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Reviewing Classical and Molecular Techniques Regarding Profiling of Probiotic Character of Microorganisms

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ABSTRACT

In recent years the roles of probiotics as functional ingredients in food has been highly adopted by the consumers and are under constant investigation by the scientific community. As a result, several probiotic-containing foods have been introduced in the market with an annual share of several billion dollars. Of particular interest in the probiotics research is the profiling of probiotic character of the microbes involving both *in vitro* and *in vivo* approaches. Initially traditional microbiological techniques were used; however they suffer by many limitations and therefore the development of new techniques, which are primarily based on the analysis of nucleic acids have been introduced. The scope of this review is to present current knowledge about the methodological approaches that are used to quantify and characterize the potential probiotic character of microorganisms. Moreover, it will focus on molecular and non-molecular tools and finally will report some new perspectives in the study of probiotics using ‘’omics” techniques.

Key words: Probiotics, Microbiota, Omics, Gastrointestinal Track, Molecular Techniques, 16S rRNA, PCR.

INTRODUCTION

Probiotic is a word derived from two Greek words “*ðñï*” and “*âéïò*” which mean “*for life*” and is associated with bacteria that have beneficial effects to the humans’ and animals’ health. Probiotics are generally defined according to FAO/WHO (2002) as: “live microorganisms which, when administered in adequate amounts confer a health benefit on the host”. This means that probiotic microorganisms should be viable during consumption; however non-viable probiotic microorganisms may also have positive health effects. In addition, the definition specifies that an “adequate amount” should be administered. However, no specific amount is defined. As a rule of thumb, 10^9 colony forming units (CFU) per dose is often used as an acceptable lower limit; although this can differ depending on the strain, food matrix and health effect[2].

Probiotics are usually bacteria of the normal human intestinal flora and include the members of the genus *Lactobacillus*, *Bifidobacteria*, *Enterococcus*, *Leuconostoc*, *Streptococcus*, but also include yeasts

like *Saccharomyces.* Moreover, other species such as *Bacillus subtilis* and *Escherichia coli* have been used as probiotics (Table 1). Lactic acid bacteria (LAB) are capable to produce lactic acid after fermentation of sugars such as glucose and lactose. *Lactobacillus*, *Bifidobacterium, Streptococcus, Leuconostoc,* and *Pediococcus* are the most often used LAB as probiotics. The consumers' demand for probiotics has increased due to the effectiveness and the therapeutic potential (Table 2) of probiotics. Many food products like fermented milks, yoghurt, freeze-dried yoghurt, cheeses, ice cream and fruit juices have been proposed as vehicles for the delivery of probiotics to consumers [3]. It is noteworthy that the world market for functional foods has been estimated at up to $50 billion annual share, whereas the global probiotic market is estimated at $15 billion [4]. The mechanisms by which probiotics influence the host and act against gastrointestinal pathogens are still poorly understood as these mechanisms may be multifactorial and strain dependent. Specific probiotic bacteria have been found to modulate local and systemic immune responses. Although the mechanism under this property is not clear, it is well known that some components of bacteria interact with specific receptors and are recognized by the immune system resulting in the modulation of immune responses [5]. In addition to that, Gueimonde and Salminen (2006)[6] presented some mechanisms of probiotic action that influence microbial flora of the gut such as lowering of cholesterol levels, pathogens inhibition by competition for nutrients, production of antimicrobial substances and finally counteraction of inflammatory processes by stabilization of a healthy microbiota and thus improvement of the intestine's permeability barrier. There are defined criteria that a microorganism should fulfil in order to be characterized as a probiotic. Besides the exact taxonomic identification (genus, species and strain), a potential probiotic should be: (i) generally recognized as safe (GRAS) for consumption, (ii) non-pathogenic or carrying antibiotic resistance genes, (ii) non-toxic and free of significant adverse side effects, (iii) able to survive through the gastrointestinal tract (resistant to gastric and bile acids) in vitro and in vivo and colonize the intestine or ability to persist in the gut, (iv) capable for a proven beneficial effect on the host, (v) able to adhere to mucus and/or human epithelial cells and cell lines, and (v) possess an antimicrobial activity against potentially pathogenic bacteria. In addition, stability of desired properties during processing, storage, and delivery is required[7,8].

