EXPLORATION OF THE INTERACTION OF PROBIOTICS AND PREBIOTICS WITH THE HOST USING OMICS TECHNOLOGIES

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Contents

Summary

Functional foods are foods that claim to promote human health over and above the provision of basic nutrition. There is not a universally accepted definition for functional foods, but a proposed working definition was given by the EU-project ‘‘Functional Food Science in Europe” (FUFOSE), stating that: ‘‘A food can be regarded as functional if it is satisfactorily demonstrated to affect beneficially one or more target functions in the body, beyond adequate nutritional effects”. Functional foods therefore comprise foods in which the composition has been changed by addition, deletion or modification of ingredients. One of the largest segments of this market comprises foods containing probiotics, prebiotics, and synbiotics. Probiotics are live microorganisms which when administered in adequate amounts confer a health benefit on the host. A prebiotic has been defined as a selectively fermented ingredient that results in specific changes in the composition and/or activity of the gastrointestinal microbiota, thus conferring benefit(s) upon host health. The term synbiotic is used when referring to a

product that uses a prebiotic and probiotic in combination.

Probiotics are predominantly, although not exclusively, bacteria from the genera *Lactobacillus* and *Bifidobacterium.* Nowadays, the genome sequencing of dozens of species of *Bifidobacterium and Lactobacillus* provides to the scientific community an excellent scenario to apply high-throughput methodologies in the emerging field of omics technologies. In this regard, genomic and proteomic research has already been extremely useful, as it provides the necessary tools for unravelling the functions of probiotics and gut-related bacteria *in vitro* and *in vivo*. Furthermore, current metagenomics research will open new avenues to understand the mechanisms for the specific effects of probiotics. The integration of massive data analysis will help in understanding the roles of probiotics and prebiotics, the processes involved in colonization, survival and the crosstalk mechanisms with the human host.

1. Introduction to Gut Microbiota, Probiotics and Prebiotics

Since 2001, when the human genome was sequenced (Venter et al., 2001), our knowledge of the genetic factors involved in health and disease has increased enormously. Nevertheless, now we know that a human being harbors more genes than those present on its genome, the genes from the microbiota, and these genes also play a critical role in human health. Therefore, a more accurate image of human biology could be drawn if the genomes of the commensal microorganisms, the so-called *Microbiome*, were taken into account (Bäckhed et al., 2005). The human gastrointestinal tract (GIT) harbors a very complex and dynamic microbial community, called *gastrointestinal microbiota*. The number of bacteria in the intestine of human adults exceeds that of eukaryotic cells in the human body. This microbiome contains more than 100 times the number of genes in our own genome, facilitating many functions that are not encoded in our genome but that we obtained through the acquisition of the intestinal microbiota, thereby forming a so-called human-microbial superorganism (Lederberg, 2000).

Each individual harbors its own microbiota throughout life, the composition and diversity of which vary depending on genetic and environmental factors, as well as on different disease states. This microbial community, containing hundreds of different species, varies all along the GIT. The stomach and the upper bowel are sparsely populated whilst the colon is heavily populated. Despite the inter-individual variation, the intestinal microbiota provides the host with a barrier against pathogenic bacteria and it has a direct impact on the morphology of the gut and the development of the immune system. Indeed, many diseases and their prevention have been linked to disturbances of intestinal microbiota. The establishment of the gut microbiota is needed, among others, for an appropriate development of the intestine and mucosal immune system, the establishment of oral tolerance and to maintain intestinal homeostasis (Ley et al., 2006). A role for the GIT microbiota on the regulation of absorption and storage of lipids has also been demonstrated, indicating a potential to fight against metabolic syndrome by means of microbiota modulation (Turnbaugh et al., 2006). Moreover, aberrancies on gut microbiota composition have been identified in different diseases, such as diarrhea, inflammatory bowel disease, allergic disease, obesity or colonic cancer. The demonstration of the importance of the intestinal microbiota on human health has attracted the attention of researchers towards the development of nutritional strategies

directed to beneficially modify the microbiota composition. This beneficial modulation of the gut microbiota forms the basis of the probiotic and prebiotic concepts.

Probiotics are defined as *live microorganisms which when administered in adequate amounts confer a benefit on the host* (FAO/WHO, 2006). This definition implies that the term probiotic should only be used to refer to strains for which specific beneficial effects have been scientifically demonstrated and the results obtained for a specific strain should not be extrapolated to others. Some of the beneficial effects attributed to specific probiotic strains are supported by good scientific evidence obtained from human intervention studies (e.g. lactose intolerance or diarrhea); whilst other effects, although promising, are merely hypothetical at the moment, requiring further confirmation through human trials.

