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Review

Correlation between *in vitro* and *in vivo* assays in selection of probiotics from traditional species of bacteria

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ABSTRACT

Background: *In vitro* selection tests such as exposure to low pH and bile salts, competitive exclusion of pathogens, adherence to cell lines and prokaryotic-eukaryotic co-cultures have been commonly used to predict the functional properties of **lactobacilli** and **bifidobacteria** for their use as **probiotics**. However, the **correlation** of *in vitro* results with ***in vivo*** performance remains obscure.
Scope and approach: To review the current state of evidence linking *in vitro* predictions to *in vivo* outcomes in selecting probiotic candidates and to discuss the advantages and limitations of the various assays presently available.
Key findings and conclusions: The successful use of lactobacilli and bifidobacteria as traditional probiotics is based on their occurrence in human milk, naturally fermented foods, in the gastrointestinal tract and feces of infants and adults as well as on their culturability, technological robustness and long history of safe use. The lack of standardized protocols for *in vitro* and *in vivo* studies hampers comparison of the potential of new species and strains. There is thus a need to conduct selection of potential probiotics in a more robust manner and to focus well-defined *in vitro* and *in vivo* studies to document health benefits.

1. Introduction

Strains of probiotic bacteria for use in humans have been historically selected mainly among species from the genera *Lactobacillus* and *Bifidobacterium*, commonly present in the intestinal tract and mucosal surfaces of healthy humans or in spontaneously fermented foods. For most current probiotics, their tolerance of various stresses has constituted a key criterion for strain selection, in practice, their resistance to industrial manufacturing processes or to gastrointestinal transit. However, such stress tolerance factors do not imply functionality and various *in vitro* functional selection criteria have therefore been proposed without a precise conception of their usefulness as predictors of the *in vivo* outcome.

Traditionally, once safety is established, the most commonly used selection criteria have included exposure to low pH and bile salts as a predictor of gastric resistance, studies of adherence to mucus or cell lines as indicators of "temporary gut colonization" and prokaryotic-eukaryotic co-cultures as prognostic factors for the immunomodulatory capacity of each strain. However, we are still far from understanding the true role of these criteria as predictors of *in vivo* effects. For instance, specific strains with health effects verified in properly-conducted clinical trials do not perform well in *in vitro* assays of stress tolerance (Dunne et al., 2001; Morelli, 2007). Then, debate on the usefulness of the traditional selection criteria continues.

Most traditional probiotics belong to well-known microbial groups (lactobacilli and bifidobacteria) with a long history of safe use. This has made possible a preliminary evaluation of their safety and functionality on the basis of the body of knowledge of these groups already available (now known as "core benefits"). The majority of *Bifidobacterium* spp. has been isolated from human or animal gastrointestinal samples and human milk, demonstrating

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their function as part of the normal microbiota with species composition varying between hosts (Rajilić-Stojanović and de Vos, 2014). Lactobacilli are found in many plant and animal sources and in the human gastrointestinal tract and human milk but also in many natural plant and cereal products. The species belonging to the order Lactobacillales are abundant in nature and thus suitable for gut microbiota modulation and incorporation to many food systems (Rajilić-Stojanović and de Vos, 2014).

2. Definition of probiotics revisited

The original WHO/FAO definition of probiotics was revised in 2014 by a new consensus panel, and enforcement of the original definition was proposed by introducing a grammatical modification only. The definition states that probiotics are live microorganisms that, when administered in adequate amounts, confer a health benefit on the host (Hill et al., 2014). In the referenced work, the idea of general or "core" benefits was introduced for certain *Bifidobacterium* and *Lactobacillus* species. On the basis of the currently available literature, which includes well-designed clinical trials, systematic reviews and meta-analyses, the consensus panel concurred that certain effects can be ascribed to probiotics as a general class, whereas many other effects of probiotics still remain strain-specific. *Bifidobacterium* (*adolescentis, animalis, bifidum, breve* and *longum*) and *Lactobacillus* (*acidophilus, casei, fermentum, gasseri, johnsonii, paracasei, plantarum*, *rhamnosus* and *salivarius*) are a core group of well-studied species likely to impart some general benefits (Hill et al., 2014).

However, the question still arises: how to select new probiotics with predictable safety and beneficial clinical outcomes?

