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Current Opinion in Biotechnology

# Application of probiotics in food products—challenges and new approaches

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The probiotic research conducted over the past 20 years has resulted in a valuable source of data related to health beneficial effects of probiotics. Nevertheless, documentation of probiotic benefits remains challenging, especially in functional foods that are designed for the generally healthy population that, however, regularly experiences episodes of 'suboptimal' health. In addition, in view of today's application of probiotics in an increasing variety of food matrixes, process optimization and product design need to take into account cell viability and probiotic function altogether. To meet this challenge, medium to high-throughput bioassays – based on the identification of active compounds and their mechanism of action – have to be developed and their predictive value established. Together with validated biomarkers for health and disease, this should help rationalize probiotic product development and associated health claim substantiation in human studies.

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**Current Opinion in Biotechnology** 2010, **21**:175–181

This review comes from a themed issue on
Food biotechnology
Edited by Dietrich Knorr and Carmen Wacher

Available online 21st April 2010

0958-1669/$ – see front matter


**DOI** 10.1016/j.copbio.2010.03.009

## Introduction from first hypothesis to scientific approach

The health-promoting use of fermented milk products started a long time before the existence of microorganisms and lactic acid bacteria was discovered. The early written records go back to 76 B.C. when Roman historian Plinio (Plinius) described their use in the therapy of various gastro-intestinal infections [1]. However, it was only after the invention of the microscope and the discovery of bacteria and especially lactic acid bacteria (LAB) in the 17th, 18th, and 19th centuries, respectively, that the scientific basis of the probiotic concept was set with the theories of Elie Metchnikoff at the beginning of the 20th century [2]. He postulated that consumption of fermented milk would suppress the growth of proteolytic bacteria and thereby reduce putrefaction in the gut, thus prolonging the life span of the host. Soon after his postulate, strains of LAB and bifidobacteria were applied as supplements and over-the counter drugs for treatment of diarrhea (e.g. *Lactobacillus* LB Lactéol in 1907, *Escherichia coli* Nissle 1917, end of 1920s) and in food products for promotion of intestinal health and prevention of disease (e.g. *Lactobacillus acidophilus* L-92 in 1910, *Lactobacillus casei* Shirota in 1935). Note that two of these early products (Lactéol and L-92) were heat inactivated. Probiotics were also introduced in animal feed in the 1970s as supplements for the promotion of animal growth and improvement of their resistance to disease [3]. Approximately one century after the initial probiotic concept, the transition from theory to scientific documentation of beneficial effects of probiotics had begun. Apart from some pioneering work in the early days, concerted efforts to demonstrate health beneficial effects of probiotics mainly started in the 1980s. The volume of research rapidly accelerated after the year 2000 such that to date, more than 700 human intervention trials have been conducted. In solely 2008, more than 1000 articles and reviews were published on the subject and more than 2000 probiotic products launched (Figure 1).

The aim of this paper is to review the probiotic development in the past years as well as the current challenges of using probiotics in different food matrixes.

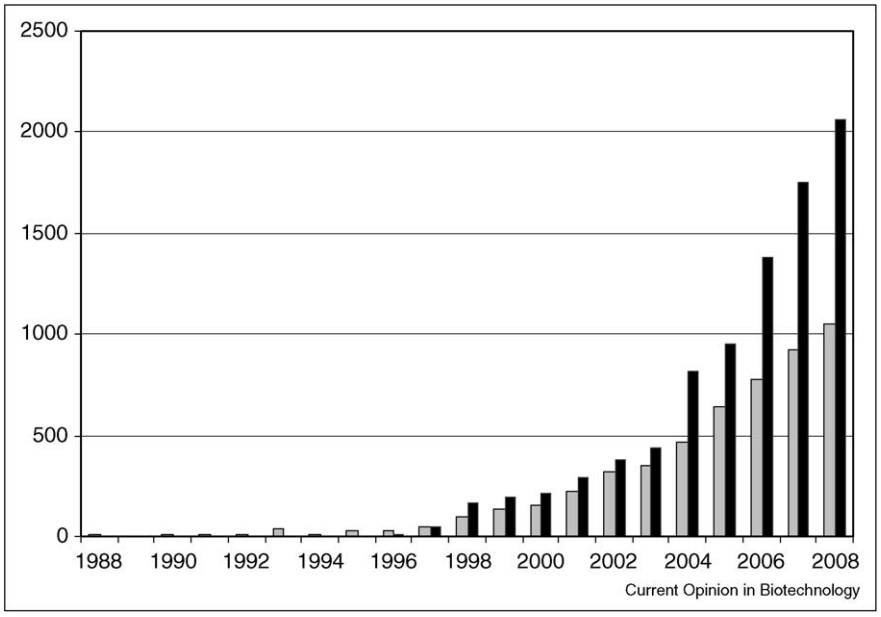
## Health beneficial effects of probiotics