A prebiotic has been defined as a *selectively fermented ingredient that results in specific changes in the composition and/or activity of the gastrointestinal microbiota, thus conferring benefit(s) upon host health* (Gibson et al., 2010). According to this definition the properties of prebiotics are based on their ability to stimulate selectively the growth of some intestinal microorganisms, in a way that has positive results for the host and it requires, as happens with probiotics, that the effects are established for each specific prebiotic compound independently. Several health-promoting properties have been attributed to prebiotics but, similarly to the case of probiotics, only a few of them have been scientifically demonstrated in humans.

Different microorganisms are used as probiotics for different applications, among them strains from the genera *Lactobacillus* and *Bifidobacterium* are the most commonly used for human applications. Most of the currently used strains have been isolated from the intestinal microbiota of healthy humans. With regard to prebiotics, oligo- and polysaccharides have been assessed, inulin and oligofructose being the most widely used.

Different prebiotics show a high variability in their monosaccharide composition, structure and degree of polymerization and therefore fermentation rates can be expected to show large differences. The effects of both, probiotics and prebiotics, on health are thought to be mediated, mainly, through modulation of the intestinal microbiota composition, establishing a clear link between probiotic, prebiotic and microbiota research. Therefore, the basis for probiotic and prebiotic research requires the understanding of probiotic strains and prebiotic substrates and their effects on the gut microbiome composition.

2. Omics Analysis in Microbial Biology

The start of the 21st century has marked an inflection point in the way of studying the biology of microorganisms. In 1995 the first bacterial genome was completely sequenced and (Fleishcmann et al., 1995), since then, more than 1,000 complete bacterial genome sequences have been completed, and several thousand bacterial genome projects are currently ongoing. In parallel, tremendous advances of DNA sequencing technologies and tools that allow the global analysis of gene expression, as

well as the development of protein (and metabolite) separation and identification techniques, have been achieved.

Furthermore, the development (an application) of computer science, statistics and specialized software, oriented to the study of molecular biology, the so-called bioinformatics discipline, has facilitated the integration of all the molecular data contained in biological systems, and has allowed the depiction of all this information in an accessible and comprehensive manner to the scientific community.

How to go from the genetic information contained in a microorganism to the explanation of its specific phenotype? This is an intriguing and challenging question that currently can be largely achieved by using omics technologies, which allow the quantitative determination of biological molecules under defined physiological states and fill the gap between the genetic information held within the structural genes of a cell and the final metabolic products.

Among these, some of the more popular omics methodologies in microbiology are genomics, transcriptomics, proteomics and metabolomics, although some others, such as interactomics, fluxomics and metabonomics, have also been described (Martin et al., 2010; Zhang et al., 2010). Genomics studies the genomes of organisms and their sequences, mapping, structural genes and non-coding sequences. Transcriptomics, however, covers the global analysis of gene expression or genome-wide expression profiling.

In contrast to genomics, transcriptomics enables the quantification of the mRNA molecules at a given physiological state, reflecting the genes that are active under specific environmental conditions (Zhang et al., 2010). Proteomics is defined as the large-scale characterization of the entire protein complement of a cell line, tissue, or organism, and its goal is to obtain a global and integrated view of a biological system by studying all the proteins of a cell rather than each one individually (Graves and Haystead, 2002).

Metabolomics seeks to identify and quantify all metabolites in a biological system and establish its metabolite fingerprint (Madsen et al., 2011). All omics analysis share a number of features that distinguish them from the more traditional molecular biology techniques, i.e. cloning, expression and characterization of a single gene or group of genes. They are high-throughput approaches generating massive amounts of data, most likely linked to powerful bioinformatic analysis which are essential to be able to properly interpret the results, and they try to integrate all the cell metabolism bringing together different molecular levels, i.e. DNA, mRNA, protein and metabolite, rather than focusing on a single molecule or group of molecules (Figures 1 and 2).

However, we have to take into account that a single omics approach is not sufficient to characterize the complexity of a biological system. That is, expression levels do not necessarily correlate with the amount of protein in the cell, nor its biological activity, and cannot be linked with further post-transcriptional modifications (Zhang et al., 2010). Therefore, the integration of different omics approaches could lead to a more

complete and accurate picture of the physiological status of a cell or cell population under defined environmental conditions.

