Research Article Omics of probiotic bacteria: which features to seek?

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ABSTRACT. Probiotics are live nonpathogenic microorganisms extensively used in food, pharmaceutical and medicinal industries. Recently, attention has focused on specific features of probiotics and on the abilities of some long known and recently described species of this group. In general, desired features of probiotics include resistance to acid and bile salts to avoid dysbiosis and induction of immune system development. The advent of next-generation sequencing technology has propelled the genomic area, allowing a search for probiotic features in a wide range of probiotic species, especially bacteria. In this context, functional genomics analyses can help interpret big data, correlating the findings with comparative genomics analyses, in a search for direct applications. To select the articles in this review, we used the following indexing terms: (probiotics OR probiosis) AND (genomics OR transcriptomics OR

proteomics OR metabolomics OR culturomics) AND bacteria. Proteomics and transcriptomics methodologies reveal important information about proteins and transcripts differentially expressed under specific conditions that mimic host environments in health and disease. In addition, new research approaches have been developed for probiotics, such as metabiotic and metagenomic analyses of host microbiota. Also, we examined probiotic related features, including bacterial safety aspects; tolerance towards digestive constraints, such as gastric juice and bile salts; bacterial pathogen exclusion mechanisms; adhesion-related genes; antimicrobial peptides; immune development and function; omics; metagenomics; culturomics; functional genomics; transcriptomics; proteomics; metabiotics and metabolomics. In summary, currently there is considerable interest in probiotic bacteria, and structural and functional genomics analyses have potential to help research in this area.

Key words: Omics approach; Probiotics; Metabiotics; Genomics; Culturomics

INTRODUCTION

Microorganisms responsible for food fermentation and to prevent putrefaction have been used by man during centuries (Salque et al., 2013; Yang et al., 2014). It is commonly accepted that the fermentation processes adapted by man appeared due to accidental contamination and appropriate climate and environment, resulting in widely used fermented products, such as *kefir, leben* and *koumiss* (Hosono, 1992). In addition to the possibility to store fermented foods, to their enhanced nutritional value and to their safety for consumption, fermented food has had great cultural importance, highlighted by citations of some of these products in the Holy Bible and sacred books of Hinduism (Bibel, 1988; Hosono, 1992; Shortt, 1999). However, it was only after the 19th century that the fermentation process was studied and that probiotic concepts were introduced by Louis Pasteur and Élie Metchnikoff (Johnson and Klaenhammer, 2014), the fathers of microbiology and innate immunology, respectively. Later on, *Bacillus bulgaricus* (currently known as *Lactobacillus delbrukeckii* subsp. *bulgaricus*) was recovered from human feces and it was shown to reduce putrefaction toxins and help in colitis treatment (Johnson and Klaenhammer, 2014).

In 1930, a Japanese microbiologist isolated a species from human feces that survived the gastrointestinal tract (GIT), identified as *Lactobacillus casei*, which was later used to develop the fermented milk product *Yakult* (Shortt, 1999). Besides the use of these probiotics in the production of fermented milk, other lactic acid bacteria (LAB) are also used in the preservation of vegetables, grains and meat (Chaillou et al., 2005). Recently, probiotics have been crossing barriers from the functional food market to pharmaceutical and therapeutic uses. This expansion is directly correlated with advances in the scientific and regulatory aspects of LAB related probiotics and the study of their protein delivery mechanisms (Bolotin et al., 2001; Foligné et al., 2013).

Nowadays, probiotics are widely known for their use in the treatment of functional gastrointestinal disorders (FGID) including irritable bowel syndrome, Crohn's disease and

ulcerative colitis, in conjunction with usual medical treatments (Bibiloni et al., 2005; Sood et al., 2009; Tursi et al., 2010). This has occurred for several reasons, but mainly because there are few options of pharmaceutical treatments for FGID, and conventional treatment options have low efficacy and serious side effects (Shen and Nahas, 2009). FGID are very common and are believed to be the cause or consequence of changes in gastrointestinal microbiota (Porter et al., 2011). Consequently, probiotics have become a useful complement to the usual treatment of such diseases.

Probiotics have been found to be a favorable option not only against FGID, but also for a wide range of disorders, because they can reinforce the gut barrier function, conferring clinical benefits at distant sites through their immunomodulatory activities (Bo et al., 2014). Some studies have shown the beneficial effects of probiotics in modulating inflammatory and autoimmune diseases, such as rheumatoid arthritis (So et al., 2008), type I diabetes (Calcinaro et al., 2005), multiple sclerosis (Lavasani et al., 2010), atopic dermatitis (Viljanen et al., 2005), and myasthenia gravis (Chae et al., 2012). Moreover, experimental results strongly suggest that selected strains of probiotics can help in the treatment of cancer, neurodegenerative diseases, metabolic syndrome and psychiatric illnesses, among other pathologies. Also, there is evidence for reduction of ventilator-associated pneumonia in intensive care units patients under mechanical ventilation (Bo et al., 2014). However, one of the challenges in preclinical and clinical use of probiotics is how to select a strain with potent immune modulating properties (Chae et al., 2012).

In view of this challenge, there is a growing interest in the study of probiotic bacteria through structural and functional genomics for the discovery of probiotic-related features. Because of the development of NGS, the bacterial whole-genome sequencing has become a low cost and suitable approach for fast and accurate screening of potential probiotic candidates for treatment of each type of disorder (Didelot et al., 2012; Senan et al., 2015). This approach allows researchers to detect and discard candidate strains that have potential risk factors, such as antibiotic resistance or virulence genes. It also facilitates the analysis and description of functional mechanisms, avoiding the difficulties of isolating and growing the microorganisms (Papadimitriou et al., 2015). This new approach using NGS techniques to screen potential probiotic candidate strains makes it imperative that we understand the genomic features that should be prioritized and sought in new strains.

