



Contents lists available at ScienceDirect

## Trends in Food Science &amp; Technology

journal homepage: <http://www.journals.elsevier.com/trends-in-food-science-and-technology>

## Review

Correlation between *in vitro* and *in vivo* assays in selection of probiotics from traditional species of bacteriaGabriel Vinderola<sup>a, \*</sup>, Miguel Gueimonde<sup>b</sup>, Carlos Gomez-Gallego<sup>c</sup>,  
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## ARTICLE INFO

## Article history:

Received 30 September 2016

Received in revised form

31 July 2017

Accepted 3 August 2017

Available online 19 August 2017

## Keywords:

*In vitro**In vivo*

Correlation

Probiotics

Lactobacilli

Bifidobacteria

## 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.

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## 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 concluded 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 i.e. 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) isolated 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 (Cuellar-García 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.

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