homeostasis of the host's defence mechanisms, both innate and adaptive immune functions [26,27]. The molecular basis of crosstalk between the luminal microorganisms and the intestinal epithelium and immune cells is beginning to emerge in recent studies [28].

The human intestinal epithelial cell lines Caco-2 and HT-29, established from colonic adenocarcinomas [29], have already been valuable *in vitro* models for studying the adherence of probiotic bacteria to host cells. Co-culture with *L. johnsonii* La1 did not alter HT-29 mRNA expression of pro-inflammatory cytokines, such as IL-8, TNF-α or MCP-1, although a non-pathogenic *Escherichia coli* did upregulate their expression [30]. In order to assess bacterial interaction with blood immune cells, an elegant *in vitro* model was established by co-cultivation of Caco-2 intestinal epithelial cells with immune cells using insert-culture technique [31•]. The immune response to non-pathogenic lactobacilli and *E. coli* was assessed by analysis of cytokine mRNA expression and protein production. Both *E. coli* and *Lactobacillus sake* stimulated production of pro-inflammatory cytokines by the Caco-2 cells, whereas *L. johnsonii* La1 only induced the expression of TGF-β. As the immunocytes had no direct access to the bacteria, epithelial–immune cell crosstalk appears to be responsible for distinguishing the bacterial type. Although the physiological significance of this differential activation is not yet clear, the development of this model and initial results are an important step towards analysis of the complex beneficial interactions between the indigenous microbiota and the host immune response.

The probiotic strains *Lactobacillus plantarum* 299v and *L. rhamnosus* GG quantitatively inhibited the adherence of an attaching and effacing pathogenic *E. coli* (EPEC) to mucus-producing HT-29 intestinal epithelial cells but not to HEp-2 laryngeal epidermoid cells [32•]. Mucin glycoproteins are the principle component of the mucus layer on the gastro-intestinal mucosa and are encoded by various mucin genes that show distinct patterns of gene expression in different epithelial tissues [33]. MUC2 and MUC3 mucins isolated from HT-29 cells could inhibit EPEC adherence to the HEp-2 cells. Furthermore, incubation of *L. plantarum* 299v cells with the HT-29 cells increased expression of MUC2 and MUC3 mRNA. Thus, the probiotic strain appears to induce intestinal mucin gene expression that in turn diminishes the binding of enteric pathogens to mucosal epithelial cells. Interestingly, sterile supernatants of *L. plantarum* 299v had a similar effect, suggesting that a cell wall or secreted component may be involved. It will be interesting to determine if increased intestinal mucin production by probiotic or intestinal LAB is a more widespread phenomenon against pathogenic bacteria and viruses as speculated [32•]. Indeed there have been previous reports of inhibition of adherence and invasion of enteropathogenic bacteria by *L. johnsonii* La1 and various human bifidobacterial strains to Caco-2 cells [34,35]. Intestinal mucins have been demonstrated to inhibit replication of rotavirus [36], and so there might be a link between the observations described and the reduction of disease symptoms and faecal shedding during rotavirus gastro-enteritis upon probiotic ingestion [37].

Molecular approaches for analysis of probiotic and intestinal lactobacilli

Strategies for the application of molecular techniques in analysis of microbial diversity, particularly those based on 16S and 23S rRNA genes, have greatly expanded in the past decade, and more recently are making an impact on the gastro-intestinal ecosystem [38]. Cloning, sequencing and phylogenetic analysis of 16S rDNA sequences from human intestinal samples has confirmed that 50% or less of bacteria have been cultured [39]. Thus, studies of this ecosystem stand to benefit significantly from this technology. Knowledge of the structure and function of the

intestinal microbiota is a prerequisite to understanding the impact of a probiotic on this community, and these novel methods can also be applied to follow the fate and activity of probiotic bacteria in the intestine.

For more routine monitoring of the diversity in the intestinal ecosystem, the rapid fingerprinting methods may be applied, including those based on sequence-specific separation of 16S rRNA PCR products, such as denaturing gradient gel electrophoresis (DGGE) and temperature gradient gel electrophoresis (TGGE) [40]. TGGE of rRNA amplicons from intestinal samples of unrelated individuals showed that each individual had a unique pattern reflecting differences in the composition of their intestinal microbiota, and also revealed that their predominant faecal bacteria remained remarkably stable over at least a six month period [41•]. In another study, this molecular approach allowed rapid assessment of the effects of probiotic consumption on the faecal microbial community of healthy individuals by comparison of TGGE patterns before and after the feeding trial [42].

