Curr. Issues Intest. Microbiol. (2000) 1(2): 59-67.

In Vitro Selection of Probiotic Lactobacilli: A Critical Appraisal

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Abstract

The problem of choosing criteria for the *in vitro* selection of lactobacilli to be used as health-promoting, probiotic ingredients, in food and pharmaceutical preparations, was apparent even in the original works of Metchnikoff, who first proposed the therapeutic use of these bacteria. In the last decade, a consensus has been reached by scientists on some criteria, with particular attention being paid to the ecological origin of the bacteria, their tolerance to the hostile conditions of the stomach and the small intestine, and their ability to adhere to intestinal surfaces. Even though these criteria have been used to select probiotic lactobacilli, some doubts still remain about the real value of these criteria. A critical rethinking of selection criteria seems necessary in order to improve the process of developing better probiotics.

Time for Some New Thinking

The possibility that the ingestion of some selected bacteria may beneficially influence the gastrointestinal tract of humans was proposed by Elie Metchnikoff, the Russian-born Nobel Prize winner, working at the Pasteur Institute at the beginning of the 20th century, who noticed: " …the different susceptibilities of people to the harmful action of microbes and their products. Some can swallow without any evil result a quantity of microbes which in the case of other individuals would produce a fatal attack of cholera. Everything depends upon the resistance offered to the microbes by the invaded organism. " (Metchnikoff, 1907, p. 164). He also stated that "The dependence of the intestinal microbes on the food makes it possible to adopt measures to modify the flora in our bodies and to replace the harmful microbes by useful microbes" (Metchnikoff, 1907, p. 162).

This sentence describes in a clear way the "probiotic concept"; the use of health-promoting bacteria able to exert a positive impact on the intestinal microflora. The word 'probiotics' was coined in the 1960s to name substances produced by microorganisms which promoted the growth of other microorganisms (Lilley and Stillwell, 1965). More recently, the meaning of this word has been refined several times and today a widely accepted definition of probiotics is: 'live microorganisms, which when consumed in adequate amounts, confer a health effect on the host' (Guarner and Schaafsma, 1998). If the complexity of the intestinal ecosystem, inhabited by more than 400 bacterial species and divided into several ecological niches, is taken in consideration, it becomes clear that the selection of the bacteria to be used as a food additive or a biotherapeutic agent (Elmer *et al.,* 1996) is not a simple task (for a review on the complexity of the intestinal flora and recent advances in this field see Tannock, 1999). It is also unlikely that each single strain belonging to one species possesses all of the characteristics that will make it a suitable probiotic. Thus strain-specific criteria have been designed, with special attention to *in vitro* assays, which have been used to perform a preliminary selection. This is followed by *in vivo* studies in which healthy volunteers are dosed with strain(s) that performed well *in vitro*.

The *in vitro* selection studies are therefore relevant to the entire selection process that follows and obviously need to be based on a sound scientific foundation. In 1991, a project focused on the establishment of selection criteria for bacterial strains to be incorporated into dairy foods was funded by the European Union, and it was, as far as I am aware, the first European attempt to obtain a consensus on procedures to be used for such a selection (Morelli, 1994). In the meantime, several authors have also published their own list of criteria to be used for a preliminary screening of potential probiotic lactobacilli (Marteau and Rambaud, 1993; Huius int'Veld and Shortt, 1996; Salminen *et al.,* 1996a; Tannock, 1997; Brassart *et al.,* 1998; Charteris *et al.,* 1998a; Klaenhammer and Kullen, 1999; Dunne *et al.,* 1999) From these publications, it appears that a kind of general agreement among scientist has been reached, at least in general terms, on the properties that a strain must have in order to be further tested for human probiotic use:

- it must be of human origin
- it must survive during gastric transit
- it has to tolerate bile salts
- it has to adhere to gut epithelial tissue.

The first criterion is based on ecological reasons, and takes into consideration the original habitat of the bacteria to be selected for probiotic use. Strains belonging to bacterial species which are generally present into the intestinal flora of the animal species (*i.e.* humans) which is to be targeted have been generally selected by authors, who assume that these bacteria have a better chance of out-competing resident bacteria and of establishing at a numerically significant level in their new host. The following two criteria are focused on the assessment of the potential of one putative probiotic strain to overcome barriers which are present in the upper part of the gut, namely the gastric environment and the action of bile salts in the upper part of the intestine. The final criterion has its rationale in the need for bacteria to counter the flow of digesta, by sticking

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to intestinal surfaces. As it is difficult to obtain samples of human intestinal epithelia, this criterion has been translated, for *in vitro* testing, into assessment of adhesion to human derived, cultured cell lines.