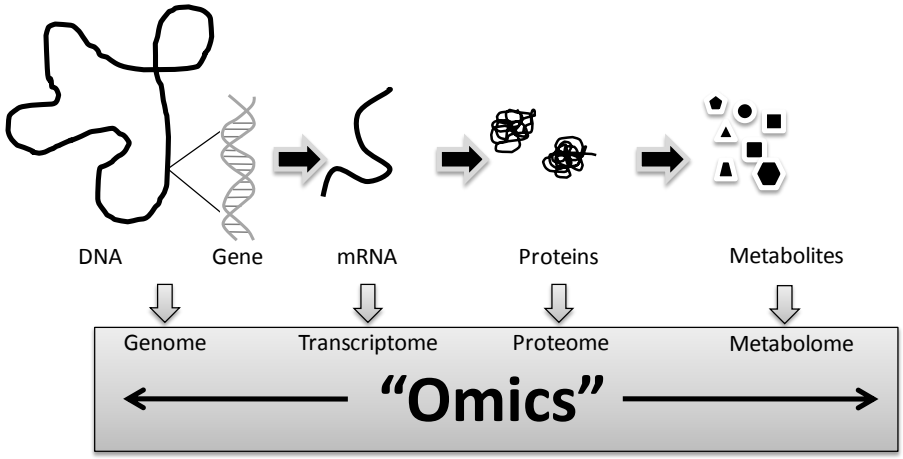

Figure 1. Workflow showing the different molecules studied by the "Omic" disciplines. From right to left, genes coding for proteins are first transcribed to mRNA. The join bioactivity of the set of proteins present in a living cell lead to the accumulation of certain metabolites. The group corresponding to the pool of all the genes, mRNAs, proteins or metabolites of a living cell/system is referred as genome, transcriptome, proteome or metabolome, respectively. The name of the "Omic" discipline responsible for their study is build by adding the suffix "–omics" to the above mentioned groups (i.e. genomics, transcriptomics, proteomics or metabolomics).

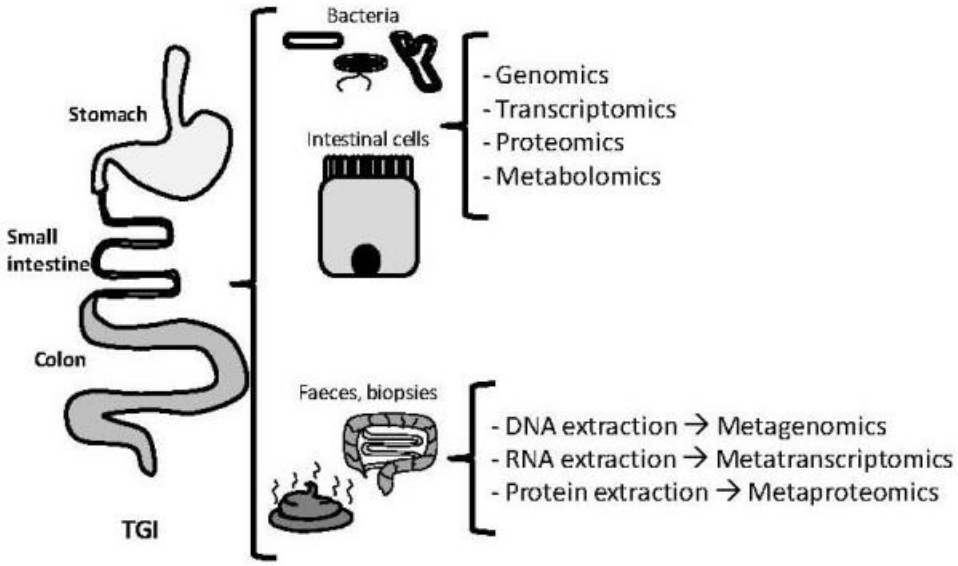

Figure 2. Application of the different "Omic" disciplines to the study of the human gastrointestinal tract. Gut bacteria or human intestinal cells can be isolated, being their genomes/transcriptomes/proteomes/metabolomes studied individually. On the contrary, the whole pool of DNA, mRNA or proteins can be obtained directly from intestinal biopsies or samples such as faeces. In this case, the technologies are named including the prefix meta- ahead of the corresponding "Omic" discipline.

3. Genomics of Human Gut Commensals, a Focus on Bifidobacteria and Lactobacilli

Genome sequencing of different probiotic strains and intestinal microbes is contributing enormously to our knowledge in this area (Salminen et al., 2005). Genomic data on intestinal bacteria are showing the genetic basis of the adaptation of these microorganisms to the gut environment and is providing data on their properties, such as mechanisms of adhesion to the gut mucosa or interaction with the immune system, and gives an idea of the potential functional properties of those microorganisms. Genomic research is also extremely useful as it provides the necessary tools, such as DNA microarrays, to unravel the *in vivo* functions of probiotics and prebiotics. At the same time, it facilitates the understanding of the microbe-host and the microbe-microbe cross-talk and provides mechanistic1 explanations for specific effects of probiotics/prebiotics on host gene expression.

Bifidobacteria and lactobacilli have been widely used as health-promoting bacteria in many functional foods. However, the molecular mechanisms as to how these bacteria positively impact on the health of the host are far from completely understood. For this reason these microorganisms represent a growing area of interest with respect to genomics, molecular biology and genetics. Recent genome sequencing of a number of bifidobacteria and lactobacilli species has allowed access to the complete genetic make-up of representative members of these bacteria. Here we will discuss how the analysis of genomic data has allowed us to understand the mechanisms by which these bacteria adapt to the gastrointestinal tract environment, while also revealing genetic functions that mediate specific host-microbe interactions.