Probiotic related features

As a first step, the simplified definition of probiotic bacteria is originally related to live cultures that help in the maintenance of a healthy and balanced intestinal microbiota (Cronin et al., 2011). Specifically, for the GIT, probiotic features have been elucidated through the fusion of structural and functional genomics techniques. In this context, three main features of the mechanisms of probiotic action deserve attention: (i) survival through GIT passage (bile salts and gastric acidity) (Bezkorovainy, 2001), (ii) competitive exclusion and antimicrobial activity, such as microcin and hydrogen peroxide production (Konuray and Erginkaya, 2018) and (iii) modulation of the immune system of the host GIT (Johnson and Klaenhammer, 2014) (Figure 1). This includes molecular associated molecular patterns (MAMP) which may be recognized by host pattern recognition receptors (PRR) (Lebeer et al., 2010). Those mechanisms will be further described in the next sections.

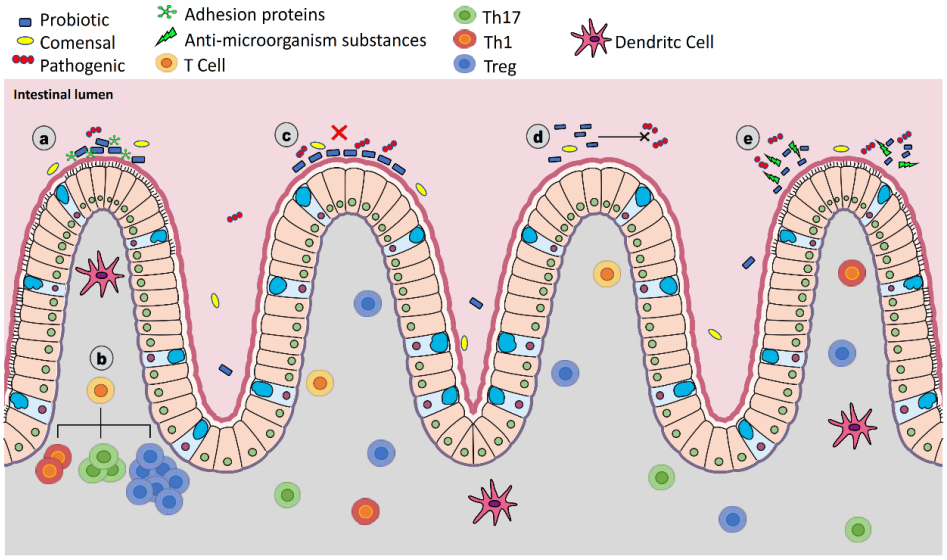

Figure 1. After surviving gastric juice and bile salts, probiotic organisms can act through specific mechanisms: (**a**) adhesion and colonization, (**b**) modulation of the immune system, (**c**) enhancement of the epithelial barrier, (**d**) competitive exclusion, (**e**) production of anti-microbial substances.

Various bacterial genera and species are used as probiotics, for instance: *Lacticaseibacillus casei*, *Lactobacillus delbrueckii*, *Lactobacillus acidophilus*, *Lactiplantibacillus plantarum*, *Limosilactobacillus fermentum*, *Limosilactobacillus reuteri*, *Bifidobacterium breve, Bifidobacterium longum, Bifidobacterium lactis, Propionibacterium freudenreichii, Bacillus subtilis, Bacillus cereus, E. coli* and *Enterococcus faecium*. All of these species have therapeutic applications in prevention and treatment of intestinal disorders, such as diarrhea in newborns (Ouwehand et al., 2002).

The first widely known scientific report about probiotic bacteria dates back to 1907 and demonstrated the correlation between the ingestion of LAB and the increase in longevity of Bulgarians and other populations (Howell, 1988). Fermented foods produced with the use of many bacteria of the *Lactobacillus* genus have been widely employed as therapeutics for the prevention or treatment of diseases due to their beneficial properties, such as relief of lactose intolerance symptoms and decrease in diarrhea due to rotavirus (Ouwehand et al., 2002).

Recently, most of studies and utilization of probiotics involve LAB, especially *Lactobacillus* isolated from the GIT. When it comes to the administration of probiotic bacteria, a certain amount of bacteria are necessary to exert a desired feature and consequent host response, which may vary according to the strain, use, and formulation; it is generally recommended to consume at least 10^7 microorganisms per milliliter in a daily dose (Corcoran et al., 2008).

Bacterial safety aspects

Over time, there are changes in the content and in order of genetic information of organisms due to genomic plasticity and evolutionary pressure (Soares et al., 2011). Genomic plasticity is the dynamic property of DNA that arises from genetic conversion and point mutations, rearrangements (through translocation and inversion, for example), deletions, and insertion of genetic material from other organisms (plasmids, transposons, bacteriophages, among others). These mechanisms alter the bacterial lifestyle, contributing to their adaptation to different environments and influencing their evolution (Schmidt and Hensel, 2004).

The detection and the analysis of phage regions plays a key role in the elucidation of the genomic plasticity of probiotic bacteria because they are used in fermented products for human consumption and therefore cannot harbor mobile elements that could be transferred to other bacteria. Phages are obligate parasites and most of them have a multiplication cycle that culminates in cellular lysis, through which hundreds of viral particles are released, ready to infect nearby cells (Summers, 2005). Moreover, phages are widely distributed all over the world; it is possible to find up to 10^8 phages in just a drop of water from the ocean (Wommack and Colwell, 2000).

All industrial or biotechnological processes that require bacterial use for the production of fermented food products or of useful molecules could be rapidly interrupted by virulent phages. They are a primary cause of failure in the fermentative process during the industrial transformation of milk (Garneau and Moineau, 2011).

