

## Original Paper

# Selection and Characterization Criteria of Probiotics Intended for Human Use from the Past to the Future

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Received: April 28, 2019

Accepted: May 10, 2019

Online Published: May 20, 2019

doi:10.22158/fsns.v3n2p73

URL: <http://dx.doi.org/10.22158/fsns.v3n2p73>

### Abstract

*The probiotic product consumption has recently increased with the prevalent intent to promote human and animal wellbeing. The complex selection process dealing with new-isolated probiotic candidates is the first challenge that has to be faced. From the isolation to the launch on the market, information about safety, tolerance to host physiological conditions, adhesion properties, genetics and interaction with the host has to be collected. Probiotics must be safe, survive to the exposition to bile salts and to gut transit, adhere to intestinal cells lining and colonize the lumen of the tract. The evaluation process of the possible probiotic health benefits is widely supported by in-vitro assays simulating the in-vivo conditions. The aim of this work is to summarize the classical models usually employed for the probiotic screening by underlying strengths and weaknesses of all models and to present some more recent analysis tools used in the probiotic field. The long term goal in new probiotic candidate selection experiencing these combined essays together would lead to the hypothetical assignment acknowledged as one strain-one function.*

### Keywords

*screening, platform, in-vitro, in-vivo, model*

## 1. Introduction

International Scientific Association for Probiotics and Prebiotics defined probiotics as “live microorganisms that, when administered in adequate amounts, confer a health benefit on the host” (Hill et al., 2014). In the recent years the global probiotic market has faced a massive growth, this expansion should be related to a robust and univocal legislation (Baldi & Arora, 2015). Nevertheless, focusing the attention on the European regulatory framework, it has to be noticed the absence of harmonized regulation. The Food Products Directive and Regulation (Regulation 178/2002/EC; Directive

2000/13/EU) and Herbal Medicinal Products Directive (2004/24/EC) are the main laws supervising probiotic products (Coppens et al., 2006). Furthermore, the European regulatory pressure in the field of probiotics led to a double negative impact by limiting the communication of their beneficial effects to consumers and by adversely impacting the market. After ten years from the enter into force of the Regulation on health claims made on food (EU 1924/2006), the market of probiotics shows a renewed interest about the health improving potential of beneficial microbes. The restored enthusiasm is mainly driven by the big pool of information derived from the omics approach for the study of the gut microbiota (Baugher & Klaenhammer, 2011). Unfortunately, the legislative framework seems to not run with the innovation associated with new analytical approaches. Actually, of the about 300 calls for health claims submitted to EFSA experts' panel on the food area none has been accepted. The only exception was represented to the use of yoghurt for lactose intolerance (Papadimitriou et al., 2015). The implementation of new and comprehensive models, exploiting the newest omics technology could ameliorate the understanding of the probiotics mechanism of action and assessing the beneficial impact on health exerted by the administration of probiotics at a quantifiable level (Rebollar et al., 2016). The integration of the traditional *in-vitro* and *ex-vivo* screening methods of microorganisms with potential probiotic features are associated with omics techniques with the intent to boost the selection process, to deeper understand their functionality and to examine the opportunity to employ cell fractions instead of viable probiotics in finished products (Franzosa et al., 2015; Lahtinen, 2012). Recent market trends concerning probiotic are interestingly targeted to the zootechnic field. For instance, it has been proposed the probiotic use to produce safe and high-quality poultry meat (Park et al., 2016). Other application fields could be retrieved in cereals, dairy products, fermented meat, baked food, and dry food productions (Competitive Market Share & Forecast, 2016). For the application related to human consumption, the validated approach applied to the selection of probiotics firstly includes the safety assessment intended to verify the absence of pathological and virulence traits (Hill et al., 2014). The next step evaluates the potential application of the isolated strain by considering the prophylactic, therapeutic use or its ability to reduce the risk to develop a certain pathological condition. Regrettably, the implication of this validation results strictly dependent on the availability of measurable biomarkers related to a certain physiological condition.