The GIT microbiota is composed of a vast array of bacteria whose composition differs depending on the different regions of the gut. Bifidobacteria and lactobacilli naturally colonize the lower regions of the GIT, i.e., the large and the small intestine, respectively (Kleerebezem and Vaughan, 2009). Notably, the intestine harbors naturally resident lactobacilli also known as autochthonous lactobacilli, but there is a plethora of additional lactobacilli that are acquired through food.

The genera *Bifidobacterium* and *Lactobacillus* are part of the phyla *Actinobacteria* and *Firmicutes*, respectively, both representatives of Gram positive microorganisms that ferment carbohydrates to acids. Bifidobacteria mainly produce acetate and lactate, whereas lactobacilli can produce a variety of organic acids including lactate. Bifidobacteria and lactobacilli are often grouped together based on the fact that these microorganisms share similar metabolic features and are both exploited by the food industry as probiotic bacteria in functional foods, although one should keep in mind that they are phylogenetically very distant.

3.1. Human Gut Microbiota and Lactobacilli-Bifidobacteria

Co-evolution has occurred between mammals and their gut microbiota for millions of years and bacteria have developed strategies to modulate the gene composition of their genomes (Bäckhed et al., 2005). It is estimated that the average human gut microbiota is comprised of at least of 10^{13} microorganisms, which largely represent anaerobic bacteria

whose metabolic inventory is still unresolved. A wide range of novel culture-independent approaches have been developed in order to obtain information on the composition of the gut microbiota, including: (a) oligonucleotide probes that target specific ribosomal RNA sequences in different hybridization techniques, such as DNA-microarray and fluorescent *in situ* hybridization FISH), (b) community profiling techniques such as PCR coupled to denaturing gradient and temperature electrophoresis, i.e. PCR-DGGE and PCR-TGGE, (c) real-time quantitative PCR for both qualitative and quantitative analyses, and (d) high throughput sequencing of taxon-discriminating PCR amplicons (so-called metagenomics approach) (for review see (Turroni et al., 2008).

All the above mentioned techniques are based on the 16S ribosomal RNA-encoding gene, which is conserved in all bacteria, and its hypervariable DNA regions makes it ideal for microbial identification at species level. A combination of 16S rRNA gene-based molecular approaches revealed that a significant proportion of the intestinal microbiota belong to *Bacteroidetes*, *Firmicutes* (the class of *Clostridia*) followed by *Actinobacteria*, *Proteobacteria* and *Archea* (Eckburg et al., 2005; Hayashi et al., 2002; Wang et al., 2005). It is suggested that each human being harbors up to 1000 different phylotypes in the intestine, formed by a small phylogenetic core of 2 % and about 80 % host-specific microorganisms (Kleerebezem and Vaughan, 2009).

The presence of lactobacilli and bifidobacteria in intestinal samples has been well documented for decades through their isolation and cultivation on synthetic media. However, the existence of unculturable human intestinal lactobacilli and bifidobacteria has recently been shown by culture-independent techniques (Ben-Amor et al., 2005; Turroni et al., 2009b).

So far, prominent intestinal lactobacilli identified from fecal samples include *Lactobacillus ruminis*, *Lactobacillus crispatus*, *Lactobacillus gasseri*, *Lactobacillus acidophilus*, *Lactobacillus casei*, *Lactobacillus paracasei*, *Lactobacillus rhamnosus*, *Lactobacillus plantarum*, *Lactobacillus delbrueckii*, *Lactobacillus salivarius* and *Lactobacillus reuteri*.

Bifidobacteria are among the first colonizers of the infant intestine, where they represent a dominant genus until weaning, at which point their prevalence in the gut drops and further decreases with age (Claesson et al., 2010; Turroni et al., 2010c). Notably, in bifidobacteria it is possible to notice an ecological niche specialization allowing the existence of an infant-type bifodobaterial species and an adult-type bifidobacterial species (Ventura et al., 2007b). In breast fed infants, *Bifidobacterium breve* is the most frequently identified species, followed by the *Bifidobacterium bifidum* and *Bifidobacterium longum* subsp. *infantis* taxa (Turroni et al., 2009a; Turroni et al., 2010c). In contrast, the bifidobacterial species detected in the adult intestine include *Bifidobacterium longum* subsp. *longum*, *Bifidobacterium catenulatum* and *Bifidobacterium adolescentis*. Such ecological niche specialization is the consequence, or the cause, of the genetic differences between the genomes of infant-type vs. adult-type bifidobacteria (see below)

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