The first description of phages infecting dairy starter dates from 1935; since then, important improvements have been made, particularly in knowledge about bacterial ecology, phage genomics and resistance to environmental factors (Brüssow, 2001). There are reports of phage regions in *Lactococcus* species, such as *Lactococcus lactis* (Cavanagh et al., 2014). However, even with all the advances in the area, phage contaminations still damage fermented milk products and reduce productivity (Moineau and Lévesque, 2004). Phages may can have various origins; therefore, it is very important to study all potential sources of contamination and their consequences for the production of fermented dairy products (Garneau and Moineau, 2011).

Another way that bacteria acquire genomic material is through genomic islands (GEI). GEI may be classified as pathogenicity islands (PAI), metabolic islands (MI), symbiotic islands (SI) and resistance islands (RI). They are large genomic regions acquired through horizontal gene transfer, harboring a large number of genes (encoding similar functions and operons) with the potential to allow the bacteria to evolve in leaps (Soares et al., 2011)

Probiotic bacteria should be analyzed for PAI and RI, which contain a high concentration of virulence factors and antibiotic resistance genes, respectively, and could be transferred to other organisms, compromising the safety aspects of the bacteria. Probiotic bacteria should only contain natural resistance, with no trace of virulence factors or antibiotic resistance genes in unstable regions, such as GEI, phages, and plasmids (Salminen et al., 1998).

There are some specific aspects that characterize probiotic action within the host GIT that will be discussed in the next section. Genomic islands related to interaction with the host and to persistence within its digestive tract have also been described by

comparative genomics (Kankainen et al., 2009). Their instability over time and in cultures evidenced the need for quality assurance and control measures targeting genome stability in probiotics (Sybesma et al., 2013).

Tolerance towards digestive constraints, gastric juice and bile salts

One of the most important attributes of a probiotic microorganism is its ability to survive the GIT environment. A study with comparative genomics analyzed the niche-based stress-responsive genes of two *Lactobacillus helveticus* strains: MTCC 5463 (a potential probiotic) and DPC4571 (a cheese starter); 5463 apparently has many genes involved in stress response. This potential probiotic strain has a larger number of genes related to heat, osmotic, cold and oxidative stress resistance than DPC 4571 (Senan et al., 2014).

Functional genomics studies have been complementing and elucidating some questions related to stress response. Using transcriptomics and proteomics, a study of the probiotic *Lacticaseibacillus rhamnosus* GG analyzed the effects of bile stress and demonstrated that 316 transcripts changed in expression level and 42 proteins (intracellular and surface-exposed), were differentially abundant. The the changes were associated with the adaptation process of this bacterium (Koskenniemi et al., 2011). Performing the same omics study on *Bifidobacterium longum* BBMN68, the expression level of 236 transcripts changed significantly and 44 proteins were differently abundant. A hypothesis involving the modification of cell membrane composition (cyclopropane fatty acid increases and transmembrane proteins decreases) was confirmed with a surface hydrophobicity assay (An et al., 2014).

Bacterial pathogens exclusion mechanisms

Competitive exclusion of pathogens is another criterion in the selection of probiotic bacteria. Among the mechanisms of action, there are, for instance, enhancement of the epithelial barrier, production of anti-microbial substances, competitive exclusion of bacterial pathogens, increased adhesion to intestinal mucosa and modulation of the immune system (Bermudez-Brito et al., 2012). Associated features may include surface compounds involved in aggregation, coaggregation, and adhesion, as well as specific biosynthesis pathways.

Adhesion-related genes

In addition to survival through the GIT, adhesion to the intestinal epithelium is another factor that may contribute to probiotic activity, through exclusion mechanisms. The interaction between microbe and host occurs via adhesion-related proteins that recognize and bind to specific receptor regions of the host cell, activating the innate response, promoting invasion or bacterial colonization. Adhesion may be mediated by pili or fimbriae extending out from the bacterial cell wall or Microbial Surface Components Recognizing Adhesive Matrix Molecules (MSCRAMMs) (Soto and Hultgren, 1999).

Preliminary *in vitro* studies using intestinal epithelial cells revealed multiple probiotic *Lactobacillus* producing adhesins (Chauviere et al., 1992; Tuomola and Salminen, 1998) and *Bifidobacterium* spp. was shown to adhere to the human intestinal mucus (He et

al., 2001). Access to the genomic sequence data combined with genomic techniques helped to elucidate the adhesion mediators. Most of these are secreted or bound to the cell wall in a sortase-dependent way, aiming to interact with the intestinal epithelium (Vélez et al., 2007; Lebeer et al., 2008). Studying 43 *Lactobacillus* strains, Harris et al. (2017) used Cluster of Orthologous Groups (COG) to identify at least one sortase A gene for each. Seven genomes among them have an extra sortase A, of which, five have a sortase C gene and a putative pilus operon.

In *L. acidophilus* NCFM, an *in silico* search on the genome allowed the identification of five cell surface adhesion proteins, including: one fibronectin binding protein (FbpA), one S-layer protein (SlpA), one mucin binding protein (Mub) and two homologous R28 proteins involved in *Streptococcus* adhesion (Buck et al., 2005).

Through mutational analyses, FbpA, SlpA, and Mub were shown to contribute to adhesion to Caco-2 epithelial cells. Similarly, one stress response protein and one aggregation promoting factor (both surface proteins) were later found in other studies, which are also contribute to adherence to Caco-2 cells (Goh and Klaenhammer, 2010; O'Flaherty and Klaenhammer, 2010).

Slp genes may indeed be involved in adhesion to host cells in various probiotics such as lactobacilli (do Carmo et al., 2018) and propionibacteria (do Carmo et al., 2017). Inactivation of the corresponding Slp gene accordingly suppresses interaction with host cells in *L. acidophilus* (Konstantinov et al., 2008) and in *P. freudenreichii* (do Carmo et al., 2017).