### The role of probiotics as functional ingredients in food

Generally, throughout life healthy individuals suffer from periods of suboptimal health. This can be caused by respiratory or gut infections or by other external stimuli that challenge the immune system [4], and often alter the microbiota and weaken mucosal barrier function. A suboptimal state of health can also be a consequence of chronic stresses, such as chronic infections, fatigue, exercise, use of medications, psychological stresses, and many other challenges resulting in downregulation or weakening of the natural defenses of the organism [4,5]. It is also well established that psychological stresses like depression or anxiety can influence digestive function and symptom perception [6] thereby resulting in gut discomfort.

The role of functional foods is to benefit human health beyond the effect of nutrients [7]. Situated between foods, which supply basic physiological functions and drugs that treat diseases, functional foods are used to

**Figure 1**




Publications and probiotic products. In gray: number of publications on probiotics published per year (source PubMed, http://www.ncbi.nlm.nih.gov/sites/entrez). In black: number of products containing probiotics launched per year (source Global New Products Database, http://www.gnpd.com).

maintain good health and counterbalance small physiological disorders that healthy hosts may experience. In addition to the well-established functional ingredients such as vitamins, minerals, and micronutrients, probiotics belong to the emerging generation of active ingredients that includes prebiotics, phytonutrients, and lipids, for example.

Historically, the development of probiotics was very much oriented toward pharmaceutical applications such as treatment of diarrhea, prevention of antibiotic-associated diarrhea, management of stomach and gastro-intestinal infections, management of chronic inflammation, and so on. However, these effects are not easily extended to the category of functional foods that are destined for the generally healthy population. Although beneficial effects of specific probiotics have been demonstrated in the treatment and prevention of several health disorders, the remaining challenge is to demonstrate long-term effects of probiotic foods as presently required by health claim regulations in Europe. As large trials of long duration are difficult to support, particularly for small and medium-sized laboratories and food companies, there is an urgent need to better identify and validate risk factors of diseases and biomarkers of health.

## Probiotics and microbiota balance

Microbiota balance is the oldest proposed probiotic benefit. Metchnikoff defined it as 'seeding' of the intestinal tract with harmless LAB that suppress the growth of harmful proteolytic bacteria. Nowadays such a benefit is usually interpreted as an increase in lactobacilli and/or bifidobacteria and a decrease in potentially pathogenic bacteria. In the past 20 years, it has been demonstrated that it is possible to transiently modify the composition of the gut microbiota of healthy individuals in favor of lactobacilli and bifidobacteria species upon ingestion of some probiotics [8]. It was also shown that infants fed probiotic infant formula have similar fecal levels of bifidobacteria as breast fed infants [9]. However, it remains difficult to link such changes with a benefit in healthy populations, although it is well established that dysbioses are associated to conditions such as chronic inflammatory disorders [5], obesity [10], or allergy [11]. One of the best studied examples of how microbiota dysbiosis affects health is seen in Crohn's disease (CD). A decrease in the global biodiversity of intestinal bacteria, particularly within the phylum Firmicutes, has been observed in CD patients [5]. Recent analyses have revealed that a lower level of *Faecalibacterium prausnitzii*, a major member of Firmicutes is associated with a higher risk of postoperative recurrence of ileal CD. Oral administration of live *F. prausnitzii* or its culture supernatant reduced the severity of TNBS induced colitis in mice indicating that counterbalancing dysbiosis might be a promising strategy in CD treatment [12••].

Although it is not known whether alteration in the microbiota is a cause or a consequence of a pathophysiological situation, the aforementioned examples underline the fact that an equilibrated microbiota is of high importance for health maintenance. However, the challenge will be to

demonstrate i) what is the composition and function of a balanced microbiota and ii) its long-term impact on health. Efforts are currently ongoing to determine the composition and functionality of the microbiota in healthy and diseased populations. A systems biology approach using techniques such as high-throughput microbiota diversity diagnostic arrays [13•], metagenomics (http://www.metahit.eu) [14], proteomics [15•], metabonomics [16], and high-throughput phenotyping of metagenomic clones [17] will progressively provide a better definition of the composition and function of a 'healthy' microbiota. Markers of 'healthy' microbiota will permit to definitively support Metchnikoff's theory of 'balanced microbiota' and its impact on health.

