Physiological and Functional Properties of Probiotics

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1. Introduction

In the last twenty years, major efforts have been invested in the proper identification and selection of probiotic strains. Today, there is widespread agreement that probiotic strains should be safe, effective and stable in the final product. This, however, is a general recommendation and does not define which specific tests should be applied when selecting a new probiotic strain. It is accepted that health claims have to be supported by well-conducted clinical trials using the final product in the targeted population. Before such trials, potential probiotic strains are to be selected from the huge natural reservoir of candidates. Most of the screening is usually performed using a variety of *in vitro* assays and animal models. However, the field lacks substantiated correlation between the results of historical methods used to select probiotic strains and clinical manifestations in humans. This situation reflects our limited knowledge of the mechanisms underlying the purported health benefits of probiotics, which renders the design of validated screening assays particularly challenging. The situation is further complicated by the fact that probiotics are intended to impact a wide array of human ecosystems (oral, stomach, small intestine, colon, vaginal tract) and a quite diverse range of physiological functions. Moreover, the interplay between the different partners of a given ecosystem is very complex and still poorly understood.

2. Project group activities and conclusions

The purpose of the project group (comprised of the experts listed in Table 1) was to determine which *in vitro*, animal or human assessments of physiological function should be recommended to determine that a microbial strain meets the minimum criteria for being considered a probiotic. The intent was to agree upon a list of physiological and functional properties required of probiotic bacteria and to establish methods to assess these functions. This effort was expected to build on the general guidelines established in 2002 by the Joint FAO/WHO working group on Drafting Guidelines for the Evaluation of Probiotics in Food (http://www.who.int/foodsafety/fs_management/en/probiotic_guidelines.pdf). The project group arrived at the following conclusions:

(1) The potential scope of physiological activities of probiotics is so broad that no unique test or battery of tests could be defined that would be considered essential to all probiotics or probiotic applications.

For example, although *in vitro* tests such as bile and acid resistance are commonly performed, results from these tests would not be necessarily relevant to a probiotic that was targeted for certain sites, such as the oral cavity [1].

As another example, adhesion to epithelial cells is often cited in the literature as an important selection criterion for probiotic strains. The underlying idea is that bacteria need to be in close contact with the epithelial barrier to exert their beneficial effect. This is a narrow and perhaps incorrect view of the *in vivo* situation with probiotics. For example, bacterial soluble molecules (metabolites, cell wall components, and DNA) are known to mediate some signaling to the host (for a review, see [2]). In addition, most *in vitro* settings used to study adhesion do not account for the complex and changing mucus lining found along the human gastro-intestinal tract. It

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has also been shown that these assays are very hard to standardize and control and that results may vary substantially from one laboratory to another [3].

(2) There is no solid documentation that strains, which perform well in physiological tests perform any better in human evaluations than strains which perform poorly in these tests.

Even for simple tests such as acid or bile resistance, there are currently no agreed cut off points for what would constitute "good" vs. "poor" performance in these tests, and no systematic comparison of *in vitro* results with human functionality have been conducted. In short, there is no solid documentation that strains, which perform well in these tests, perform any better in human evaluations than strains which perform poorly in these tests. Under these circumstances, there is no basis for requiring these tests be conducted for all probiotics. Nevertheless, they might still be used as part of a thorough characterization of the candidate probiotic strain. Moreover, it cannot be excluded that in the future, when supported by a systematic correlation to *in vivo* biological effects, *in vitro* tests may help classify strains into "functional groups". As it stands at present, it should be made very clear though that they are neither sufficient nor necessarily indicative of a "probiotic status".

(3) Establishing Standard Operating Procedures for limited numbers of functional assays is premature considering the current status of research.

A large number of *in vitro* assays and animal models attempting to mimic - at least in part - the human situation are presently in use or under development. This reflects the complexity and variety of:

i. ecological niches targeted for probiotic administration;
ii. action site and mediation of effects;
iii. targeted health benefits;
iv. target populations; and
v. impact of the final probiotic formulation/product on functional effects.

The knowledge of the mechanisms underlying the cross-talk between the probiotic strain and the host is currently limited (although the field is rapidly moving), which makes the selection of any one particular assay difficult to support. In addition, validated biomarkers or surrogate markers that can be followed to substantiate specific effects in humans are still under development.

The PASSCLAIM EU network and ILSI Task Forces [4]; [5]; [6] have recently reviewed a variety of assays used to study the effects of probiotics on the host, with a special focus on the impact probiotics may have on the host immune system. Their recommendations certainly represent a move forward. Along the same line, the PROSAFE EU network evaluated the relevance of specific *in vitro* and *in vivo* assays to evaluate the safety of probiotic and dairy strains [7].

(4) Concerning health claims, it is recommended today to conduct two independent double-blind placebo-controlled clinical trials with the final probiotic product in the targeted population.

Establishing efficacy in humans requires studies in humans. Existing worldwide regulatory guidelines on the level and type of information needed vary on this subject [8], and in some locations such as the European Union recommendations are still under discussion. From a scientific perspective, clinical trials or human intervention studies should follow the principles of "good clinical practice". This includes a study population with sufficient numbers of individuals to reach statistical power/significance. Such criteria will likely be required by regulatory agencies for support of health claims in the future anyway (http://eur-lex.europa.eu/LexUriServ/site/en/oj/2007/l_012/l_01220070118en00090018.pdf).