Comparative genomics was used with two *L. rhamnosus* strains and revealed the presence of genomic islands; one of them, predicted in *L. rhamnosus* GG, harbors genes coding for three secreted proteins, sortase-dependent pili, encoded by a *spaCBA* operon that were later confirmed through experimental analyses as pili encoding genes (Kankainen et al., 2009). Functional annotation was used to characterize the probiotic potential of *Bacillus coagulans* HS243, in which 11 genes were predicted as adhesion-related proteins, among them: enolase, fibronectin binding protein and flagellar hook associated proteins (Kapse et al., 2018).

The adhesion mechanism is an important property to select a probiotic strain and using *in silico* analyses it is possible to determine more details of the adhesion sites, such as mucin and binding to fibronectin (Papadimitriou et al., 2015)

Antimicrobial peptides

The LAB action in the conservation of food is due to both medium acidification (pH 3.5 to 4.5) and the production of numerous bacterial agents, such as organic compounds and bacteriocins (Van de Guchte et al., 2001). Bacteriocins are bacterial produced peptides, which act against other microorganisms and to which the producer has specific immunity mechanisms (Cotter et al., 2005).

The first work reporting the mechanism of action of bacteriocin mediated inhibition reported the discovery of antagonists among *Escherichia coli* strains (Gratia, 1925). Although the use of bacteriocins was formally proposed later (Hirsch et al., 1951), it is probable that humans were already benefitting from bacteriocin production for ~8,000 years since the first production of cheese and fermented food (Cotter et al., 2005).

Bacteriocins were first classified in 1993 (Klaenhammer, 1993); since then, modifications of this classification have been proposed (Cotter et al., 2005). Bacteriocins are divided into classes I, II, III and IV. Class I harbors lantibiotics or thermostable peptides with a molecular weight below 5 kDa produced by gram-positive bacteria and which present atypical amino acids, such as lanthionine (Lan), methyl lanthionine (MeLan) and others (Karpiński and Szkaradkiewicz, 2013). The class II bacteriocins are represented by non-lanthionine bacteriocins; they are thermostable and have 10 kDa molecular weight, slightly heavier than class I. Due to differences in the structure of class II bacteriocins, they are divided into subclasses: pediocin (IIa), lactacin F (IIb), enterocin (IIc) and lactococcin A (IId) (Cotter et al., 2005; Karpiński and Szkaradkiewicz, 2013). Using comparative genomics to characterize the potential probiotic feature of *L. plantarum* ZJ316, a study showed that this strain is an important producer of bacteriocins, since it is capable of producing at least two classes of bacteriocins, IIb and IIc (Li et al., 2016).

Bacteriocins weighting more than 30 kDa are classified in class III. They are thermolabile and are mainly produced by gram-positive bacteria (van Belkum and Stiles, 2000). Class III bacteriocins are also divided into subclasses, where a group is represented by bacteriolytic enzymes (bacterial lysins), which lyses sensitive strains, and the non-lytic group of antimicrobial proteins, represented by lysostaphin and enterolysin A (Cotter et al., 2005; Karpiński and Szkaradkiewicz, 2013). Using comparative genomics, a potential probiotic strain of *Lactococcus lactis* was analyzed for bacteriocins. Based on an annotated and curated genome, strain NCDO 2118 presented one bacteriocin for each of three classes, of which two were not previously predicted in the genome sequence (classes I and III) (Oliveira et al., 2017).

Class IV includes bacteriocins that require carbohydrates or lipids in their molecule to have a complete activity (Jack et al., 1995). Compared to the use of antibiotics in infection treatments, bacteriocins are more target-specific, have low or no toxicity to eukaryotic cells, and are active against antibiotic-resistant strains. However, there is still a lack of evaluation about their effect on the gut microbiota and also their role in probiotic effects in healthy animals (Umu et al., 2016).

Besides the gut, the skin and other mucosal tissues are in direct contact with external aggressive agents and consequently are continuously exposed to huge numbers of pathogenic microorganisms. To combat these pathogens, the epithelial/mucosal surface and the microbiota induce various mechanisms that directly kill or inhibit the growth of the pathogens (Gallo and Hooper, 2012; Dickson et al., 2013; Bao et al., 2017). The bacteria of the microbiota also produce bacteriocins and these molecules are also essential for host protection in health and disease.

Immune development and function

Coevolution between microbes and mammals, including humans, has brought many mutual benefits, which are affected by the diversity and niches of these microbes, especially the gut bacteria, which aid in the prevention of many human diseases. One of the various benefits of this coevolution for humans is that the microbiota assist in the development of the human immune system (Francino, 2014). One of the clearest types ofevidence of this role is that germ-free animals, are severely affected deficiencies in the development of the immune system in the gut (Kabat et al., 2014). Animals depleted of gut

microbiota have smaller Peyer's patches, fewer antimicrobial peptides, antibodies and B cells, as well as other immunodeficiencies (Round and Mazmanian, 2009). Also, immune system development induced by the gut microbiota is associated with host protection against inflammatory disorders (Belkaid and Hand, 2014) and infectious diseases (Duan et al., 2010).

Besides their role in the effective development of the immune system, probiotics present potent immunomodulatory and anti-inflammatory activities (as shown in Figure 1), acting in the prevention and treatment of inflammatory and autoimmune diseases. Because of these properties, various dairy products are popular and widely consumed, especially fermented milk (de Moreno de LeBlanc et al., 2011).