### Probiotics and immune system

Since the early studies of mucosal immunity in the 1970s, a lot of progress has been made in understanding the mode of interaction between the gut microbiota and the immune system (e.g. see review [18]). The ability of exogenous probiotics to improve clinical outcomes through modulation of the immune response has been demonstrated in subjects with chronic and acute diseases. For example, the probiotic mix VSL#3 was shown to reduce pouchitis relapse [19,20] and to improve clinical scores in ulcerative colitis patients [21,22] through improvement of the inflammatory status of the patients. *Lactobacillus rhamnosus* LGG given to infants during episodes of acute rotavirus diarrhea resulted in greater increase in non-specific antibody secreting cells and specific anti-rotavirus antibodies in the circulation than that seen in the placebo group and resulted in shorter duration of the diarrhea [23,24]. Furthermore, beneficial immunomodulatory effects of specific probiotics have been observed for *H. pylori*-associated gastritis [25], development of allergies, or reduction of allergy symptom scores (see [26]).

Immune stimulatory effects have been established in generally healthy population as well. An improved specific immune response to a *S. typhi* oral vaccine has been described in individuals consuming a probiotic mix containing *L. johnsonii* La1 and *B. lactis* BB12 [27] and to influenza vaccine in subjects receiving *L. fermentum* CECT5716 [28]. Furthermore, Natural Killer (NK) and phagocytic cell activity was increased in healthy elderly or adults upon ingestion of *B. lactis* HN019 [29] or *L. johnsonii* La1 [30,31], respectively. Finally, a few studies have shown a link between activated immune markers and improved resistance to infections. For example the probiotic mix containing *L. gasseri* PA16/8, *B. longum* SP07/3 and *B. bifidum* MF 20/5 shortened the duration of common cold episodes and reduced fever, while at the same time increasing the number of leucocytes, lymphocytes (specially T-lymphocytes) and monocytes [8]. Studies such as the latter, which demonstrate a link between immune markers and improved resistance to disease will greatly help in proving the benefit of probiotic foods for the healthy population as well as in validating immune markers.

### Intestinal discomfort

Periods of gastro-intestinal discomfort are very frequent in otherwise healthy individuals. Irritable bowel syndrome (IBS), one of the most common disorders seen by primary care physicians, affects 7–10% of the world population [32]. In the absence of an efficient therapy with no side effects, well selected probiotic strains might provide a valuable alternative. Certainly, a significant reduction in IBS symptoms has been observed after intervention with probiotics such as *B. infantis* 35624 [33•] or with a probiotic mix [34]. As another example of intestinal discomfort, constipation that represents the most prevalent complaint among the general adult population was shown to be regulated by a probiotic fermented milk containing *B. animalis* DN-173010 [35,36]. Finally, successful treatment of colicky symptoms, frequent in newborn infants, was achieved using probiotic *L. reuteri* ATCC55730 [37•].

### Probiotic effects beyond the gut

It is important to note that a positive impact of probiotics is progressively being demonstrated beyond the gut. To cite only a few examples, oral intake of *Lactobacillus rhamnosus* GR-1 and *Lactobacillus fermentum* RC-14 had a positive impact on vaginal health [38], *Lactobacillus paracasei* ST11 improved recovery of skin immune homeostasis [39•], and *Streptococcus salivarius* K12 improved oral malodour parameters [40]. Signalling of intestinal microorganisms to the gut–brain axis is an actively emerging field of research [41•]. Apart from animal studies that showed impact of probiotics on anxiety, mood, and behavior, the first human trials suggest that probiotic interventions may modulate mood and stress induced gastro-intestinal symptoms [42,43].

## Probiotic production process and probiotic functionality

### Cultivation of probiotics

In addition to providing added value to food, probiotics need to be cost effectively produced, which implies maximizing substrate-to-biomass yield and stability during processing and shelf life.