These criteria have been used to select strains that have been successfully tested *in vivo* and are nowadays the active ingredients of probiotic products that have been proven to perform well for the well-being of consumers. However, I feel that problems caused by the selection procedure of probiotic lactobacilli, that were present from the very beginning of the history of probiotics (see the following section), are not yet fully resolved and more research effort is needed. This paper is intended to describe the need to critically rethink the criteria for selecting probiotic strains.

In the Beginning There Was Great Intuition, but Also Confusion

The problem of strain selection was apparent even in the work of Metchnikoff. In chapter five (Part III) of his frequently cited, but probably rarely read, book, Metchnikoff reviewed the pros and cons of various fermented dairy products. Then he drew attention to a bacterium previously isolated from Bulgarian yoghurts by a Swiss scientist who later studied at the Pasteur Institute. This strain was the most active bacillus in causing souring of milk and, even before Metchnikoff's studies, was already used in experiments with human volunteers. This bacterium was named the 'Bulgarian Bacillus'.

It is probably a mistake to identify this bacterium with the species named, in modern times, *L. bulgaricus* or *L. delbrueckii* subsp. *bulgaricus* and used for yogurt production. The original description by Metchnikoff deals with a bacterium able to produce 25 g/l of lactic acid when grown in milk. This high level of acidity is not typical of the current *'bulgaricus*' strains and more closely resembles the values recorded for *L. helveticus* strains (Kandler and Weiss, 1986).

In addition, in international culture collections (ATCC cat. N. 521 = CIP 76. 19 =JCM 1003 = NCFB 87 = NCIB 2889= DSM 11445), there is only one strain identified as "one of the original Metchnikoff strains". It was deposited as *Lactobacillus jugurti*, a species nowadays recognised as a biotype of L. *helveticus* (Dellaglio *et al.,* 1973; Kandler and Weiss, 1986). This strain however, "also gives a disagreeable taste of tallow" and Metchnikoff suggested associating it with another lactic acid bacterium, the so called 'paralactic bacillus' (whose real taxonomic position is unclear), to obtain a more pleasant flavour. Metchnikoff supported the assumption of Dr. Cohendy, another scientist at the Pasteur Institute, who stated that the Bulgarian Bacillus was "able to take its place in the intestinal flora of man". It is now clear, however, that species of lactobacilli inhabiting the human intestine are different from those used to produce fermented milk (Klein *et al.,* 1998). These historical observations show that difficulties in selecting probiotic bacteria were present from the origin of the probiotic concept.

These historically based problems could also, at least partially, explain the inconsistent results often reported by scientific studies and the scepticism about probiotics showed by many members of the scientific community (Tannock, 1990; O'Sullivan *et al.,* 1992).

Selecting the Right Probiotic Bacterium Remains a Difficult Task

Human Origin of Strains

The misidentification of the Bulgarian Bacillus as an intestinal species probably provides a good explanation for using true intestinal isolates in products. It was already attempted by Metchnikoff who suggested that a starch-digesting microorganism could be used instead of dairy bacteria. Unfortunately, this bacterium was isolated from a dog and this origin was regarded unfavourably by the public (Bibel, 1988). Studies carried out at Yale University in the 1930's showed that the Bulgarian Bacillus was killed when it passed through the stomach (Rettger, 1935). A suitable substitute was considered to be *Lactobacillus acidophilus* which was believed to be an inhabitant of the human gut and was found to tolerate gastric conditions.

Strains described as "*acidophilus*" have been isolated not only from the intestinal tract of humans, but also from other animals, including rodents and birds. *In vitro* assays (Morishita *et al.,* 1971; Suegara *et al.,* 1975; Barrow *et al.,* 1980; Tannock *et al.,* 1982; Mayra-Makinen *et al.,* 1983), suggested that adhesion to epithelial tissues could be 'host-specific'; lactobacilli isolated from mammals adhered only to cells obtained from mammals. *In vivo* experiments however, did not produce clear-cut results. Dosing germfree mice with 19 *Lactobacillus* isolates demonstrated that, at least in these special animals, all of the strains were able to maintain substantial gastrointestinal populations for at least three weeks. Only *Lactobacillus* isolates from mice and rats, however, were able to form a continuous layer on the non-secretory epithelium of the forestomach (Lin and Savage, 1984). However, it must be noted that among these biofilm-forming strains, there was also one *L. reuteri* strain, isolated in my laboratory from calf faeces.