3. Isolation of potentially probiotic bacteria

The genus *Lactobacillus*, which belongs to the Firmicutes phylum, is widely distributed in nature and is particularly heterogeneous, comprising over 200 recognized species and subspecies. *Bifidobacterium* belongs to the phylum Actinobacteria, and its distribution is limited to the gastrointestinal tract of mammals. So far, these two genera include the probiotics most commonly used in food and pharmaceutical preparations. Naturally occurring environments have constituted the main source for the isolation of traditional probiotics. Adaptation of microorganisms to specific environments, such as the human intestine, constitutes an opportunity and a limitation at the same time, since comparative genomics have revealed a trend to genome size reduction due to adaptation (Makarova & Koonin, 2007); (Sun et al., 2015). For instance, comparative functional genomics of vaginal *Lactobacillus* spp. have revealed a reduced genome size compared to intestinal lactobacilli and possible mechanisms for specialization to the vaginal environment (Mendes-Soares, Suzuki, Hickey, & Forney, 2014). Analyses of the significant genomic differences across LAB species may provide relevant information for specific applications reinforcing the evidence that specific traits associated with probiotic properties are still strain-dependent (Lukjancenko, Ussery, & Wassenaar, 2012). observed enrichment in the case of genes belonging to translation, ribosomal structure, post-translational modification and chaperones in the core genome of *Bifidobacterium* and *Lactobacillus*, among other genera, with the hypothesis that genes overrepresented in the core genome would mostly contribute to their probiotic or fermentative lifestyle. At the same time, a limited size genome suggests a relatively reduced potential adaptation to other environments, underlining the importance of selecting future probiotic strains from the same niche in which they are presumed to be active ie. the gastrointestinal tract, breast-milk and skin depending in the proposed application. In this context it is not surprising that most of the new probiotic microorganisms proposed for treating or preventing gut disorders are indeed inhabitants of the healthy human gut.

Human milk represents a continuous supply of commensal bacteria from the mother to the infant gut (Civardi et al., 2013; Rautava, Luoto, Salminen, & Isolauri, 2012). Human milk can be a source of new probiotics such as bifidobacteria and lactobacilli, but it also contains other microorganisms such as *Streptococcus* or *Staphylococcus*, which are in fact dominant in breast-milk and therefore represent a natural high exposure of the healthy breast-fed infant to these genera. However, natural occurrence is not a prerogative for their use as probiotics, as safety issues must be always considered. Additionally, lactobacilli occur in many traditional or artisanal fermented foods (Farnworth, 2008), together with other lactic acid bacteria of potential probiotic interest. However, of all the possible sources of potential probiotic bacteria mentioned, breast-milk is particularly attractive (Sánchez, Margolles, Ruas-Madiedo, de los Reyes-Gavilan, Gueimonde, 2010; Arboleya et al., 2011a). Microbes present in breast-milk might be possibly of various origins. They may derive from the mother's intestinal microbiota through the enteromammary circulation, from the breast skin or from the infant oral cavity by cross-contamination during suckling (Latuga, Stuebe, & Seed, 2014; Rautava et al., 2012). A more recent study also suggests the possible presence of a distinct resident microbiota in the mammary gland even in women without a history of lactation (Urbaniak et al., 2016).

It may be hypothesized that bifidobacteria and lactobacilli present in breast-milk display putative functional properties, making them potential candidates for the development of probiotic cultures especially for infants and children. However, for a strain to be marketed it must also evince certain technological features and resistance to the transit through the gastrointestinal tract. Zacarías et al. (2017) isolatedd three strains of bifidobacteria from human breast-milk (*Bifidobacterium animalis* subsp. *lactis* INL1, *Bifidobacterium longum* LM7a and *Bifidobacterium dentium* LM8a'). However, only *B. animalis* subsp. *lactis* INL1 displayed resistance to freeze-drying, to long-term storage and to simulated gastric digestion, suggesting that not all isolates from breast-milk, although putatively functional, might possess characteristics enabling them to be produced in a large scale and exploited for commercial purposes.

Reports accumulated during the last 30 years in favor of *Lactobacillus* and *Bifidobacterium* as genera with probiotic properties have been revisited and assessed through meta-analyses. The basic tenet of a meta-analysis is that there is a common truth behind all conceptually similar scientific studies. In relation to lactobacilli and bifidobacteria, several meta-analysis studies demonstrate that they are effective, always in a strain-dependent manner, against different microbiota-associated diseases. Such disorders include pediatric antibiotic-associated diarrhea (Goldenberg et al., 2015), deviated blood lipid concentrations (Cho & Kim, 2015), allergies (Cuello-Garcia et al., 2015), overweight and obesity (Zhang, Wu, & Fei, 2016), antibiotic-associated diarrhea in adults (Jafarnejad et al., 2016) or *Clostridium difficile*-associated diarrhea (Lau & Chamberlain, 2016). In addition, medical societies and food based nutrition guidelines recommend probiotics for many gut disorders and beyond (Ebner, Smug, Kneifel, Salminen, & Sanders, 2014); (Smug, Salminen, Sanders, & Ebner, 2014). In this sense, the isolation of new probiotic candidates from the genera *Lactobacillus* and *Bifidobacterium*, with a long tradition of safe use in humans, still remains promising and is encouraged by meta-analysis supported evidence.