In the present state of knowledge, it remains difficult to anticipate to which extent growth conditions of probiotics may affect their functional properties. Therefore, the consequences of changes in growth conditions to achieve higher biomass yield, which may for example alter bacterial components with purported probiotic activity, may be overlooked. For instance, Gitton *et al.* [44] highlighted that the global proteomic pattern differed when *L. lactis* was cultivated in M17Lac broth, milk microfiltrate, or milk. Moreover, the time of harvesting may also influence the exerted functional properties. Fayol-Messaoudi *et al.*

[45] demonstrated that apart from growth temperature the *in vitro* anti-pathogen activity of the studied probiotics depends largely on the growth stage at which the cells were collected. Similarly, the expression profiles of human mucosa (duodenal biopsies) displayed differences in modulation of NF-κB-dependent pathways after consumption of *L. plantarum* harvested at different growth phases or when heat treated [46••].

Thus it would be of great advantage to identify bioactive components of probiotics that can be measured under different growth conditions in order to ensure that probiotic functionality will be optimized.

### Stability throughout process and storage

For production of probiotics in dried form, which provides a longer shelf life than liquid products, the challenge is to master loss of viability due to removal of water, exposure to oxygen, and eventually high temperature during drying. Further, the stability over shelf life period, which is dictated by physical parameters of the final product matrix and the storage conditions, has to be ensured. In most cases viability loss during storage is more drastic than during processing. Improvement of stability in powders can be achieved for instance by use of protective agents/encapsulation material, application of mild environmental sublethal stresses either during or after fermentation as recently reviewed by Muller *et al.* [47•].

Use of protective agents is a well-known strategy to increase the drying tolerance of strains. However, the potential impact on the physiology of the bacterial cells should also be considered. For instance, Reddy *et al.* [48] reported variation in *in vitro* cholesterol assimilation and acid tolerance upon spray drying of probiotics with different types of protective agents or carriers. Finally, different matrixes may influence the survival and functionality of probiotics. Corcoran *et al.* [49•] published that the presence of a metabolizable sugar could markedly enhance survival of *L. rhamnosus* LGG in simulated stomach conditions.

Alternative strategies to address this type of questions start to emerge. For example, accelerated evolution was applied to select a heat-shock resistant 'spontaneous' mutant of *B. longum* NCC 2705 [50•]. The differential transcriptomic profile of mutant and wild type strains highlighted the constitutive overexpression of the classical heat-shock regulon dnaK in the mutant, which has further proven to exhibit higher survival during down stream processing (W. Sybesma, unpublished data). Importantly, the mutations introduced in the strain did not influence its functionality, as far as was demonstrated in a rotavirus induced diarrhea mouse model (N. Pagé, L. Hammarström, personal communication).

With regard to liquid probiotic application, beyond maintenance of high levels of viable cells, also post-acidification and taste deterioration have to be prevented. Among several factors, oxygen has been identified as the key one that influences survival in liquid products. Strategies to protect probiotics against oxygen were discussed elsewhere [51].

### Rehydration

A last topic related to probiotic stability and viability assessment, which is often neglected, is the influence of the rehydration step. Several studies established that depending on the applied reconstitution conditions such as buffer [52], pH, duration, sugar content [53], and rehydration temperature [54], the difference in the final cell count could vary up to 1 log cycle. These observations indicate that a large proportion of the probiotic bacteria may be killed or made unculturable depending on the rehydration conditions. Hence, the conditions of the applied enumeration methods are relevant for the interpretation of the stability and viability data of probiotics.

Furthermore, it remains to be answered to what extent the living, the non-culturable and/or non-replicating probiotics contribute to delivering a functional benefit. In the past years studies have reported that non-replicating probiotics may still deliver specific health benefits [55•]. It is noteworthy that probably all products containing live probiotics will also contain a portion of dead or damaged cells. Many of these products are down stream processed in presence of at least part of the spent culture medium. Hence, it cannot always be ascertained if the claimed functional effects of probiotic preparations are delivered by the biomass, intracellular or extracellular cell components, and/or media derived bioactive compounds (Table 1).

For these reasons, it is presently recommended to conduct clinical trials with the final formulation of the ingredient or product until predictive and validated functional bioassays can be applied.

## Safety of probiotics

The application of probiotic microorganisms in foodstuffs requires a thorough safety assessment. Several guidelines are available on how to assess the safety of probiotics used in food applications [56–59].