Further evidence of host-specificity was provided in the 1980s by taxonomic studies. The use of a genetic approach to taxonomy revealed that the "*L. acidophilus*" strains belonged to at least six different species, as determined by DNA-DNA hybridisation (Johnson, 1980; Lauer *et al.,* 1980). These species are now named *L. acidophilus, Lactobacillus crispatus, Lactobacillus gallinarum, Lactobacillus amylovorus, Lactobacillus johnsonii, Lactobacillus gasseri* (Lauer and Kandler, 1980; Cato *et al.,* 1983; Fujisawa *et al.,* 1992). As a consequence of the taxonomic reorganisation of the *acidophilus* group of species, attempts were made to correlate the new taxonomic position of the strains with their original habitat (*i.e.* the intestinal tract of humans or other animals) (Sarra *et al.,* 1980; Sarra *et al.,* 1985; Axelsson and Lingren, 1987; Pryde *et al.,* 1999; our unpublished results). The first results were obtained for strains isolated from animals (Table 1) and, even if further evidence is needed, they suggested that a host-related distribution of these six species did occur and could be used to support (at least for animals other than humans) the concept of host-specific colonisation properties. These data suggested that *L. amylovorus* and *L. crispatus* are the species-specific homofermentative lactobacilli in pigs and poultry respectively, while *L.*

Table 1. Host Specificity of the "Acidophilus" Species

Animal source	Identified as:	Updated taxonomy	References
Pigs	*L. acidophilus* A3	*L. amylovorus*	Axelsson and Lingren, 1987
Pigs	*L. amylovorus*	*L. amylovorus*	Pryde, 1999
Calves	*L. acidophilus*	*L. johnsonii*	Sarra *et al.,* 1980
Poultry	*L. acidophilus*	*L. crispatus*	Sarra *et al.,* 1985 and our unpublished results
Poultry	*L. acidophilus*	*L. johnsonii*	

johnsonii seems to be widespread in the intestinal tract of animals.

Lactobacilli isolated from human faecal samples and identified by molecular genetical methods (Morelli *et al.,* 1998; Dunne *et al.,* 1999; Song *et al.,* 1999 and 2000; Tannock *et al.,* 2000) suggested that the most frequently encountered lactobacilli belonged to species that are not commonly isolated from other animals. The *Lactobacillus casei* group of species (formed by *L. casei, Lactobacillus paracasei, Lactobacillus rhamnosus, Lactobacillus zeae*), and the obligatory homofermentative species *L. gasseri* and, to a lesser extent, by *L. crispatus* and *L. johnsonii* were represented. *Lactobacillus reuteri* seemed to be the dominant heterofermentative species present in the human gut (Table 2). Comparing Tables 1 and 2, it appears that *Lactobacillus* species that are frequently encountered in the human gut are different from those isolated from other animals. If confirmed, this observation could provide scientific support to the selection criterion: "use a strain isolated from humans as a probiotic for human beings".

Further reflection, however, suggests more prudence and some doubts. If we consider *L. johnsonii* strains, it appears that they have been found in humans, poultry and calves. How can we discriminate the human biotype (if one exists) of this species from the others? As far as I know there are not any tests able to provide an answer. *L. gasseri* is present, not only in the human gut, but also in the urogenital tract (Giorgi *et al.,* 1987; Boris *et al.,* 1997). Is it possible to distinguish the strains of these bacteria according to their original site of isolation? Is it worthwhile undertaking this scientific effort, or has the 'human origin' to be taken in a broad sense? Again, in this case, there is not an answer. If we move our attention to heterofermentative lactobacilli, *L. reuteri* strains have been isolated from humans, poultry, calves and pigs (Sarra *et al.,* 1980 and 1985; Stahl *et al.,* 1994; Pryde *et al.,* 1999). A recent paper (Jacobsen *et al.,* 1999) has shown that one *L. reuteri* strain isolated from a pig was one the best performing strains during a human colonisation trial, together with two lactobacilli isolated from human sources but belonging to the *L. rhamnosus* species. Unfortunately, there was not a comparison with a human isolate of *L. reuteri*. The question arises if it is really necessary to use 'human' *L. reuteri* isolates as probiotic additives. The *L. casei* group is widely present in dairy foods and in the human gut. From the taxonomic point of view, strains isolated from dairy products are indistinguishable from those of human origin (Ferrero *et al.,* 1996; Fitzsimons, 1999). It could be of interest, though, to investigate if it is possible to cluster these isolates according to their sources.