4. Requirements for safety and efficacy: the European approach

Identification and characterization of novel species for probiotic use may pose new risks, such as their infective potential and those related to interference with the host metabolism. Up to now infective capacity and resistance to antibiotics have comprised the main focus in traditional safety criteria. In the European Union the novel food legislation imposes specific requirements also on novel microbes (EFSA 2016).

According to the EU regulation on novel foods (2015/2283), the main objective is to guarantee a high level of protection of human health and consumer interests. New probiotics should thus clearly demonstrate safety and health benefits (Kumar et al., 2015) The information required for a product containing novel probiotics should include: 1) specification of the strain, including origin and metabolism and verification of the species and strain identity according to internationally accepted methods; 2) history of the organism used as source of the novel probiotic; 3) production process; 4) potential for toxigenicity and pathogenicity; and 5) allergenic potential (EFSA 2016). Safety assessment could also be based on the microbial species having previously been assessed in the Qualified Presumption of Safety (QPS) system by the European Food Safety Authority (EFSA). Others species not belonging to the group possessing QPS status need to be extensively evaluated. Probiotic status assessment requires demonstration by human studies and a scientific and technical guidance in presenting applications for health claims on food has been provided by the EFSA (Reg. EC 1924/2006). The EFSA has rejected all health claim applications on probiotics since 2008, which reflects the need for more scientific evidence and well-designed human intervention studies.

Contrary to the European standard, Canada has a positive list of species that can be marketed as probiotics. This list represents a core group of well-studied species likely to contribute to a healthy gut microbiota (Hill et al., 2014).

5. Traditional function assessment criteria. Limitations and challenges

5.1. Acid and bile tolerance

Tolerance to gastrointestinal transit is a functional selection criterion commonly used in probiotic research. Ingested microorganisms have to cope with a particularly acidic gastric environment, to be then exposed to high concentrations of bile salts in the first portion of the small intestine. Formerly, *in vitro* tolerance has been assessed as resistance to gastric acidity and bile salts as two independent assays with pure cultures of the potential probiotic strain exposed to low pH hydrochloric acid solutions containing pepsin and sodium chloride. Thereafter, in a separate assay, a fresh culture of the same strain would be exposed to bovine or porcine bile salts solutions. This approach does not account for the successive exposure to gastric acidity followed by bile salts and intestinal secretions *in vivo*. The use of bovine or porcine bile salts is also a matter of debate. Whereas the majority of studies has been conducted using bovine bile salts, their porcine counterpart displays a chemical composition much closer to that of human bile salts (Begley, Gahan, & Hill, 2005) but more inhibitory when compared to the former (Burns et al., 2010). Few studies used human bile salts for the screening and selection of probiotics candidates (Dunne et al., 2001; Thornton, 1996), may be due to the difficulties for their obtention compared to dehydrated commercial animal-counterparts. In the majority of studies, cells have been exposed to a constant low pH, ranging from 1.5 to 3. However, *in vivo*, the pH of the stomach displays a gradual increase of acidity (pH diminution) throughout the digestive process (Blanquet et al., 2004). Additionally, gastric emptying is a dynamic process with a rate which depends on age (Bhutto & Morley, 2008) and on the consistency of the food consumed (Martinez et al., 2011). For instance (Berrada, Lemeland, Laroche, Thouvenot, & Piaia, 1991), showed that almost 30% of an ingested fermented milk containing bifidobacteria, left the stomach after 20 min. By that time, the pH of the stomach may range from 2.8 to 4.2, depending on whether it is a fast or a slow passage (Blanquet et al., 2004) or higher than 3 (Mainville, Arcand, & Farnworth, 2005). Whereas in *in vitro* assays all microbial cells are exposed to the same pH for the same time span, *in vivo*, gastric emptying takes place while pH drops and not all cells are exposed to the same pH for the same period of time. Thus a gradient of cells stressed to different levels is expected to enter the small intestine. In this sense, *in vitro* static experiments might be much more inhibitory than a real gastric digestion process. In order to better reproduce the gradual drop of pH which takes place in the stomach during digestion (Burns, Lafferrierer, Vinderola, & Reinheimer, 2014), performed a gradual and manual pH reduction (in batch) in *in vitro* experiments to study the influence of dairy practices on the capacity of probiotic bacteria to overcome simulated gastric digestion.

5.2. Eukaryotic cells for the study of adherence

Adherence of probiotics to the intestinal epithelium may contribute to their persistence on the mucosal surface. However, colonization of the gut by orally administered probiotics appears to be only temporary. Numerous studies show that probiotic elimination in feces follows the interruption of their oral administration. Nevertheless, transient but no permanent colonization was observed, even by early administration of probiotics to the infant gut, after intake of *Lactobacillus rhamnosus* GG for 6 months after birth (Gueimonde, Kalliomäki, Isolauri, & Salminen, 2006). It appears that adhesion of probiotics, at least transiently, is necessary for their interaction with the gut epithelium and with the lymphoid tissue. For example, such ability was found necessary for the induction of Th17 cells (Atarashi et al., 2015).