## 2. Classical *in-vitro* Approaches for New Probiotics Screening

Guidelines for the evaluation of the probiotic use in food have been published several years ago (FAO/WHO, 2002). The principal focus identified was defined as a selection scheme for the conventional assessment of probiotic properties and for the characterization of functional bacterial strains as probiotics in food. Despite their time-consuming application, the *in-vitro* assays are routinely used due to their presumed predictive value to predetermine, the putative association between the isolated probiotic candidate strain and a specific health claim, targeting its potential general or specific application (Kumar et al., 2013). The major limitation of these assays is represented by the high

variability in terms of reproducibility even if a certain degree of standardization has been suggested. The natural continuation of these essays would be theoretically represented by *in-vivo* tests that are laborious, expensive and not feasible for ethical reasons.

### 3. Persistence and Survival in the Human Gut

A peculiar required probiotic feature is represented by the ability to survive the passage through the gastrointestinal tract remaining in a viable state and with the sufficient number of cells necessary to exert the probiotic effect. To verify this ability simulated gastric and pancreatic juices (Charteris et al., 1998; Lavermicocca et al., 2008) and porcine or bovine bile have been extensively used, in fact these assays are easy and cheap if compared to more recent approaches including GIT simulators. Unfortunately, those *in-vitro* approaches are affected of some biases dealing with the unrealistic conditions simulated during benchtop assays. Moreover, potential probiotic candidates are tested against severe environmental parameters not really miming the stomach acidity and lower intestine conditions during food consumption (Papadimitriou et al., 2015).

### 4. Safety for the Use in Human Nutrition

The Joint Working Group FAO/WHO (2002) underlined the relevance of some safety issues for the use of probiotics in food. The EFSA panel of experts suggested the precise determination of the minimal inhibitory concentrations (MICs) towards 9 listed antibiotics and also stated the microbiological cutoff values to evaluate a new probiotic candidate as susceptible or resistant to antibiotics and the reference methodology to assess it (EFSA; 2012). Safety aspects usually assessed by *in-vitro* tests also include aggregation to platelets, to fibronectin and to fibrinogen, hemolytic activity, the synthesis of certain enzymes and the production of biogenic amines. The major concern related to these essays is based on the fact that the expression levels of the *in-vivo* traits could significantly vary from the *in-vitro* conditions inducing to an over- or an under-estimation of some vexing features of probiotics (Papadimitriou et al., 2015).

### 5. Colonization Ability

The colonization ability is an indicator of the probiotic cellular features to interact with the host. The adhesion of bacteria to mucus and epithelial cells is an important parameter evaluated with several *in-vitro* assays; this trait could both represent positive connotations associated with pathogens displacement and negative implications for instance the increasing risk of translocation (Sanders et al., 2010). Furthermore, Caco-2, HT-29 epithelial cell adhesion assays often produce weak results due to the low reproducibility maintenance of the tested conditions. In order to circumvent the problem, resected gut tissue or whole tissue models including the mucus layer have been taken into consideration but reliability bias are still present (Vesterlund et al., 2005; van Tassell & Miller, 2011). To evaluate the ability of a strain to interact with the host, some physical features of probiotics have been considered,

for instance, the hydrophobicity of the cell surface (Yadav et al., 2014). Unfortunately, the scientific significance of these tests is only considered partially because of the lack of the peculiarity of this trait. The enzymatic activity expression such as GAPDH (glyceraldehyde-3-phosphate dehydrogenase) promoting the probiotic attachment to the mucus has been evaluated as a potential screening parameter even if with scarce results (Kinoshita et al., 2008).

The assay conditions, even if optimized could not reproduce the complexity of the bacterial community structure and the possible interactions among different microorganisms. The adhesion assays are performed on a single and purified strain without considering different microbial species leading to doubts about the results, significance and their possible extensive transposition to the gut microbiota.