There have not been any other studies comparing probiotic effects of strains of lactobacilli belonging to the same species but isolated from different ecological habitats. Due to the significant presence of lactobacilli forming the non-starter flora of several dairy products (*e.g.* fresh cheese, mozzarella) it could be of relevance to compare the *in vivo* behaviour of a *Lactobacillus* strain isolated from cheese and another strain of the same species obtained from human faeces. This comparison could also lead to a better understanding of the phenotypes that allow a strain to survive and remain in a complex environment such as the gut.

Genetic tools have also allowed us to find out that there are humans harbouring a relatively simple *Lactobacillus* population, in which a very few strains were numerically predominant isolates for several days or weeks. This observation, originally made with infants by Reniero *et al.,* (1991), was recently confirmed by Tannock and co-workers (Kimura *et al.,* 1997; Tannock *et al.,* 2000). It could open the way to a new and interesting method of selection: to select predominant strains, naturally occurring in human beings. The rationale behind this new criterion is, as we lack a complete understanding of the mechanisms which allow a strain to persist in the gut, to exploit a strain which has been shown to remain in this environment without continuous administration of the bacteria. What is clear, is that more attention must be paid to the correct taxonomy of the strains. It is surprising that even today (Chou and Weimer, 1999) strains that have been classified in the 1980s as *L. johnsonii* and *L. helveticus* (Dellaglio *et al.,* 1973; Johnson *et al.,* 1980; Lauer *et al.,* 1980) are still presented in scientific papers as *L. acidophilus*, causing confusion and possibly mistakes.

In conclusion, it is my opinion that the criterion of

Table 2. *Lactobacillus* Species Identified by Means of Genetic Tests and Most Frequently Encountered in the Human Gut

References	Species
Morelli *et al.,* 1998	*L. paracasei, "acidophilus"* groupB of Johnson (*L. gasseri* and *L. johnsonii*)
Dunne *et al.,* 1999	*L. salivarius, L. paracasei*
Tannock *et al.,* 2000	*L. casei* group of species, *L. plantarum*
Song *et al.,* 1999 and 2000	*L. gasseri, L. salivarius, L. paracasei, L. crispatus*

human origin still holds true, but more for 'historical reasons' (the need to avoid strains similar to those used by Metchnikoff) and 'common sense' (as we are lacking knowledge about the real mechanisms which make a probiotic bacterium able to persist in the gut, it seems reasonable to use a strain naturally present in the selected environment) than for scientifically-based reasons which are still not evident.

Survival During Gastric Transit

To reach the intestine, strains must first pass through the stomach, which secretes hydrochloric acid and enzyme. More than two litres of gastric juice is secreted each day, with a pH as low as 1.5 providing a barrier to the entrance into the gut of bacteria. A clear quantitative measurement of the destructive action of this environment was provided in 1987 by Conway *et al*. They showed, using gastric juice obtained from human volunteers, that strains belonging to species used to produce yoghurt were extremely sensitive to killing by gastric juice while enteric species of lactobacilli were more resistant, with significant strain to strain variations. Interestingly, the best performing between the two *L. acidophilus* strains used in that study (strain ADH) has been reclassified as *L. gasseri*, the species of homofermentative lactobacilli which is thought to be typical of the human gut (Raya and Klaenhammer, 1992). Is it possible that the observed differences could be explained in terms of species and host-specificity? Another study conducted using human gastric juice (Goldin *et al.,* 1992) showed that *L. rhamnosus* GG was unable to survive when challenged at pH 1 but there was no loss of viability at pH 3 or higher. *In vitro* results (Hood and Zottola, 1988; Charteris *et al.,* 1998b) showed that enteric lactobacilli could tolerate exposure to pH 2 for several minutes, while higher pH slightly affected their viable counts and pH 1 was destructive for all of the lactobacilli that were tested. During the FLAIR project, the Irish group co-ordinated by K. Collins, confirmed that human gastric juice at very low pH (about 1-1.2) was a potent killing agent for lactobacilli, as three out six strains tested were killed after 30 min of exposure, while the other three were able to survive, but with a decrease in viability of 4-5 logs (Collins and Thorton report of the UCC activities, included in Morelli, 1994). The puzzling point is that *L. paracasei* F19, one of the strains with was found to be 'sensitive' when challenged either with human (K. Collins and G. Thorton, included into: FLAIR final report, Morelli, 1994) or simulated gastric juice (Charteris *et al.,* 1998b) was recently shown to be an excellent coloniser of the human gut during *in vivo* trials (Fonden *et al.,* 2000). The same remark could be made concerning data published recently by a Danish group (Jacobsen, 1999). Strains of lactobacilli which have a documented ability to survive and reproduce in the human gut scored poorly when challenged *in vitro* for 4 hours at pH 2.5. Furthermore, when data obtained by *in vitro* experiments are compared, it appears the lactobacilli of the "*casei*" group of species are the most sensitive. This observation does not correlate with the high rate of isolation from human faeces of this group.