Bacterial cell surface hydrophobicity has been used as a *in vitro* predictor of the interaction of microbes with the host intestinal epithelial cells (Pérez et al., 1998); (Kotzamanidis, Kourelis, Litopoulou-Tzanetaki, Tzanetakis, & Yiangou, 2010; Kouidhi, Zmantar, Hentati, & Bakhrouf, 2010). (Burns et al., 2008) (Burns, Reinheimer, & Vinderola, 2011); correlated a higher hydrophobicity with enhanced immunostimulation using a low-hydrophobic bile-salt resistant derivative obtained from *Lactobacillus delbrueckii* subsp. *lactis* 200 by progressive exposure to growing amounts of bovine bile salts (Frece, Kos, Beganovic, Vukovic, & Suskovic, 2005). correlated adhesion to porcine ileal epithelial cells *in vitro* with adhesion to mouse ileal epithelial cells *in vivo*. The wild-type *Lactobacillus crispatus* M247, which displays cell aggregation phenotype, and its spontaneous non-aggregating mutant, *L. crispatus* MU5, were compared for their *in vitro* adhesion to human ileal mucus and to Caco2 cells, and for their *in vivo* colonization and adhesion potential, with colonoscopy patients as volunteers, in feeding trials. The wild-type strain adhered better to mucus or to Caco2 cells than did the mutant (Voltan et al., 2007). assessed the effects of the strains on gut colonization and immune modulation. After 14 days of supplementation, the aggregating strain *L. crispatus* M247 was recovered from feces at higher levels and was found to increase Toll-like receptor 2 (TLR2) mRNA levels, and to reduce TLR4 mRNA and protein levels in the colonic mucosa, whilst the non-aggregating MU5 strain was ineffective. Additionally, daily administration of *L. crispatus* M247, but not *L. crispatus* MU5, reduced the severity of DSS colitis in an animal model in a

dose-dependent manner (Castagliuolo et al., 2005). Although several studies demonstrate the *in vitro* correlation of hydrophobicity to *in vivo* adhesion, environmental conditions may alter hydrophobicity (Borecká-Melkusová & Bujdáková, 2008) (Högfors-Rönnholm & Wiklund, 2010); and caution should be taken with *in vitro* prediction of adhesion through this parameter.

Despite their widespread use, Caco-2 and HT-29 are tumor cell lines and are far from perfect as *in vitro* models for studying adhesion and the mechanisms of action of probiotics. They possess mixed large- and small-bowel phenotype and they have different sugar composition on the cell surface compared to normal cells. It is therefore important to note that results on the attachment ability of probiotic bacteria to epithelial cells cultivated as monolayers on plastic surfaces may not always give the real picture about adhesion ability *in vivo*. For example, *Lactobacillus rhamnosus* GG or *Lactobacillus casei* Shirota were found to adhere poorly to Caco-2 cells grown on plastic surfaces, whereas in the normal intestinal functional cell model, the same strains were found to bind almost to 50% (Cencič & Langerholc, 2010). In the context of the *in vitro* to *in vivo* correlation of cell adhesion (Sugimura, Hagi, & Hoshino, 2011), studied the adhesion of 8 probiotic candidates for fish using intestinal mucus derived from healthy carps. They reported that two strains displayed adhesion values higher than 15%, whereas the remainder displayed adhesion values of less than 8%. The strains were administered to carps for 12 days whereafter administration was interrupted. The authors reported that only the two strains which performed well in *in vitro* adhesion assays were observed to be present in the intestinal content of carps after 20 days from discontinuation of their administration.

The specific adhesion model used may markedly affect the results obtained. It is quite common to find strains showing, for instance, a high adhesion to the model of epithelial cell lines, but low adhesion in other study systems such as intestinal mucus (Arboleya et al., 2011b). Adhesion can be also assessed using human intestinal tissue pieces with local microbiota to mimic conditions in different parts of the intestinal tract (Ouwehand et al., 2002). Such an approach could be used for selecting site-specific or function specific, even disease-specific probiotics. However, these examples are scarce and in most cases the *in vitro* models do not consider the presence of the normal microbiota.