An important strategy of mammals to maintain the homeostasis of the intestinal environment is to minimize contact between gut lumen microorganisms and the surfaces of intestinal epithelial cells (IEC) (Llewellyn and Foey, 2017). Various types of pattern recognition receptors are expressed by intestinal immune cells, including Toll-like receptors (TLR), NOD-like (nucleotide oligomerization domain) receptors and G protein-coupled receptors (GPCR), which recognize microbial compounds (PAMPS, pathogen-associated molecular patterns); but this recognition or activation is limited (Hill and Artis, 2010; Llewellyn and Foey, 2017). On the other hand, concerning microbiota and immunity, these live microorganisms are able to increase or modulate the activity of gut dendritic cells, monocytes, macrophages, natural killer cells and T cells, controlling the production/activity of chemokines, cytokines and antibodies (Klaenhammer et al., 2012; Frei et al., 2015; La Fata et al., 2018).

Evaluating the probiotic potential of *Lactobacillus jensenii* TL2937 in pigs via the extracellular proteome, six proteins with potential immunogenic properties were found, incluiding chaperonic protease ClpB, Rpf protein (possesses a G5 protein family domain – present in various extracellular peptidases, responsible for cleaving human IgA) (Bateman et al., 2005; Gilad et al., 2011).

In *L. acidophilus*, the surface layer protein SlpA was shown to mediate the key immunomodulatory effect of this probiotic by interacting with DC-SIGN receptors at the surface of dendritic cells (Konstantinov et al., 2008). Propionibacteria, recently identified as human commensals with a key immunomodulatory role, mitigate intestinal inflammation. Accordingly, their presence in infant gut microbiota correlates with reduced incidence of necrotizing enterocolitis (Colliou et al., 2017). In *P. freudenreichii*, these immunomodulatory properties were evidenced *in vitro* and *in vivo* (Foligné et al., 2010). They rely on specific surface proteins (Le Maréchal et al., 2015), are highly strain-dependent (Foligné et al., 2013) and require a set of Slps (Deutsch et al., 2017).

Based on these considerations and other published data, especially in the last five years, we may state that the microbiota is involved in the immune response to virtually all diseases already studied, whether they are of infectious origin or not. That is, dysbiosis of microbiota, whether in the intestine and/or in other tissues, is closely related to prevention and/or treatment of infections, inflammatory diseases, autoimmune diseases, cancers, neurodegenerative diseases, depression, anxiety, diet, trauma, metabolic syndrome and related diseases among other disorders (Kang and Im, 2015; Sander, 2017; Westfall et al., 2017; Lee et al., 2018).

OMICS APPLIED TO PROBIOTIC BACTERIA

Genomics

The first completely sequenced genome of the LAB group was *L. lactis* subsp. *lactis* IL1403 strain, published in 2001. This study revealed biosynthetic pathways, phages and some of the components that participate in aerobic metabolism (Bolotin et al., 2001).

In 2002, a program intended for the mass sequencing of LAB genomes was announced by Lactic Acid Bacteria Genome Sequencing Consortium (Klaenhammer et al., 2002). Currently, more than 100 *Lactococcus* genomes are available on the NCBI database (National Center for Biotechnology Information - http://www.ncbi.nlm.nih.gov/genome/genomes/156), of which 39 are complete genomes.

LAB have small genomes, approximately 2Mb in length on average, coding for approximately 2000 genes, but range from 1600 to 3000 genes, according to the species. This variation results from LAB evolution through gene loss, duplication, and acquisition (Khalid, 2011).

Bolotin et al. (2004) showed that dairy streptococci have undergone reductive evolution, resulting in divergence between them and pathogenic species of streptococci. The most remarkable example is *Streptococcus thermophilus*, which diverged from other species of *Streptococcus* through the loss of virulence factors, such as those involved in adhesion and antibiotic resistance.

Many studies highlight the importance of genomic sequencing in the discovery of new features related to the LAB, such as the identification of genes encoding proteolytic enzymes (which participate in cheese maturation) in *L. helveticus* (Smeianov et al., 2007). The sequencing of the first *Lactobacillus* species: *L. plantarum* WCFS1 (Kleerebezem et al., 2003), *Lactobacillus johnsonii* NC533 (David et al., 2004; Denou et al., 2008) and *L. acidophilus* NCFM (Altermann et al., 2005), revealed some interesting characteristics, such as lifestyle adaptation islands, lack of biosynthesis pathways, and unique structures named potential autonomic units (PAU).

Bioinformatics approaches have helped identify the citrate catabolic pathway in *L. casei* (Díaz-Muñiz et al., 2006), and other studies have identified genes responsible for decarboxylation of a branched-chain alpha-ketoacid of *L. lactis* (De La Plaza et al., 2004; Smit et al., 2005). Genomic sequencing has also played a role in the elucidation of LAB probiotic effects; for instance in the study of antimicrobial compounds and immunomodulatory mechanisms of *Lactobacillus reuteri* (Saulnier et al., 2011), comparative analysis of pilus-associated genes and metabolic pathways in *Lactobacillus rhamnosus* and *Lactobacillus casei* (Douillard et al., 2013), and identification of adhesion-associated proteins (*cwaA*) in *L. plantarum* (Zhang *et al.*, 2015).

Assessment of the probiotic properties of a selected microorganism requires many experiments *in vitro* and *in vivo*. This takes considerable time. The omics approach allows one to speed up these studies by enabling the identification of potential probiotic microbes. Recently, Salvetti et al. (2018) studied 269 species of Lactobacillaceae and Leuconostocaceae with a phylogenetic approach. Twenty-nine ribosomal proteins and housekeeping genes were compared. They conclude that the *Lactobacillus* genus has various subclades, which may lead to reclassification of the lactobacilli. This grouping facilitates development of accurate molecular markers to help avoid problems sucha as the

the misidentification of probiotic strains. More recently, 2459 high-quality whole genomes were analyzed and, based on a polyphasic phylogenetic approach, reclassification of the *Lacatobacillus* genus into 25 genera and fusion of the Leuconostocaceae and Lactobacillaceae families was proposed (Zheng et al., 2020).