The European Food Safety Authority (EFSA) has developed the QPS (Qualified Presumption of Safety) approach as a tool for the safety assessment of microorganisms used in food. This is based on a documented history of use and knowledge of potential pathogenic or toxicogenic properties associated to a particular genus and species. In the US, probiotic microorganisms would be assessed via the GRAS (Generally Recognized As Safe) system. For example, *B. lactis* BB12, *L. rhamnosus* LGG, *L. reuteri* DSM 17938 strains have over recent years been

**Table 1**

**Different concepts of probiotic production processes and related potential probiotic derived bioactive compound.**

| Production Process | Potential Probiotic Derived Bioactive Compounds | | | | | Products/Examples |
|---|---|---|---|---|---|---|
| | Living probiotic cell | Fermented medium component | Intracellular components | Extracellular components | Killed probiotic cell | |
| Fermented product as a whole | Yes | Yes | Yes | Yes | Yes | Fermented yogurt drinks with probiotics |
| Cultivated and down stream processed* without culture medium | Yes | No | Yes | Yes | Yes | Dried culture powders |
| Cultivated and down stream processed* without cells | No | Yes | No | Yes | No | Spent culture medium, for example, see [64] |
| Cultivated, killed and downstream processed with or without culture medium | No | Yes | Yes | Yes | Yes | Heat treated lactobacilli, for example, see [65] |
| Potential mode of action/main active component | Signalling through metabolites or cell components or activity through direct contact | Lactic acid derived from carbohydrates, bioactive peptides derived from milk proteins or raw materials | DNA, cellular proteins or peptides | Bacteriocins, antimicrobial peptides, cell wall components | Activity through direct contact or by cell components | |

* For example, frozen, spray dried, freeze dried, encapsulated.

**Table 2**

**Comparison of assessment schemes for microorganisms used in food by US FDA GRAS and EU EFSA QPS systems.**

| GRAS guidelines | QPS guidelines |
|---|---|
| Applies to food additives in general | Applies to microorganisms only |
| Determination of GRAS status by FDA and/or external experts | Determination of QPS status by EFSA |
| Open list | Positive list |
| Based on common use | Based on history of use and adverse effects |
| Describes specific substance or microorganism | Describes taxonomic unit (e.g. genus, species, or strain) |
| Case-by-case assessment | General assessment |

Adapted from Wassenaar 2008 [66].

accepted in the US as GRAS for their intended use. The main differences between the two approaches are summarized in Table 2. EU-funded research projects such as ACE-ART [60] and PRO-SAFE [61] addressed the issue of antibiotic genes in probiotic and starter strains, and initiated an evaluation of assays that can be used to assess biosafety of probiotics.

Data requirements to assess the safety of probiotics can vary depending on the bacterial species of interest, the intended application/use and/or the target populations. Parameters such as taxonomy and identification, phenotypic characterization, history of food use, and human exposure, and so on, are generally considered important [62,63]. If no history of safe use can be demonstrated, extensive preclinical studies, including standard 90-day toxicity studies as defined in OECD Testing Guideline 408, should also be considered. Clinical studies should include parameters to demonstrate safety in use or tolerability in the target population(s).

Rare cases of adverse effects linked to probiotic administration have been documented in individuals having serious underlying disease. Thus, special care must be taken with particularly vulnerable target population(s) such as neonates, immunocompromised subjects, or critically ill/hospitalized patients [63]. Overall the vast amount of available data and long history of use in foodstuffs have not indicated any safety concerns for currently used probiotics (mainly lactobacilli and bifidobacteria) in healthy populations.

## Conclusion and outlook

The role of functional foods is to promote the host's health or to restore it when it is transiently affected. Demonstration of the health beneficial effects of probiotics in generally healthy humans currently remains a challenging task due to the lack of validated biomarkers of health and risk factors of diseases, and the need to undertake generally large and long-term human trials. An additional challenge lies in probiotic production in a cost

effective way that will ensure probiotic viability over shelf life. One should keep in mind that inclusion of probiotics in increasingly diverse food vehicles requires product and process modifications that may bear the risk of modulating the probiotic functionality, as suggested so far mostly by *in vitro* testing.

Therefore research and development in the probiotic area should dedicate sustained efforts in the development and validation of biomarkers of health and disease, the design of functional assays with predictive value, and the identification of bioactive molecules in probiotic products. This will strongly depend on continuous efforts to identify mechanisms of action of probiotics. With these tools in hands it will be able to rationalize and optimize selection of probiotic candidates and downstream processing, and to evaluate potential effects of the food matrixes on probiotic functionality. This in turns should help to better design the human trials required for health claim substantiation.

## Acknowledgement

We would like to thank Dr. Anne Constable for critical reviewing the safety section of the manuscript.

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- • of special interest
- •• of outstanding interest


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