5.3. Immunomodulative properties

Besides adherence studies, cell lines have been used to study the immunostimulative capacity of potential probiotic strains. The predominant common type in the epithelial layer is the enterocyte. However, other cell types can be found in this tissue, including entero-endocrine, goblet, Paneth, M and cup cells (Cencič & Langerholc, 2010). Below the gut epithelia, the gut-associated lymphoid tissue (GALT) is the largest collection of lymphoid tissues in the body, consisting of both organized lymphoid tissues such as mesenteric lymph nodes (MLN) and Peyer's patches (PP), and more diffusely scattered lymphocytes in the intestinal lamina propria (LP), including large numbers of IgA + plasmablasts (Forchielli & Walker, 2005). Other immune cells such as dendritic cells and macropages are also found within PP and the lamina propria (Pabst & Rothkötter, 2006). One possible limitation attending the use of epithelial cell lines (Caco-2, HeLa, HT-29, CaSki) for the study of the gut immune modulating capacity of probiotic candidates is that these cell lines behave as isolated cells when compared to epithelial cells in the gut, which is a much more complex system with many interacting cell types and also the presence of the microbiota. In this sense, epithelial cell lines may be considered an extremely simplified version of the gut, with a possibly limited or biased response when compared to a normal epithelial cell inserted in the complex system of the gut. Another possible limitation of most cell lines is that their response may be conditioned by their origin. The Caco-2 cell line, originating from a colon carcinoma, is a widely used *in vitro* model for the small-intestinal epithelium. Cancer cells have an altered metabolism, making it difficult to infer whether they in fact represent the tissue from which they are derived. Proteomic assessment has been used to compare the protein expression pattern of Caco-2 cells with intestinal epithelial cells from the human small intestine. Several biologically significant proteins were expressed at comparable levels in Caco-2 cells and small-intestinal scrapings, indicating the applicability of this *in vitro* model. Caco-2 cells, however, appear to over-express as well as to under-express certain proteins, a circumstance which needs to be considered when using this cell line. Hence, care should be taken to prevent misinterpretation of *in vitro* obtained findings when translating them to *in vivo* situations (Lenaerts, Bouwman, Lamers, Renes, & Mariman, 2007). Fortunately, epithelial cell lines immortalized from normal (non-carcinogenic) epithelial tissues have also been reported to be available (Cencič & Langerholc, 2010).

Primary cell cultures of the intestine involve the culture, under controlled conditions, of a piece of tissue or explant. Tissue pieces have been successfully used (Ouwehand et al., 2002). The advantage here is that the response may be closer to that of normal tissue compared to immortalized cell lines, but the primary cultures tend to have a very limited lifespan. Primary cultures of the small intestine have been used to study the interaction capacity of *L. casei* CRL 431 and *L. helveticus* R389 with the gut. In one assay, different concentrations of viable and non-viable lactobacilli were co-incubated with primary cultures of the small intestine of mice for IL-6 determination by ELISA in the supernatant. In another assay, mice received orally both strains as viable or non-viable cultures and the small intestine was recovered, treated for primary culture and left to incubate for IL-6 secretion to the supernatant. A qualitative correlation was found between both assays (*in vitro* stimulation of primary cultures of the small intestine versus *ex vivo* incubation of the primary cultures of small intestine of mice stimulated *in vivo*), especially when compared the response of non-viable versus viable cells (Vinderola, Matar, & Perdigón, 2005).

Whereas the co-culture of eukaryotic cells, either epithelial or immune, with potentially probiotic microorganisms, with subsequent determination of the cytokines produced, is a test traditionally used in identifying immunomodulating strains, and such tests have been employed as predictors of the immunomodulating capacity *in vivo*, their main limitation is the lack of the response derived from other immune cell linages. The simultaneous presence of a variety of immune cells and a competing and complex microbiota is almost impossible to reproduce unless explants of gastrointestinal tissue are used (Randall, Turton, & Foster, 2011). The development of indefinitely propagating human derived “mini-guts” may offer new perspectives for probiotic research providing a novel model of intestinal organoids and enteroids, as well as colonoids, to study the interactions between probiotics, pathogens and the host. This development was recently reviewed by (In et al., 2016) and offers new ways of characterizing future probiotics.

5.4. Intestinal transit

Several different *in vitro* approaches have been used, with very different methodologies applied in simulating gastrointestinal tract transit (Burns et al., 2014). The buffering capability of the food matrix and the resulting enhanced probiotic survival along the gastrointestinal tract has often been neglected or underestimated. Moreover, the gastrointestinal stress (acid, bile) would appear to act as an intestinal signal triggering the expression of genes related