Besides the genomic approach (Figure 2), other omics have been providing information to help understand the divergence and evolution many species over time (Pfeiler and Klaenhammer, 2007). Using an omics approach, it is possible, for example, to correlate protein data with survival within the host under stress conditions or secreted proteins that may exert a specific role in probiotic effects of certain strains, through analysis of bacterial-host interactions.

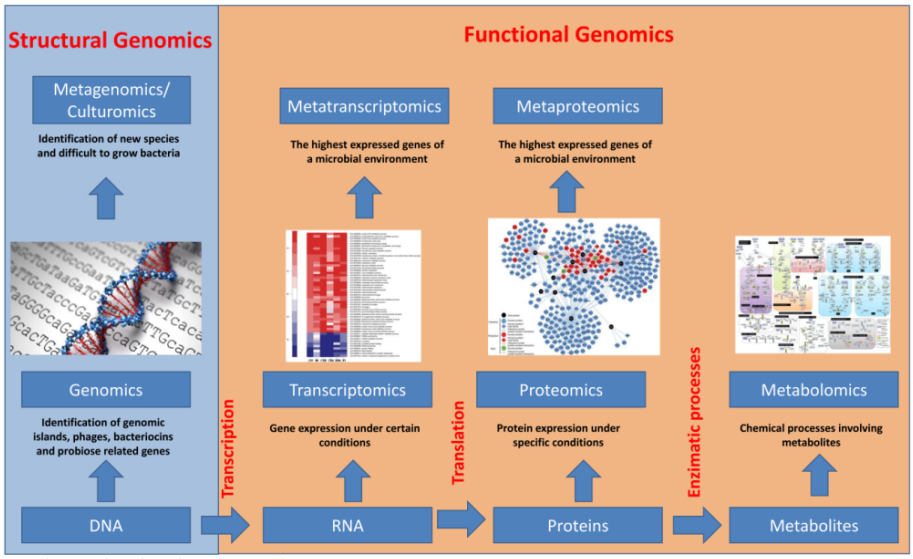

Figure 2. The goals of omics approaches.

Metagenomics

Through metagenomic (Figure 2) analyses, it is possible to access physiological and genetic information about uncultured organisms, such as the human GIT microbiota, through the sequencing of 16S rRNA genes and by shotgun sequencing (Handelsman, 2005). This type of tool gives important genetic information for uncultured organisms, helping develop novel hypotheses of microbial function. Before metagenomics, the methodology consisted of cloning DNA from environmental sources, followed by functional expression screening (Handelsmanl et al., 1998; Handelsman, 2005), which is time consuming and limits the number of strains and species that can be studied.

A pioneer study in this area consisted in the large-scale metagenomics projects in in the Sargasso sea, in which a massive microbial population was characterized through 1,045 billion base pairs from seawater samples. This large number of sequences provided important information on the diversity, gene content and the relative abundance of the organisms (Venter, 2004).

The development of metagenomics, mainly with the advent of next-generation sequencing technologies (NGS), and the creation of the International Human Microbiome have both boosted the field and opened a new door in analyses of bacteria/host interactions. The culture-free methodology used by NGS technologies expanded the analyses of microbial composition and may now be used not only to predict new probiotics from a comparison of the microbiota from healthy and diseased individuals, but may also be used to analyze the composition of the microbiota before and after administration of a given probiotic bacterium (McFarland, 2014).

In a study of the microbiota of genetically obese mice and their lean littermates, Turnbaugh et al. (2006) demonstrated through metagenomics analyses that the obesity was associated with an abundance of two groups of bacteria: Bacteriodetes and Firmicutes.

Nobutani et al. (2017) administrated *Lactobacillus gasseri* strain CP2305 or a placebo to patients with irritable bowel syndrome. They identified 87 genera, among which 13 differed in frequency; the genera *Dorea, Enterococcus,* and *Dialister* decreased in the CP2305 group.

Culturomics

Some studies have demonstrated the usefulness of a culturomics approach (Figure 2) for probiotic analyses (Dubourg et al., 2014). A culturomics approach consists in growing under multiple culture conditions, followed by matrix-assisted laser desorption/ionization-time of flight mass spectrometry and 16 rRNA analysis for the identification of less representative species in the sample (Lagier et al., 2016, 2018). This technique has helped identify new organisms that are generally not found through metagenomics techniques (Pfleiderer et al., 2013; Dubourg et al., 2014).

As an aid to identify a larger number of organisms, culturomics can identify populations with a concentration of less than 10^3 CFU per mL, which is below what can be detected in large-scale metagenomics studies (Lagier et al., 2012). Culturomics was reborn with studies of environmental microbiologists. For instance, Bollman et al. (2007) created a new method that was able to isolate almost numerous microorganims present in a specific aquatic environmentthat were not detected with other methods.

The first study of the microbial composition of the gut microbiota using culturomics dates from 2012. Lagier et al. (2012) grew microorganisms under 212 different culture conditions and used mass spectrometry and 16S rRNA amplification and sequencing to help identify the colonies found. the culturomics analyses of the microbiome resulted in 31 new species in addition to more than 100 species never described from the human gut before.

Culturomics and metagenomics leverage the potential of identification of new species. They complement each other, providing greater knowledge and understanding of new and/or difficult-to-grow bacteria. A database was created in order to group several prokaryotic species associated with human beings (commensals or pathogens), highlighting the importance of culturomics and metagenomics. The 2172 species listed were classified into 12 different phyla, most of them being Proteobacteria, Firmicutes, Actinobacteria and Bacteroidetes (Hugon et al., 2015). More recently, it was reported that the number of species had increased, totalng 2776 species, due to culturomics techniques that facilitated the identification of new bacterial species (Bilen et al., 2018).