Fluorescent *in situ* hybridisation (FISH) with rRNA-targeted oligonucleotide probes has become an important technique for the *in situ* identification of individual cells in complex environments [43]. By designing probes specific for phylogenetic groups, FISH has been used to detect, locate and enumerate various bacteria, including lactobacilli and bifidobacteria, in faecal samples [44,45]. A recent systematic study of the accessibility of the *E. coli* 16S rRNA for fluorescently labelled probes was carried out, which will be a valuable contribution to the design of 16S rRNA probes for *in situ* hybridisation [46•]. The exciting development of molecular beacons, DNA probes that fluoresce only upon hybridisation to their target sequence and possess enhanced specificity, should also have an impact on this area [47]. The discovery that enhanced specificity is a general property of conformationally restrained probes can also be a useful feature to take into consideration when designing probes, and also primers for PCR experiments [48]. Techniques to monitor the activity of individual bacteria at the single cell level in the intestinal tract are also essential for understanding their role and physiological state in their environment. Approaches including *in situ* reverse transcription PCR have been used to quantify specific mRNAs in single *Salmonella typhimurium* cells [49•], and may be applied to *in situ* analysis of probiotics in intestinal samples.

In addition to these molecular methods that allow *in situ* analysis of natural communities which can immediately be applied to human samples, several recent approaches include the use of reporter genes in intestinal bacteria. The transcriptional fusion of promoters with the luciferase genes has been proposed as a general method for studying promoter strength and physiology of bacteria in the gastrointestinal tract [50]. Furthermore, by coupling the green fluorescent protein (GFP) reporter to the *nisA* promoter, which is controlled by extracellular nisin via signal transduction [51], and subsequent introduction in *Lactobacillus plantarum* carrying the nisin sensor and regulatory genes, significant GFP production was observed upon nisin induction [52•]. These fluorescent lactobacilli are being successfully used to follow the fate of the bacteria in both *in vitro* and *in vivo* animal experiments.

Designing functional health microbes

The considerable advances in the genetic accessibility and protocols for lactobacilli [53], and the anticipated genome sequences for *Lactobacillus* strains (see OP Kuipers, this issue pp 511–516; RD Pridmore, AC Pittet, MC Zwahlen, B Mollet, unpublished data), will provide substantial opportunities for the development of strains with safe and effective health-promoting effects. Modulated gene expression and secretion systems are essential for further industrial development [53,54]. Currently, the most notable novel recombinant probiotic is a derivative of *L. johnsonii* La1. The presence of D-lactate in yoghurt following fermentation or subsequent growth of this probiotic strain in the intestinal tract poses no adverse risks to the vast majority of consumers. D-lactate-producing lactobacilli colonising the intestine, however, cause D-lactate acidosis and encephalopathy in patients with short bowel syndrome or intestinal failures. Inactivation of the single-copy D-lactate dehydrogenase gene of La1 resulted in a re-routing of pyruvate to mainly L-lactate with no D-lactate production [55]. This novel strain has the same beneficial properties as the parent probiotic but the absence of D-lactate makes it a safer alternative for specific consumers.

Other studies hint at further possibilities to design recombinant strains with novel properties that confer competitive advantages to their survival. A recent example involves colicin V, a narrow host range toxin produced by *E. coli* whose export depends on an ABC transport system that recognises a double-glycine-type leader peptide on the immature colicin V. Replacement of the colicin V leader peptide by a signal peptide from the bacteriocin divergicin A of *Carnobacterium divergens*, which is exported via the cell's general secretion pathway, allowed the expression and secretion of the Gram-negative antimicrobial in this LAB [56].

Lactobacilli and other LAB in general have a variety of properties that make them attractive candidates for oral vaccination purposes, such as a long history of safe use, ease of oral administration, low intrinsic immunogenicity and extensive industrial experience of handling [52•]. Specific strains may be selected that confer health-promoting effects and exhibit immunoadjuvant properties with the capacity to colonise the site threatened by infection. Antigens from pathogenic bacteria or viruses, including several relevant for human health, such as tetanus, rotavirus, HIV-1, and the nontoxic subunit B of *E. coli* heat labile toxin, have now been produced either intracellularly, secreted or displayed on the cell surface of various *Lactobacillus* species

[57,58]. Immune responses following mucosal administration of the studied antigens were encouraging and demonstrated that well-expressed antigens could induce systemic and local immune responses after nasal administration [52•]. Recently, a series of expression vectors were constructed that allowed secretion of human myelin basic protein by *Lactobacillus casei* into the growth medium [59]. Parenteral immunisation with this medium induced antibodies against the myelin basic protein, and it is proposed that the recombinant strain may be useful in oral tolerance induction for intervention in the autoimmune disease multiple sclerosis.

Conclusions

The growing realisation by consumers that our food profoundly influences our health has fuelled the introduction of food products with health claims such as probiotics into the market. Research to support the claims must take into account the effects on and activity of our gastro-intestinal microbiota, of which there is an estimated 10 times more than our tissue cells. Novel molecular technologies are transforming our approaches for analysis of the complex intestinal ecosystem. Although, acknowledging the strides made in elucidating the activity of pathogenic microbes in disease [60••], we can appreciate that an understanding of probiotic functionality in intestinal ecology will require continued concerted and interdisciplinary research efforts.

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