to survival in the intestinal environment (Gueimonde, Garrigues, van Sinderen, de los Reyes-Gavilán, & Margolles, 2009; Ruas-Madiedo, Gueimonde, Arigoni, de los Reyes-Gavilán, & Margolles, 2009). Acid and bile exposure induce changes on the bacterial surface, which may affect other properties such as adhesion to the gut epithelium or the immunomodulating capacity of bifidobacteria (de los Reyes-Gavilan et al., 2011). Another example is provided by *Lactobacillus plantarum* WCFS1 and its host interactions; in this strain *in vitro* gastric stress was found to increase the expression of adhesion factors and several genes involved in survival and interaction with the host were found to be upregulated during *in vivo* gastrointestinal transit (van den Nieuwboer, van Hemert, Claassen, & de Vos, 2016). Such considerations may throw doubt on the adequacy of laboratory growth conditions for the functional screening of probiotics, when acting in the human gut. Bacterial physiology would appear to be different in cells grown in laboratory media compared to cells exposed to the gastrointestinal environment (Sánchez, Ruiz, Gueimonde, Ruas-Madiedo, & Margolles, 2013), yet some properties may vary significantly due to technological processes or to spontaneous mutations in the strains used (Douillard et al., 2014; Grześkowiak, Isolauri, Salminen, & Gueimonde, 2011).

5.5. Complete intestinal models

More sophisticated and reliable computer-controlled *in vitro* dynamic settings for the study of resistance to gastrointestinal digestion were reported as well (Blanquet et al., 2004; Mainville et al., 2005). A study was conducted to validate a dynamic model of the stomach and small intestine to quantify the survival of lactic acid bacteria and to assess the influence of gastrointestinal secretions (Marteau, Minekus, Havenaar, & Huis in't Veld, 1997). The survival of single strains of *Bifidobacterium bifidum*, *Lactobacillus acidophilus*, *Lactobacillus bulgaricus* and *Streptococcus thermophilus* was measured under physiological conditions and compared with data obtained from humans. No significant differences were found between the *in vitro* and *in vivo* data, indicating that the model is of predictive value for the survival of these bacteria in humans. 'SHIME' is an acronym for the Simulator of the Human Intestinal Microbial Ecosystem and since 2010, the name has been jointly registered by ProDigest and Ghent University. The SHIME® is one of the few gut models that mimics the entire gastrointestinal tract incorporating stomach, small intestine and different colon regions (Van de Wiele, Van den Abbeele, Ossieur, Possemiers, & Marzorati, 2016) and its *in vitro* to *in vivo* correlation has been validated through several studies (Molly, Vandewoestyne, Desmet, & Verstraete, 1994; Possemiers et al., 2006; Van den Abbeele et al., 2013).

6. Correlation between *in vitro* and *in vivo* (animal) studies

Foligne and associates (Foligne et al., 2007) demonstrated that the *in vivo* protective capacity of lactic acid bacteria and bifidobacteria could be predicted based on their cytokine profile established *in vitro*. A PBMC-based assay was used as primary indicator to narrow down the number of candidate strains to be tested in murine models for their anti-inflammatory potential. In the study in question, the authors aimed to develop a simple and standardized *in vitro* test allowing preliminary classification of candidate probiotic strains according to their immune modulation capacity, which would be predictive of their *in vivo* effect in a mouse model of TNBS-induced colitis. To this end, thirteen lactic acid bacteria and bifidobacterial strains were ranked with reference to the IL10/IL12 cytokine ratio induced on human PBMCs, and their protective effect further assessed against TNBS-induced colitis in mice. In both *in vitro* and *in vivo* results, it was observed that strains displaying the highest *in vitro* anti-inflammatory profile (a high IL-10/IL-12 ratio) were most protective in the *in vivo* colitis model. Although this link could not be expressed as an "exactly linear" association between the percentage of protection and IL-10/IL-12 ratio, it was found to be highly significant using the Spearman rank correlation coefficient.

Another example of a good correlation between *in vitro* findings and *in vivo* outcomes was observed in a study reported by Grangette et al. (2005). A wild-type and a mutant strain of *L. plantarum* were used, the latter displaying an impaired capacity to synthesize teichoic acids. These acids are among the main immunostimulatory (proinflammatory) components in pathogenic Gram-positive bacteria. Less proinflammatory activity was observed for the mutant in co-culture with PBMC or monocytes, compared to the wild type strain, and this correlated well with a higher efficiency in controlling inflammation in a murine model of colitis.

The mechanisms of action of probiotics in the gut are most probably multi-factorial, involving a variety of effector signals, cell types and receptors. Hence, probiotic strains may differ in their ability to trigger these signals in terms of both immunocompetent and intestinal epithelial cells (Foligne et al., 2007). Prevention of enteropathogen infections by probiotic administration may be mediated by immune and/or non-immune mechanisms. The former involve probiotic antagonism to intestinal pathogens mediated by excreted inhibitory compounds. For instance, it is conceivable that *Lactobacillus* strains may moderate colitis by other non-immune related modes of action, for example by acting on barrier integrity or influencing the oxidative pathway (Foligne et al., 2007). The well-diffusion inhibition assay of probiotic candidates against food pathogens is widely used in screening for lactic acid bacteria and bifidobacteria isolates in order to find possible strains controlling pathogens by direct antagonism. In many cases, isolates displaying *in vitro* inhibitory activity against classic food pathogens (mainly *Salmonella*) are used in the well-established model of murine infection. Murine models of systemic salmonellosis have been set up to understand the pathogenesis of typhoid fever, using different strains of the closely related species *Salmonella enterica* serovar Typhimurium. The mouse model is widely used not only to study the mechanisms underlying the pathogenesis, but also to study the capability of probiotic bacteria in the prevention or treatment of enteric infections caused by *Salmonella* (Zacarías, Reinheimer, Forzani, Grangette, & Vinderola, 2014).