A further issue to be considered is the food matrix or the other ingredients which are used to incorporate or protect cells of lactobacilli that are to be ingested by consumers. It was specifically demonstrated for probiotic bacteria that food intake could protect bacteria during gastric passage (Conway *et al.,* 1987, Charteris *et al.,* 1998b). This is a factor that should be taken into consideration during *in vitro* evaluation. Testing tolerance to gastric juice using pellets of centrifuged cells or freeze dried preparations, and not the final product in which these cells have to be incorporated, could lead to misleading results. While this criterion remains valid when used to eliminate extremely sensitive bacteria (such as *S. thermophilus* and *L. delbrueckii* subsp. *bulgaricus*) from the number of potential probiotic species, it is doubtful, in my opinion, whether it can predict that a strain is able to tolerate gastric transit in real life. A critical evaluation of results obtained by *in vitro* assays of tolerance of lactobacilli does not indicate, in my opinion, that scores obtained using these tests could be really predictive of *in vivo* behaviour of these strains. Intestinal strains of lactobacilli have never been found to be extremely sensitive in these assays.

Tolerance to Bile Salts

The ability to survive the action of bile salts is an absolute need of probiotic bacteria, and it is generally included among the criteria used to select potential probiotic strains. The sensitivity of dairy isolates of lactobacilli to bile salts was demonstrated in the 1970s by the Gilliland group (Gilliland and Speck, 1977). They also suggested the importance of assessing bile tolerance in the selection of lactobacilli for probiotic use (Gilliland 1979). In a further study (Gilliland *et al.,* 1984), it was shown that bile resistance could differ among members of the same species of enteric lactobacilli and that this difference could account for differences in the ability of strains to colonise the intestinal tract of calves. Two strains of *L. acidophilus,* isolated from calves, exhibiting a different degree of bile resistance when assayed *in vitro,* were used in a feeding trial with calves. Results indicated that both strains were able to increase the total counts of lactobacilli in treated animals. Comparison with the control group of animals, however, revealed that statistically significant differences in the number of lactobacilli were achieved only in the jejunum of calves administered the bile resistant strain. In the ileum, numbers of lactobacilli were significantly higher for groups of calves receiving both strains of lactobacilli and the difference between the treated and untreated groups was not significant. In the large intestine, the treated animals did not show an increased number of lactobacilli compared to the control group. These results are a good example of how it is possible to demonstrate the *in vivo* relevance of a phenotypic trait associated with specific strains.

The bile preparation used by Gilliland to assay strains *in vitro* was 'oxgall', a product derived from bovine bile, at a concentration of 0.3% (w\v). The core of the assay was the measurement of the lag phase in the growth curve caused by the presence, in liquid medium, of oxgall. Similar methodologies have been used by several authors (see Table 3) to assess the bile resistance of potential, or already commercialised, probiotic lactobacilli. Results reported in all of these papers showed that the amount of delay detected in the growth curve of lactobacilli challenged with oxgall was strain, and not species, dependent. This

Table 3. Strain-Dependent Bile Tolerance. List of papers in which at least two strains of the listed species have been tested for their bile tolerance

Species.	References
L. acidophilus	Gilliland *et al.,* 1984; Gilliland and Walker, 1989; Chateau *et al.,* 1994; Walker and Gilliland, 1993; Jacobsen *et al.,* 1999)
L. rhamnosus	Chateau *et al.,* 1994; Jacobsen *et al.,* 1999
L. plantarum	
L. fermentum	Chateau *et al.,* 1994
L. gasseri	USMAN and Hosono,1999
L. crispatus	Jacobsen *et al.,* 1999
L. casei - L. paracasei	

observation was confirmed in other species of lactobacilli isolated from humans, or intended to be used for human consumption (Table 3), and can be considered a well established observation.