## 6. Antimicrobial Potential

It is known that probiotics could exert competitive inhibition towards pathogens exploiting several mechanisms, for instance the production of metabolites showing antimicrobial activity. This feature has been proven influence other bacteria, viruses and fungi at a large spectrum, taking advantage of different compounds like organic acids or bacteriocins (Denkova et al., 2013). Several *in-vitro* models have been experienced, from the agar diffusion test to more advanced systems targeting the release of antimicrobial substances using indicator strains (Szweda & Szweda, 2016). Probiotics exhibit other features as co-aggregation with pathogens and/or by reinforcing the integrity and functions of the gut inner barrier. Briefly, probiotic cells bind pathogens causing a sort of clumping effect limiting the pathogen interaction with the surfaces of the host and facilitating the excretion of pathogens through biological fluids (e.g., saliva, feces) (Janković et al., 2012). The gut barrier could exert its function only if the tight junctions integrity is maintained, this condition represents a key issue to partially prevent the bacterial translocation, pathogens included. It has been proved that probiotics may improve the barrier integrity as measured by specific enzymatic activities (Putala et al., 2008).

The evaluation of probiotics antimicrobial potential is affected by some biases since the laboratory conditions only partially reproduce the *in-vivo* situation. Similarly to what has been observed for the described above *in-vitro* models, the level of expression of the real antimicrobial potential of tested probiotic strains is not completely defined. Substances as exopolysaccharides (EPS), biosurfactants, bacteriocins and organic acids represent the main antimicrobial compounds produced by probiotics (Evivie et al., 2017).

## 7. Immunomodulatory Actions

Probiotics could stimulate the secretion of antibodies by host cells and activate cell-mediated immune responses (Haghighi et al., 2005). Usually, immunomodulatory effects are promoted by M cells from the Payer's patches or dendritic cells (DCs) and the response to the occurrence of bacterial cells is assessed by co-culture of probiotics and immune cells and by the detection and the quantification of cytokines (Gad et al., 2011). These co-incubation models are frequently applied to the selection of

probiotics showing anti-inflammatory features useful in the management of chronic inflammatory diseases (e.g., IBD) (Rutella and Locatelli, 2011) or to be employed as adjuvant in the treatment of allergic symptoms (Ozdemir, 2010). However, the center of the debate still remains the *in-vivo* modality of action of probiotics and on the possible solution to translate experimental findings to the concrete evaluation of the probiotic strains efficacy in the modulation of the gastrointestinal and systemic immunity.

### **8. *In-vitro* Models of Cardiovascular Diseases**

Another trait that is required from probiotic candidates is represented by the expression of the bile salt hydrolase activity (BSH). This enzymatic activity allows the deconjugation of bile acids and it is pretty easy and quick to obtain qualitative and quantitative results (Cani & Van Hul, 2015; Zheng et al., 2013; Tomaro-Duchesneau et al., 2014). However, these routinely used models are sometimes considered relatively insufficient to estimate the real probiotics contribution to reduce cardiovascular diseases risks associated (Papadimitriou et al., 2015), albeit the beneficial effects seem to be highlighted by scientific evidences (Ebel et al., 2014).

### **9. *In-vitro* Anti-cancer Models**

It has been proven that probiotics reinforce natural host body protection functions by improving metabolic and immunological parameters (Ashraf & Shah, 2014). Furthermore, these beneficial bacteria could exert antigenotoxic and antimutagenic activities and promote nitrosamines and heterocyclic amine degradation whose accumulation within the intestinal ecosystem could lead to potential negative effects (Duangjitcharoen et al., 2014; Faridnia et al., 2010). Excluding the ability of withdrawing dangerous metabolites, the data support the probiotics ability of releasing short chain fatty acids (SCFA) within the intestinal lumen (Table 1). These substances have been proven to exert some anticarcinogenic effects *in-vivo* (Burns & Rowland, 2004; Castro et al., 2010). Nevertheless, despite a supportive background is already available, researchers consider these *in-vitro* tests as not sufficient to provide consistent probiotic anticancer activity information and, for this reason, the *in-vivo* validation is compelled (Chong, 2014).

To easily complete the probiotic profile of beneficial bacteria, a pool of other *in-vitro* model has been proposed, among them the measurement of B-galactosidase activity to monitor lactose intolerance symptoms (Vonk et al., 2012), B-group vitamin production (Leblanc et al., 2011), oxalate-degrading features (Abratt & Reid, 2010) and inhibition of pathogen oral strains responsible for volatile sulfur compounds (VSCs) production (Lee & Baek, 2014).