Probiotics have evinced a significant potential as preventive or therapeutic options for a variety of intestinal conditions, but the mechanisms responsible for these effects have not been fully elucidated. Several important modes of action underlying the antagonistic effects of probiotics on various microorganisms include modification of the gut microbiota, competitive adherence to the mucosa and epithelium, strengthening of the gut epithelial barrier and modulation of the immune system to confer an advantage on the host (Bermudez-Brito, Plaza-Díaz, Muñoz-Quezada, Gómez-Llorente, & Gil, 2012).

Problems related to the high complexity of the human gut environment in the study of probiotic-host interaction could be partially solved with the use of gnotobiotic animals, with the advantage that in these models it is possible to combine the metabolic activities of a whole organism and an intestinal microbiota of known composition (Becker, Kunath, Loh, & Blaut, 2011). In addition, the use of gnotobiotic mice allows the selection of a microbiota environment to test the probiotic, with the possibility to employ a healthy or an altered human microbiota. Issues due to inter-individual responses were also addressed in an *in vitro* gut model (Geirnaert et al., 2015).

7. Outcomes of human clinical trials compared to *in vitro* prediction

The correlation of *in vitro* assays with results of human clinical trials was also studied. The PBMC IL-10/IL-12 ratio used to predict the anti-inflammatory potential of probiotic candidates in mice has also been used to determine the immunomodulatory properties of probiotics in a clinical study of irritable bowel syndrome (IBS) (O'Mahony et al., 2005). Concomitant with alleviation of the main IBS symptoms, the authors showed normalization of the PBMC IL-10/IL-12 ratio in patients receiving probiotics versus placebo, showing that *in vivo* probiotic efficiency can be in line with that expected from the *in vitro* assays (O'Mahony et al., 2005). However (Flinterman et al., 2007), reported a different immunomodulatory potential *in vitro* versus *ex vivo* upon oral administration of probiotics in children with food allergy. In the referenced trial, thirteen children were enrolled in a study where 7 of them received 10^9 CFU/day for 3 months of a mixture of *Lactobacillus acidophilus* W55, *L. casei* W56, *L. salivarius* W57, *Lactococcus lactis* W58, *Bifidobacterium infantis* W52, *B. lactis* W18 and *B. longum* W51 (Winclove Bio Industries, Amsterdam, The Netherlands), whereas 6 children received a placebo. At baseline, and after 1 and 3 months, peripheral blood mononuclear cells were stimulated with crude peanut extract, anti-CD3, or anti-CD40 and IL-4 in the presence (*in vitro* response, 10^5 CFU/mL) or absence (*ex vivo* response) of probiotics. The proliferation and production of IFN-γ, IL-5, IL-13, IL-10, TNF-α, IL-6 and IgE were analyzed. The *in vitro* co-culture of probiotics with peripheral blood mononuclear cell cultures resulted in enhanced proliferation and production of IFN-γ, IL-10 and TNF-α. After oral treatment, proliferation in the presence of probiotics increased, whereas *in vitro* IgE production decreased in the probiotic group, compared to baseline. The *ex vivo* production of IL-10, TNF-α and IL-6 tended to decrease. Th1 and Th2 cytokines were not altered. The authors concluded that probiotics enhanced the production of Th1 and regulatory cytokines *in vitro* whereas oral administration of the same product resulted in a slightly decreased *ex vivo* production of IL-10, TNF-α and IL-6, suggesting that probiotics have a different potential to modulate the immune response *in vitro* versus *ex vivo*. Again, the different dynamics of probiotic-eukaryotic cell interaction may interfere with the correlation, while in *in vitro* assays probiotics are co-cultured for less than 24 h with a unique eukaryotic cell type, in *in vivo* assays probiotics are administered for a longer period of time and they interact with different populations of eukaryotic immune cells on their transit along the intestine. More sophisticated *in vitro* models that may account for this dynamics, today neglected by available static models, is still an enormous challenge for researchers in refining and establishing more accurate predictive *in vitro* models.

Marked inter-individual variability in intestinal microbiota composition may also explain the relatively limited correlation observed between clinical studies and *in vitro* data. Such microbiota variability is often not represented in the *in vitro* screening procedures applied to probiotics selection. This limited variability may also hamper the predictive capability of such *in vitro* models.