In these papers, bile preparations derived from bovines was always used to assay lactobacilli selected for human use. The composition of bile preparations was not taken into consideration. When this problem was addressed during the FLAIR project (Reports of The University College Cork Ireland and of NIZO-The Netherlands, cited in the Project Final Report, Morelli 1994), it was observed that commercially available bile preparations contained variable proportions of conjugated and deconjugated bile salts and that assessment of sensitivity gave different results according to the commercial product used. Two commercial preparations were compared for their bile salt composition and their ability to inhibit the growth of lactobacilli. One was found to contain 97.2% of conjugated bile salts whereas the latter was composed of only deconjugated bile acids. Results showed that all tested strains exhibited resistance to the preparation containing conjugated bile salts and were more sensitive to the deconjugated bile salts. It was also shown that porcine bile was more inhibitory than bovine bile, but that "regardless of the resistance patterns observed in the presence of either bovine or porcine bile, all the assayed bacteria (all of human origin) were capable of growth in a physiologically relevant concentration of human bile (approx. 0.3%)" (Dunne *at al.,* 1999).

It was also recently shown that bile is a potent inhibitor of strains used in probiotic preparations and belonging to the genus *Bacillus* (Table 4). These results prompted the authors to suggest that any claimed probiotic effect of these preparations must be due to spores and not to vegetative cells (Spinosa *et al.,* 2000). All of the above observations suggest that this selection criterion is capable of discriminating the enteric origin of the strains, but not to predict the real *in vivo* behaviour of strains. However, a recent paper (Kimoto *et al.,* 1999) has reported that some isolates of dairy lactococci have a tolerance to bile salts comparable to that of enteric lactobacilli. The same authors conclude that this *in vitro* observation needs an *in vivo* confirmation.

An interesting development in the evaluation of the role played by bile resistance in defining the probiotic capabilities of strains is seen in the study of bile salt hydrolases at the genetic level. Deconjugation of bile acids has been studied in lactobacilli since the 1960s (Hill and Drasar, 1968) and the gene encoding a bile salt hydrolase has been cloned and sequenced (Christiaensen *et al.,* 1992). Disruption of the gene was accomplished (Leer *et al.,* 1993) and the implication for bacterial survival in the gut has been addressed in one paper (De Boever and Verstraete, 1999). Data obtained from the comparison of the wild type, deconjugating strain with the mutant clone, suggested that bile tolerance *in vivo* could be the result of complex interactions of lactobacilli with other members of the intestinal microflora. No doubt this kind of study will lead to a better understanding of the real behaviour of lactobacilli in the gut. They are also, however, reducing the relevance of assaying bile tolerance *in vitro* with procedures that seem to be too simplified and still need improved standardisation.

Table 4. Minimal Inhibitory Concentration of Bile Salts for *Bacillus* Species Used in Probiotic Preparations Compared to Enteric Species (adapted from Spinosa *et al.,* 2000)

Species µg/ml (mM)	MIC of TDOC[1] µg /ml (mM)	MIC of DOC[2]
Bacillus subtilis	195 (0.4)	78 (0.2)
Bacillus thuringensis	98 (0.2)	78 (0.2)
Bacillus clausii	≤ 24 (≤ 0.05)	78 (0.2)
Enterococcus faecalis	≥ 25000 (≥51.2)	625 (1.6)
Enterococcus faecium	3125 (6.4)	625 (1.6)

[1]= Taurodeoxycholic acid; [2]=deoxycholic acid

Adhesion to Intestinal Cell Lines

The gastrointestinal tract, especially the small intestine, is a dynamic environment and the flow of digesta washes out any bacterium unable to counter the flow either by rapidly multiplying or by attaching itself to intestinal surfaces. It is generally agreed that adhering probiotic strains are more likely to have an increased opportunity to colonise the intestine. Results obtained with probiotics used in farm animals have shown that some strains of *Lactobacillus* are able to adhere, *in vitro*, to small pieces of intestinal tissues in a species-dependent way and that it is possible to observe a positive correlation between results obtained *in vitro* and colonisation potential tested *in vivo* (Fuller, 1975 and 1978; Barrow *et al.,* 1980; Savage, 1984). However, these studies were performed using freshly harvested tissues, and raised some questions. Savage (1984) concluded that ". . . the capacity to adhere to the surface is undoubtedly insufficient by itself to ensure that the microorganisms can colonise the epithelial habitat".