The scope of this review is to present current knowledge about the methodological approaches that are used to quantify and characterize the potential probiotic character of microorganisms. Moreover, it will focus on molecular and non-molecular tools and finally will report some new perspectives in the study of probiotics using ''omics" techniques.

Overview of molecular and non-molecular tools for characterization of probiotics

The identification of probiotic strains requires the use of internationally accepted methods such as DNA–DNA hybridisation or DNA sequencing encoding 16SrRNA and strain identification with techniques such as pulsed field gel electrophoresis or randomly amplified polymorphic DNA [9]. These techniques are combined with specific cultivation methods for the identification of microbial phenotype

Table 1: The most commonly used probiotic species

Lactobacillus sp.	Bifidobacterium sp.	Enterococcus sp.	Streptococcus sp.	Pediococcus sp	References
L. acidophilus *L. casei* *L. cellobiosus* *L. curvatus* *L. fermentum* *L. lactis* *L. plantarum* *L. brevis* *L. reuteri*	*B. bifidum* *B. adolescentis* *B. animalis* *B. infantis* *B. thermofilum* *B. longum*	*Ent. faecalis* *Ent. faecium*	*S. cremoris* *S. salivarius* *S. diacetylactis* *S. intermedius*	*P. acidilactieri*	[3-4], [7-8], [10-11], [19]

and profile. The use of selective media for the cultivation of diluted faecal samples is possible, however, it is not the solution since their selectivity is at best relative and the results are usually either false-positive or false-negative, while the currently available techniques are not suitable for all microbes to be cultured[10]**.**

The characterization of probiotic strains and more specifically their physiological characteristics, microbial phenotype and profile is mainly made using methods such as their ability to ferment specific carbohydrates and their enzymatic activity. In this perspective a very important step is the selection of substrates or enzymatic activities that will be evaluated and should be relevant to the expected functional effects of each strain. For example the ability to hydrolyze bile salts or to produce antimicrobial substances are very interesting tests but at all cases depend on the proposed use of each strain[5,6]. In the last decades the characterization of probiotic profile is based on the comparison of the highly conserved molecules, *i.e.*, genes encoding ribosomal RNA (rRNA). Main progress in molecular biology methods has relied on the sequencing of the 16s and 23s rRNA subunits and the generation of databases of sequences of desired probiotic strains. Furthermore, similar strains have been distinguished by the application of molecular biology methods based on like plasmid profile, restriction enzyme analysis, ribotyping, random amplified DNA, and pulsed electrophoresis[11].

Table 2: Some beneficial effects of probiotics

Claimed health benefits	References
Pediatric intestinal disease	[3],[8],[102]
Infectious gastroenteritis and diarrhea-celiac disease	
Necrotising enterocolitis	
Lactose metabolism and food digestion	
Antimycotic effects	
Enhancement of short-chain fatty acid (SCFA) production	
Protection against vaginal or urinary tract infections	
Maintenance and reestablishment of a well-balanced indigenous intestinal and respiratory microbial communities	
Antibiotic-associated diarrhea	
Ulcerative colitis	
Pouchitis	
Diverticular disease of the colon	
Allergic and atopic diseases	
Production of antimicrobial peptides and control of enteric infections	
Anticarcinogenic properties-prevention of colon cancer	
Antiatherogenic and cholesterol-lowering attributes	
Maintenance of epithelial integrity and barrier	
Clostridium difficile colitis	
Crohn's disease	
Irritable bowel syndrome	

The use of 16S rRNA enables enumeration of microbes which either cannot be cultured by the current cultivation techniques or have died during transport and storage. These methods have proved successful in the characterization of the gut microbiota, the ‘‘pool” of probiotic origin. The 16S rDNA gene contains highly conserved regions, present in all bacteria, and highly variable ones that are specific for certain microbes. Fluorescent in situ hybridization (FISH), polymerase chain reaction techniques (PCR amplification followed by cloning and sequencing), Temperature gradient gel electrophoresis (TGGE), Denaturing gradient gel electrophoresis (DGGE), Terminal restriction fragment length polymorphism (T-RFLP) analysis, real-time quantitative PCR (qRT-PCR), metagenomic and metaproteomic approaches have also been applied to characterize the probiotic profile[10,12].