8. Importance of *in vitro* assays and future needs

Targeted assays must be performed in order to predict (*in vitro*) and then to confirm (*in vivo*) the functional traits of a strain which may lead to health benefits in human subjects and subsequently to a potential health claim approved by the regulatory authorities. If an isolate displays properties acceptable with regard to technological handling (biomass production, resistance to freezing or dehydration and survival to storage as a supplement or into a given food), it is then worth to perform *in vitro* and *in vivo* biological and functional assays. Taken together, even with their biases, *in vitro* assays are nonetheless still of value. They are suitable for preliminary screening of isolates to narrow down the number of strains to undergo more sophisticated studies, or in order to determine the protective effect of a food matrix or the choice of a suitable protectant for freeze-drying (Vinderola et al., 2012), among other applications. There is a lack of consensus as to what conditions to use when assessing *in vitro* the resistance to simulated gastrointestinal digestion (Burns et al., 2014). In order to produce more comparable data about probiotic resistance to *in vitro* static gastrointestinal digestion, a standardized protocol is still needed and might be welcomed by the scientific community, as happened for the study of food macronutrient digestion (Minekus et al., 2014). This protocol, with international consensus, could also be used for the characterization of probiotic bacteria with a view to producing more comparable *in vitro* data.

9. Omics-based screening

Since omics-based technologies are becoming more and more accessible, they may replace traditional screening methods in the future. Genomic sequencing could aid to detect strains possessing desirable and undesirable traits also constituting the best method for strain identification, therefore very likely becoming a standard procedure in the next years. High throughput transcriptomics and proteomics will allow a fast screening of functional properties, facilitating also the design of more efficient *in vitro* and *in vivo* tests. Likewise, omics are allowing understanding microbiome complexity and have showed a vast field of potential new probiotics candidates.

10. Conclusions

For more than 30 years now researchers have been isolating and characterizing candidate probiotics, mainly from the genera *Lactobacillus* and *Bifidobacteria*. The success of their use in food and pharmaceutical products has been based on a combination of factors such as occurrence, abundance and culturability in human breast milk and faeces (bifidobacteria and lactobacilli) and in naturally fermented foods (mainly lactobacilli). Technological robustness and the European Union QPS system paved the way for their massive use by the food and pharma industries. Several successful probiotics have been isolated using the presented criteria. Major findings of the *in vitro* to *in vivo* correlation of studies reviewed in this work are shown in Table 1. Some common *in vitro* tests in the selection of potential probiotic strains used globally include evaluation of resistance to gastrointestinal digestion, adhesion to cell lines and prokaryotic-eukaryotic co-culture for immunomodulation. Some concerns about these tests include the fact that changes in gastric pH and gastric emptying along digestion are difficult to mimic in simple *in vitro* tests, unless more sophisticated approaches (SHIME, for example) are used. Prokaryotic-eukaryotic co-culture is a simplified model compared with the complex system of the gut. Additionally, tumorigenic cell lines have an altered metabolism and different surface sugar composition than normal cells, which can result in a different response compared to a healthy epithelium, but could be replaced by patient specific “mini-guts”. Anyway, for the characterization of novel probiotics, a fair correlation has been demonstrated, in a case-dependent manner, through *in vitro* tests of adhesion to the gut epithelium and immune stimulation along with animal models of

Table 1
Correlation between *in vitro* and *in vivo* studies for specific probiotic functions.

Function	Correlation *in vitro* and *in vivo*	Potential improvements
Resistance to gastrointestinal digestion	Differences in the use of bovine or porcine bile salts (porcine has a chemical composition more similar to human bile salts) Changes in gastric pH and gastric emptying are difficult to mimic. *In vitro* static experiments might be much inhibitory than real gastric digestion.	Agreement on a standardized protocol to study the gastrointestinal resistance of probiotic bacteria
Adhesion to gut epithelium	Variable correlation depending on the bacterial strain and the cell line or mucus employed. Tumorigenic cell lines have an altered metabolism and different surface sugar composition than normal cells. Prokaryotic-eukaryotic co-culture is a very simplified model compared with the complex system of the gut.	Use of epithelial cell lines from normal epithelial tissues and primary cultures from small intestine explants, use of intestinal tissue segments
Immunomodulation	Case-dependant correlation between *in vivo* protective capacity of lactic acid bacteria and bifidobacteria and their cytokine profile established *in vitro*. Limitations in the time of exposure and the type of eukaryotic cells employed in *in vitro* assays.	Development of more sophisticated *in vitro* models.

prevention of enteric infections and inflammation or human trials on physiological benefits or disease risk reduction. However, the lack of standardized protocols hinders the comparison of the potential of different strains. Such protocols are needed to render the selection of potential future probiotics more rational and more predictable for human intervention studies.

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