Table 5. Lactobacilli with an Adhesion Index of at Least One Bacterium per Caco-2 Cell.

Strain	Isolated From:	Adhesion Index	Reference
L. acidophilus BG2FO4	Human	2.3	Cocconier *et al.,* 1992
L. johnsonii LA1		1.55	Bernet *et al.,* 1994
L. acidophilus LB		2.1	Chauviere *et al.,* 1992
L. rhamnosus GG		1.25	
L. acidophilus C7	Chicken	1.5	
L. helveticus CNRZ 239	Dairy	1.4	
L. helveticus CNRZ 240		2.1	
L. delbrueckii subsp. *lactis* CNRZ 239		1.9	
L. delbrueckii subsp. *lactis* ATCC 7830	Unknown	2.3	Sarem *et al.,* 1996
L. delbrueckii subsp. *lactis* LY	Yoghurt	1.5	

He showed that one *Lactobacillus* strain, isolated from a pig, and another strain derived from a mouse, adhered *in vitro* to mouse tissues in equally high number, but only the latter was able to colonise the same kind of tissue surface *in vivo*. Fuller (1978) noticed that strains that did not adhere *in vitro* to chicken crop cells scored negative results during an *in vivo* feeding trial. Of the two strains that scored positively both *in vitro* and *in vivo*, the best performing *in vitro* (adhesion index of 22) was less able to colonise *in vivo* than the strain which scored an adhesion index of 2 *in vitro*. If *in vitro* assays for probiotic bacteria to be used in animal feeds are not fully reliable, the problem of assessing, under laboratory conditions, the potential for adhesion *in vivo* of probiotic strains intended for human use is, in my opinion, far from resolution.

Two approaches have been followed in adherence assays. The first was based on intestinal cells collected from ileostomic patients (Conway *et al.,* 1987) whilst the second used a human foetal intestinal cell line (Kleeman and Klaenhammer, 1982; Hood and Zottola, 1987, 1988, 1989). Adhesion to human ileal cells gave striking results as it was possible to establish clear differences between strains. Two human isolates scored much better than two dairy isolates that were tested. The best adhering human isolate (*L. gasseri* ADH) was able to attach at a density of 120-205 bacterial cells per ileal cell, while the other strain (*L. acidophilus* N2) ranged from 51 to 69 bacteria per intestinal cell (Conway *et al.,* 1987). Adhesion assayed by means of a human foetal intestinal (Kleeman and Klaenhammer, 1982) cell line was also able to establish differences between human and non-human isolates and also between strains. Unfortunately, in this paper, adhesion was measured subjectively and it was not possible to make a comparison with results reported by other authors. These methodologies suffer severe limitations since the use of freshly isolated intestinal cells yields fluctuating results because of the use of different donors, and the foetal cell line was poorly differentiated with ill-defined brush borders.

At the beginning of 1990s, some groups turned their attention to two tumor cell lines, Caco2 and HT29, in order to have a better model system to assay adhesion of lactobacilli *in vitro*.

These cells show morphological and functional differentiation, having the characteristics of mature enterocytes. They have been extensively used for assessing attachment of pathogenic bacteria. The use of these cell lines, especially the Caco-2 cells, resulted in the publication of a number of papers aimed at assessing the adhesion potential of lactobacilli belonging to a range of species that were isolated from different sources. Methodologies used for this assessment were not standardised so it is difficult to compare the results obtained in the different studies. In at least four of these papers, it was possible to calculate the ratio of bacterial cells adhering to one Caco-2 cell (Table 5). For the best scoring strains, the ratio was about two bacterial cells per Caco-2 cell. This figure is in striking contrast with results obtained by Conway *et al.,* (1987) where lactobacilli adhering to ileal cells ranged from 51 to 205 bacteria per cell. The difference in adhesion levels of *Lactobacillus rhamnosus* GG detected with Caco-2 cells compared to that obtained with ileal cells (Gorbach and Goldin 1989) also raise doubts about the reliability of this model. We might assume that freshly harvested intestinal cells would be a closer model to the *in vivo* situation than a tumor cell line. It must be added that these results are probably overestimates because the procedure included the use of acid, culture supernatants. The results in Table 5 were obtained without the use of chelating agents. These chemicals have been shown to artificially increase the adhesion potential of lactobacilli. Even if non-enteric lactobacilli seem to be influenced more by these parameters than intestinal isolates, it seems clear that reported differences among strains of lactobacilli are very small (1 vs. 1.5 bacteria per Caco-2 cell) which are unlikely to be able to predict significant behavioural differences *in vivo*. Furthermore, the *in vitro* adhesion index system has been shown (Greene and Klaenhameer, 1994, Lehto and Salminen, 1997, Tuomola *et al.,* 1998, Blum and Reniero, 2000) to be extremely sensitive to factors such as (Blum and Reniero, 2000):