Characterization of probiotic bacteria with molecular techniques

Detection and identification of probiotic bacteria became an easier task after the introduction of molecular techniques and especially PCR in the 1980s. The main disadvantage of culture based techniques, that cannot detect non-culturable cells, has been overcome by the introduction of techniques that are based on the analysis of amplified nucleic acids by PCR in the sample[13,14]. Generally, the detection ability of such techniques is based on the detection of DNA polymorphisms between species or strains and differs in their dynamic range of

taxonomic discriminatory power, reproducibility and standardization[15]. Genetic fingerprinting techniques that are used, nowadays, for probiotics are described below.

Fluorescent in situ hybridization (FISH)

FISH involves whole cell hybridisation with specific bacterial groups and species having fluorescent oligonucleotide probes targeted against them. The bacteria cell samples in order to become capable for studying are immobilised on microscope slides and made permeable for fluorescently labelled oligonucleotides with subsequent microscopic observation of the hybridisation signal intensities[16,17]. Nowadays, FISH of the 16S rRNA gene can be used for the enumeration of almost 20 dominant phylogenetic groups in the human microbiota using a comprehensive set of probes. Application of these probes to faecal samples and comparison of the results with those received applying culture-based techniques indicated that bifidobacteria in faeces were culturable for the greatest part. Therefore, these results support that within the human intestine bifidobacteria have a predominant role[16]. In addition, bifidobacteria are estimated to comprise 4.4 ± 4.3% of faecal microbes according to extensive studies in the north European adult population's faeces[18]. In the field of Lactobacillus-specific FISH probes, LAB158 was found to hybridize to lactobacilli and enterococci and effective detection often requires permeabilization of the cells prior to hybridisation.

The influence of *Bifidobacterium longum* and *Lactobacillus fermentum* individually or in

Table 3: ISO methods available for the enumeresion of LAB and bifidobacteria

ISO methods	Target time	Response	Specificity versus other LAB	Comments	Ref. [67]
ISO (1998)	Mesophilic lactic acid bacteria	72 h	Low	Non mesophilic lactic acid bacteria can grow and produce false positive results. Confirmation of of results is needed	
ISO (2010a)	Bifidobacteria	72 h	High		
ISO (2003a)	Streptococcus thermophilus, Lactobacillus	48 h-72 h	Low	This method works on yoghurt containing only the mix of S. thermophilus and *L. bulgaricus*. Other microorganisms can grow if they are present in the sample to be analysed.	
ISO (2006b)	Lactobacillus acidophilus	72 h	Low	This ISO method produces false positive results if the proportion of presumptive *L. acidophilus* is much lower than that of other microorganisms (i.e., *L. hamnosus*, *L. reuteri*, *L. plantarum*, *L. helveticus*) in a mix.	
ISO (2006a)	Citrate-fermenting lactic acid bacteria	72 h, 96 h up to 120 h	Medium	Some other strains like S. thermophilus can grow and produce false positive results. Confirmation of results is needed.	

combination with two prebiotics on the gut microbiota of elderly individuals using faecal batch cultures and three-stage continuous culture systems was studied with the FISH method, indicating that synbiotic combinations the Bifidobacterium and Lactobacillus count might occur[19].

In addition, the insufficient automation for high sample throughput[20] and the extensive knowledge of the community is required since the probes need to be designed beforehand. Moreover, during a hybridization run, not all but only a limited number of probes can be used and this is the main drawback of FISH especially when it is used for community analysis on a high level of phylogenetic resolution[13].