Table 1. Participants in the project group on Physiological and Functional Properties of Probiotics of the IDF/ISO Joint Action Team on Probiotics

Name (Country)				
B Bianchi Salvadori (IT)	S Blum Sperisen (CH)	P Breeuwer (CH)	E Brockmann (DK)	C Cavadini (CH)
M Danielsen (DK)	B Degeest (BE)	F Dellaglio (IT)	D Ellekaer (DK)	J Foy (US)
M Gueimonde (FI)	KJ Heller (DE)	G Huys (BE)	BL Jacobsen (DK)	I Lenoir-Wijnkoop (FR)
R Lodi (IT)	A Mercenier (CH)	V Ninane (BE)	M Pineiro (IT)	T Sako (JP)
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3. Recommendations

3.1 Establishing probiotic properties of strains

With the current state of the science, the best approach to establish probiotic properties of a candidate strain would start with defining target human physiological function to be impacted by the probiotic, defining the target populations (e.g., infants, elderly, healthy, at risk), and finally applying the best available methods to study this function. In this process, well-controlled *in vitro* tests would reduce the number of strains to be studied in animal models and so help selecting the best candidate strain to perform clinical trials. One flow diagram for the approach to document physiological functionality for a commercial strain follows:

i. Definition of the targeted health benefit, target population and intended product format.
ii. Identification of most relevant *in vitro* selection tests, usually more than one, in order to select most appropriate candidate strains. The test(s) based on a mechanistic hypothesis may later serve as supporting evidence for the substantiation of the health claim.
iii. Verify technological and organoleptic properties of the strains.
iv. Conduct animal studies; if possible or if relevant, use more than one recognized model.
v. Deposit strain in international strain collection for research purposes, restricting access, if desired, for commercial purposes.
vi. Perform pilot clinical trial in the target population followed by at least one double blind placebo controlled (DBPC) intervention study to support purported health claim.
vii. Build scientific dossier and present dossier to regulatory authorities.
viii. Translate scientific evidence into a claim that is properly understood by the target population.

3.2 Good Practice Guidelines

Whilst it was considered premature under existing knowledge to establish standardized procedures for any one specific physiological or functional test, a series of good practice guidelines could be established. While some of these guidelines might not be applicable for every specific case of use for a probiotic microorganism, they could provide a framework for evaluating the strength of support of physiological function. The working group came up with a number of recommendations concerning microbial aspects that are too often insufficiently taken into account or described in publications (Table 2).

Table 2. Requirements to fulfill when conducting *in vitro* and *in vivo* studies with probiotic strains and probiotic containing products.

Requirements
Work with properly identified strains.
Specify the correct name and number of the strain and from what source it was isolated.
Check purity of the strain and when relevant precisely describe probiotic mixes/combinations (identification and relative counts of the individual strains).
Proper assessment of safety based on the characteristics of the genus, species and strain, intended use and target consumer of any microbe newly proposed as a probiotic must be conducted. One component of this assessment is determination of the potential risk of transferability of phenotypic antibiotic resistances observed for the strain.
Describe growth conditions and growth phase at which strains were collected as well as final counts/dose administered in the study.
Standardize these conditions when comparing strains to each other.
Specify conditions of the probiotic preparation or post-harvesting treatments (e.g., washes, freezing, buffer).
Use reference strains (or reference compounds) that should ideally be included in successive tests when the assays show high technical variability.
Determine degree of strain-specificity of effects by including additional (number depending on the complexity of the assay) strains in the assay.
Run dose-response curves.
Include appropriate controls (e.g. to eliminate effect of acidification by the strains).
Perform statistical analysis of the results and ensure statistical power and significance.

4. Conclusion

The development of standard criteria for probiotic selection is difficult due to the diversity of microbes used as probiotics, the range of conceivable physiological endpoints, the different mechanisms that may operate to achieve an overall result and different possible end users. The colonized environments of the human body remain incompletely described. Additionally, characteristics of the cross-talk between a probiotic strain and the intestinal microbiota and the host, which takes place into a dynamic and complex ecological niche, must be elucidated. However, the use of well-controlled *in vitro* and *in vivo* assays is important for selecting the best candidate strain for a specific intended use, being aware that the ultimate proof of health promoting effects will come from human intervention trials. In the course of pre-clinical studies, the physiological properties of a candidate strain should be investigated in-depth and should be well-documented. This information will enable a thorough characterization of the strain and could be helpful support for obtaining approval of ethical committees when preparing clinical trials, and when building the scientific dossier necessary to support health claims. Preliminary recommendations concerning the process and practice of establishing the probiotic properties of a strain are provided. Unraveling the mechanisms, identifying relevant biomarkers, standardizing microbial methods and investing efforts to establish a correlation between *in vitro* and *in vivo* assays, and human trials, will all help this field to progress. To this end, the IDF work programme was carried forward to the ILSI Europe Task Force and Expert Group on Probiotics that are currently preparing guidance for assessing probiotic beneficial effects, which will be reviewed in a scientific workshop before publication in a peer-reviewed journal.

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