Stool samples of malnourished and healthy patients from Senegal and Niger were analyzed using culturomics and metagenomics. Besides finding a decrease in the diversity of fecal microorganisms and an enrichment of potentially pathogenic bacteria, they identified some probiotic bacteria only in healthy children. Also, new species were identified, including Propionibacteriaceae and Bacillaceae species (Tidjani Alou et al., 2017).

Functional genomics

Transcriptomics

The area of transcriptomics (Figure 2) may elucidate how genes are involved in adaptation for specific conditions. Van der Meulen et al. (2016) identified 375 novel regulatory mRNAs in *L. lactis* MG1363 involved in stress response and metabolic processes, such as internal promoters, operon structures and novel ORFs. Using probiotic preparations containing *L. acidophilus, L. casei* and *L. rhamnosus*, an *in vivo* experiment was performed with volunteers to analyze the gene-regulatory networks and pathways in the human mucosa. A significant variation was observed among the transcriptomics results of the volunteers; however, various factors, such as the resident microbiota, diet, genetic background and lifestyle can affect a probiotic response, (van Baarlen et al., 2011).

Functional genomics may also contribute to refine phylogenetic studies due to the high homology among some bacteria, such as *L. acidophilus.* Using core and transcriptomic data it was possible to identify small ORFs that are highly conserved and transcribed in various species of this group, highlighting new possibilities to characterize and present new probiotics to the market (Crawley and Barrangou, 2018). Studying the transcription profile of genes associated with adhesion and stress response of the probiotic *L. acidophilus* NCFM, Weiss and Jespersen (2010) used specific conditions to mimic the GI tract *in vitro*. During gastric digestion, the genes encoding GroEL, ClpP and DnaK showed considerable up-regulation. The genes encoding mucin-binding and fibronectin-binding proteins were up-regulated in the incubation process (duodenal juice and bile).

A major breakthrough in this field was *in vivo* transcriptomics. Introduction of a pure culture of a probiotic bacterium, constrained in a dialysis bag, within the colon of a rabbit, allows monitoring upregulation of genes related to digestive constraint adaptation. First described in an *in vivo* proteomic study of *Bifidobacterium longum* in rabbits (Yuan et al., 2008), it was then adapted to an *in vivo* transcriptomic study of *P. freudenreichii* in the colon of pigs (Saraoui et al., 2013). This study revealed over expression of key genes involved in specific carbohydrate catabolisms, in alternative pathways to produce NADH, NADPH, ATP and precursors (utilizing of propanediol, gluconate, lactate, purine and pyrimidine and amino-acids), as well as genes specifically expressed during cell division.

Proteomics

Proteomics (Figure 2) allow the study of the expression of a large range of proteins from a specific organism. A proteomic analysis comparing a wild strain of *L. plantarum* with a mutant one under physiological and heat stress conditions showed an induction of proteins related to re-folding of proteins subject to cellular damage, elucidating the

importance of CtsR regulon control in lactic acid bacteria (Russo et al., 2012). Another study using proteomics comparison of three *L. plantarum* strains confirmed the bile resistance characteristics of *L. plantarum* 299V, already known as a probiotic. The analyses were made using strains with different levels of bile resistance and helped to understand how these strains modulate their metabolism to survive in stress environments (Hamon et al., 2011).

In a study of a long-chain carbohydrate known to be a prebiotic, called inulin, it was observed that *L. plantarum* was able to use this compound and an operon (*fosRABCDXE*) for inulin metabolism was identified in this genome (Buntin et al., 2017).

Proteomic analyses of *Bifidobacterium longum*, isolated from stool, was performed to evaluate protein expression under the effect of bile salts. Using different degrees of exposure to bile, it was possible to identify 34 different proteins differentially regulated, amongst them: general stress response chaperones and some enzymes of pyruvate and glycolysis catabolism (Sánchez et al., 2005). *B. longum* was also used in an *in vivo* proteomic study. Incubation of this probiotic bacterium within the colon of rabbits led to the induction of a set of specific proteins (Yuan et al., 2008). This set included proteins related to the metabolism, to translation, and to intestinal adaptation, such as EF-Tu which is a Bifidobacterium adhesin-like factor, and bile salt hydrolase (BSH).

Concerning probiotic propionibacteria, proteomics was used to reveal mechanisms induced in acid (Jan et al., 2001), bile salts (Leverrier et al., 2003) and thermal adaptation (Leverrier et al., 2004). Some of these key stress proteins were also induced in a Swiss-type cheese matrix (Gagnaire et al., 2015), in accordance with the protective role of this food matrix towards digestive constraints

Metabiotics and metabolomics

Another omic strategy recently applied to probiotics analysis is metabolomics (Figure 2). Through this approach it is possible to determine and quantify the metabolites present intracellularly (Mozzi et al., 2013). Some metabolites promote health, and are named Metabiotics. They are metabolites from the structural components, metabolites or signaling molecules of probiotic bacteria, such as lactic acid, short chain fatty acids (SCFAs), linoleic acid, some glycoproteins/peptides and potentially carcinogenic metabolites. Metabiotics have beneficial bioactive substances that act in host-specific physiological functions, regulatory, metabolic and/or behavior reactions (Shenderov, 2013; Sharma and Shukla, 2016). Among these, SCFAs are the most studied, being a source of energy for colonocytes and the modulators of various metabolic activities (Shenderov, 2013).

Metabiotics-producing bacteria include not only the well-known probiotic species of *Lactobacillus*, *Escherichia,* and *Enterococcus*, but also other species in the dominant human intestinal phyla (Bacteroides, Firmicutes, Proteobacteria, Actinobacteria, and Archae) for nutrition and medical purposes (Shenderov, 2013).