**Table 1. Short Chain Fatty Acids (SCFA) Produced by Probiotics**

Probiotic microorganisms	Produced SCFA	Reference
<i>L. salivarius</i> spp <i>salcinius</i> JCM 1230, <i>L. agilis</i> JCM 1048	Propionate/butyrate	Meimandipour et al., 2010
<i>L. acidophilus</i> CRL 1014	Acetate/butyrate/propionate	Sivieri et al., 2013
<i>L. rhamnosus</i> LGG	Propionate	Le Blanc et al., 2017
<i>L. gasseri</i> PA 16/8	Propionate	Le Blanc et al., 2017
<i>B. longum</i> SP 07/3	Propionate/acetate	Le Blanc et al., 2017
<i>B. bifidum</i> MF 20/5	Propionate/acetate	Le Blanc et al., 2017
<i>Bifidobacteria</i>	Acetate/lactate	Pessione et al., 2012

## 10. Development of Innovative Screening Platforms

The rationale underlying standardized *in-vitro* assays to study several aspects of probiotic effectiveness is driven by the necessity of set reproducible experimental protocols and consequently decrease the percentage of false positive and negative results.

*In-vivo* models should be considered integrative for *in-vitro* ones with the purpose of choosing the appropriate probiotic for a potential application.

Several animal models including invertebrates (e.g., insect, worm) and vertebrates (e.g., rodents, dogs, monkeys and swine), have been tested even though the predictivity for the situation in humans seems to be powerless and for this reason, a critical approach should be applied when results are transposed to humans. In the latest years the conventional screening pipeline for probiotics (*in-vitro* assays—animal model—clinical trial) including mice or rat models has strongly been debated and there was the tendency to directly check the findings from *in-vitro* evidences on small number human cohorts. However, animal models will certainly be exploited, particularly for studying specific pathological conditions, both induced by chemicals administration or using animal with genetic predispositions, or pathogenic infection conditions. Some probiotic positive modulation effects of intestinal inflammation have been successfully studied thanks to animal models. For instance, the co-occurrence of probiotics and carcinogenesis prevention, metabolic disorders control or auto-immunological conditions have been experienced. On the contrary, immune-deficient animals carrying human tissue or genes also known as humanized mice or rat models, are basically used more for pharmaceuticals purpose instead of probiotic validation (Papadimitriou et al., 2015).

The combination of *in-vitro* and *in-vivo* test represents the new frontier, Europe coupled the two tests in some *in-silico* models to examine from a mathematical point of view the interaction between nutrients, epithelial cells, gut communities and host (Tan & Liong, 2014). These applications are supposed to mimic the complex gut environment processes by proposing predictive functions and taking in consideration the possible interactions between probiotics with multiple factors.

The omics approaches with the advent of new molecular methods greatly contributed to the comprehension of the usefulness of probiotic towards hosts, mainly quantifying the expression variation of certain housekeeping genes of probiotic cells in response to specific environmental conditions. The resistance to stressful conditions, modulation of the immune system, adhesion to host tissues, production of antimicrobial or noble nutrient compounds, degradation of prebiotics and quorum sensing are areas of interest investigated with special consideration (Papadimitriou et al., 2015).

Omics technologies are assumed to explicate the complexity of the communication dynamics inside microbiome or between it and host tissues and to provide new insights on the impact of a foreign probiotic strain meeting those structured communities (De Melo Pereira et al., 2018).

## 11. Conclusions

The number of available assays to validate probiotic efficacy *in-vitro* and *in-vivo* is rather complex. The major limitations linked to those approaches are that they consider variables explanatory and not easily reproducible. As a consequence, often the efficacy of new-isolated probiotic candidates could be accomplished only by combining several assays.

At the state of the art, on the one hand, there is an urging need of defining a clear workflow for the probiotics selection in order to obtain reliable and unquestionable proof of efficacy, and on the other, the design of appropriately designed clinical trials. Human studies are often associated with significant costs, consequently probiotics are still screened following the “funnel-like” approach, firstly using *in-vitro* tests and subsequently by expensive and appropriate validations on selected strains.

The objective sought in new probiotic candidate selection would lead to the development of screening platforms, reducing from a large pool of strains to a restricted group intentionally selected for a specific human need to achieve the ideal aim of *one strain-one function*.

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