- pH
- the presence of calcium ions
- the number of lactobacilli
- the presence of culture supernatant
- the growth phase in which the bacteria were harvested

Observations that the adhesion of lactobacilli to cell lines is concentration-dependent (Greene and Klaenhameer, 1994, Lehto and Salminen 1997, Tuomola *et al.,* 1999) and that a saturation end-point was never reached, suggested that non-specific adhesion is measured by this *in vitro* procedure. High adhesion indices scored by dairy isolates (see Table 5) further strengthen this suggestion. Studies using subjective evaluation of adhesion to Caco-2 cells by lactobacilli report high levels of adhesion for non-enteric lactobacilli (Lehto and Salminen, 1997, Jacobsen *et al.,* 1999). It is surprising to note that in one of these papers *Lactobacillus rhamnosus* GG, whose *in vivo* colonising ability has been reported (Alander *et al.,* 1999), was found to be less adherent than a dairy *Lactococcus* (Lehto and Salminen, 1997).

Jacobsen *et al.,* (1999) using a subjective index, have scored three well studied probiotic strains in the following way: *L. johnsonii* LA 1=17, *L. rhamnosus* GG= 630, *L. plantarum* 299v=355. Despite these different *in vitro* scorings, all of these strains have been shown to persist in the human gut using doses of equal magnitude (reviewed by Salminen *et al.,* 1996b). It seems to me that cell line models are unlikely to be able to provide an absolute differentiation between adhering and non-adhering strains. Available data suggest that a strain, of human enteric origin, scoring a positive result in the *in vitro* system, is likely to perform well *in vivo*. But it cannot be assumed that a negative result in this model means a lack of colonisation potential *in vitro*. Some new approaches have been introduced, such as the use either of human intestinal tissue, maintained viable for short periods *in vitro* (Sarem-Damerdij *et al.,* 1995), or human mucus glycoproteins (Tuomola *et al.,* 1999). Even if some of the results seem to be reliable, the lack of knowledge about the molecular adhesion mechanisms of lactobacilli does not allow firm conclusions about the significance of these observations to be made. New data, obtained at the genetic level, as regards proteins secreted by lactobacilli and able to adhere to mucus (for a review see Vaughan *et al.,* 1999) may provide a breakthrough in this field in the future.

Conclusions

Interest in the field of probiotics has boomed in recent years, paralleling the renewed interest in studies focusing on microbial ecology of the gut and powered by the use of randomised, blind or double-blind human trials. Molecular biology has provided good tools to assess the real behaviour of a specific strain *in vivo* and to learn more about the composition of the intestinal microflora. The development of new probiotic products has produced new scientific achievements and a strong demand for improved and scientifically-based selection criteria (Tannock, 1997). The ability of a specific probiotic strain to survive and reproduce in the hostile environment of the gut is the most relevant feature to be checked during the selection procedures. But *in vivo* testing is time-consuming and expensive. *In vitro* selection is therefore the first approach used to select a few strains that can be further evaluated *in vivo.* Criteria used at the moment have been defined without a clear knowledge of the bacterial characteristics that are important in the proliferation of probiotic bacteria in the intestinal tract. Nevertheless, results achieved are positive and promising, but clearly show the scientific limitations of current selection criteria. Further development of probiotic products requires a refinement of these criteria. A deeper understanding of the molecular mechanisms that are used by bacteria to tolerate and persist in the harsh environments of the upper part of our gastrointestinal tract is required to achieve new and improved criteria.

Acknowledgements

The author is indebted to all partners of the EU FLAIR Project AGRF-CT91-0053 and the EU FAIR Project CT96-1028, whose excellent research work has provided the basis for this paper. I am particularly in debt to the late A. Weerkamp, who introduced me to a critical assessment of criteria used for strain selection. I wish also to thank my former PhD student R. Reniero, now at the Nestlè Research Centre, who shared with me many years of fruitful research in this field.

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