Probiotics produce several bioactive substances with beneficial effects in combatting GIT diseases, which help in homeostasis and competitive exclusion of pathogens (Verma and Shukla, 2013). More interestingly, the multifunctional SCFA acetate plays an important role in epithelial cell division, ileal motility and other functions (Hong et al., 2005). Moreover, an increase in the colic content of propionate, another SCFA, as a

result of probiotic propionibacteria consumption, was correlated with enhanced apoptotic depletion of colon cancer cells in human microbiota inoculated rats (Lan et al., 2008). At the cell level, it was shown to induce apoptosis by acting on cancer cell mitochondria, triggering the intrinsic cell death pathway, leading to caspase activation (Jan et al., 2002) and increased susceptibility to induced cell death (Cousin et al., 2016).

The most widely studied metabiotic is SCFA butyrate, produced by *Faecalibacterium prausnitzii* and *Eubacterium rectale* in the gut (Zhong et al., 2014), which has the potential to differentiate between cancer and normal cells to exert epigenetic effects and inhibit the growth of cancer cells. Butyrate has been associated with the induction of apoptosis in colon cancer cells due to its ability to convert procaspase 3 to active caspase 3 (Medina et al., 1997).

Among beneficial metabolites produced by probiotic bacteria, recent studies have drawn attention to gamma-aminobutyric acid (GABA) in the context of the gut-brain axis and of altered brain function (Janik et al., 2016; Dinan and Cryan, 2017), and to trimethylamine/trimethylamine N-oxide (TMA/TMAO) in the context of cardiovascular disease (Bu and Wang, 2018).

Some technologies are extremely useful for metabolomics studies; the most current and integrated methods related to separation and detection processes are liquid chromatography (LC: high-performance, HPCL and ultra performance, UPLC) and mass spectrometry (MS). HPLC technique is able to separate multiple compounds according to stationary phase and UPLC gives results similar to HPLC; however, this technology has greater capacity, resolution, sensitivity and higher speed (Mozzi et al., 2013). Major progress has furthermore been achieved by the use of nuclear magnetic resonance spectroscopy (Dunn and Ellis, 2005; Sugahara et al., 2015; Janik et al., 2016).

Integrative omics and enrichment approaches

Identification of a single specific factor associated with a probiotic effect might have limited value for selecting bacterial strains and therapeutic rationales. In this case, a multi-omics approach compared to a single-omic analysis may offer greater advantages, as they cover a broader range of information through the identification of associated factors from different biological processes, such as gene expression, protein synthesis, post-translational modifications and cellular metabolic processes (Perakakis et al., 2018). The integration of data from genomic, transcriptomic and metabolomic analysis can be a powerful tool to investigate and rapidly validate new molecular probiotic features. In this context, promising achievements have been made by studies investigating molecular resistance features towards stressful conditions that bacterial probiotic strains face during the manufacturing process. For example, Bianchi et al. (2020) identified altered amino acid production, as well as metabolic and health-promoting changes in probiotic strains of *L. paracasei, Streptococcus thermophilus,* and bifidobacteria as a result of different conditions of manufacture and formulation through an integrative approach of functional proteomics, metabolomics, and *in vivo* analyses.

Although promising, integrative omics currently presents great challenges such as a need to development and employ bioinformatics pipelines and algorithms to associate and harmonize large amounts of data generated by the different high-throughput platforms (Jiménez-Pranteda et al., 2015). Recently, feature-annotation enrichment analysis has been

extensively used to identify biological processes by comparing *de novo* data with accumulated biological data deposited in public databases. This approach offers a rapid solution to systematically classify large feature lists into pathways, cellular localization and function categories (Gandhi and Shah, 2017). A plethora of enrichment tools have emerged in the last 20 years, and are being improved to become more suitable as data-mining exploration tools (Huang et al., 2009).

CONCLUSIONS

Probiotic bacteria have been used by humans for a long time in the maturation of cheese and the production of fermented food. However, their importance has been only recently highlighted with the study of their safety aspects, exclusion mechanisms, survival through the host GIT and production of immunomodulatory and anti-inflammatory proteins. With the advent of NGS technologies for structural and functional genomics, coupled with whole proteomics analyses using mass spectrometry, there are several new possibilities for probiotic identification using metagenomics of GIT microbiota to investigate microbial changes under disease conditions and after probiotic administration. We can highlight the importance of auxiliary techniques, such as culturomics for the identification of bacteria not detected in metagenomics, for example. In addition, both approaches may be used to elucidate problems with identification of probiotic strains.

Omics will allow seeking for probiotic features in new and promising strains of probiotics. Based on the set of data acquired from established probiotic strains, omics will allow screening strains for expression of key molecules involved in beneficial interactions with the host. These include MAMPs such as Slps and pili, key metabolites such as GABA, as well as proteins involved in adaptation to the host gut.

Genomic studies may also be used in the analyses of genome plasticity of probiotic and non-probiotic related strains, for the identification of genes related to each of the probiotic features. Finally, transcriptomics and proteomics may help in the identification of differentially expressed genes in probiotic and non-probiotic species for the later elucidation of metabolic pathways and protein-protein interactions analyses. Future improvements in the area may involve the identification of probiotic-pathogenic and bacterial-host protein-protein interactions from a wider systems biology perspective. The omics approach brought new paths and means to analyze characteristics of future potential probiotic bacteria and broaden our understanding of the different ways theh interact with the gut microbiota of the host; this was only possible with an integrative omics approach.

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AUTHOR CONTRIBUTIONS

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CONFLICTS OF INTEREST

The authors declare no conflict of interest.

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