Denaturing and Temperature Gradient Gel Electrophoresis (DGGE/TGGE)

Both denaturing gradient gel electrophoresis (DGGE) and temperature gradient gel electrophoresis (TGGE) examine microbial diversity. Electrophoresis of small PCR-amplified DNA fragments (200–700 bp) on an acrylamide gel having a low to high denaturant gradient, is used in both cases. The main difference between DGGE and TGGE is the DNA denaturation

Table 4: Separation techniques used in metabolomics

Separation techniques	References [90], [91]
High-performance liquid chromatography (HPLC): HPLC enables to separate different types of compounds, using appropriate columns packed with 3–5 μm porous particles of a stationary phase, with which they interact differently.	
Ultra-performance liquid chromatography (UPLC): Innovations in pump systems enable operations at high pressures, using 1.7-μm porous particles packed in long capillary columns combined with high operating pressures. The UPLC technology provides a higher peak capacity, greater resolution, increased sensitivity, and higher speed than HPLC.	
Gas chromatography (GC): This is an analytical technique for separating compounds mainly based on their volatilities; thus, GC is limited to volatile metabolites and those that can be derivatized to yield volatile and more thermostable products.	
Capillary electrophoresis (CE): CE separates species based on their mass-to-charge ratio into a small capillary filled with an electrolyte; in contrast with GC and HPLC, which operate based on differential interaction with a stationary phase.	
Ion mobility spectrometry (IMS): IMS has been applied for metabolomic analysis of bacterial metabolites as indicator of microbial growth, cheese and beer production, and food packaging material.	

procedure. More specifically in DGGE, chemicals like urea or formamide are used while in TGGE a temperature based denaturation is used. This results in TGGE, DNA fragments to be separated based on their melting properties. These properties depend on the products' length, GC content and the sequence of nucleotide[21].

Both methods (DGGE and TGGE) are ideal for comparison of structural changes in microbes and monitoring of population dynamics. Several regions of the 16S rRNA gene have been used for DGGE or TGGE fingerprinting. In addition, increasingly DGGE profiles of mRNA-derived PCR products are generated to describe the diversity in metabolically active populations. DGGE and TGGE have numerous applications in analysis of human faecal microflora[13]. More specifically, DGGE may apply on the antibiotic therapy of hospitalized patients and particularly assess its effect on their faecal microflora[22]. DGGE also applies in the study of LAB and Bifidobacteria temporal variation, mainly due to effects of diet or probiotic consumption[23]. It is well known that bifidobacteria are dominant in humans and therefore DGGE gels often present bands that have their origin from bifidobacteria. This was also proved by the comparison of DGGE profiles of bifidobacterial strains, which also confirmed previous culturing approach and diagnostic PCR on colony DNA studies that has suggested their vertical transmission from parents to offspring[24].

Table 5: Detection techniques used in metabolomics

Detection techniques	References
Nuclear magnetic resonance (NMR): This method detects the specific resonance absorption profiles of metabolites in a magnetic field (which is dependent on chemical structure). One major advantage of NMR analysis is its non-invasiveness and non-reliance on analyte separation, thus the samples can be recovered for further analyses.	90, 91
Mass spectrometry (MS): This technique affords high sensitivity and selectivity; its greatest advantage is that it allows the comprehensive evaluation of various molecules, as it can discriminate some compound classes, depending on the ionization type used.	
High resolution magic angle spinning (HRMAS) NMR spectroscopy (HRMAS): NMR is a rapid and accurate alternative technique, which retains the advantages of both classical solid- and liquid-state NMR, allowing the direct examination of the whole food product without component extraction.	
Fourier transform infrared spectroscopy (FTIR) :FTIR has been proposed as a metabolomic fingerprinting tool for rapid and non-destructive analysis of the quality and composition of a large number of different products.	

These two methods are used to determine the constituents of complex microbiota such as gut flora. Specifically, the diversity and novelty identified by these methods reveal the existence of limited knowledge for these important habitants in this ecosystem[15].

Major limitation of both methods is that sequencing is required for correct identification of individual bands seen in the gel. As reported by Schmalenberger et al. (2001), genetic profiles in microbial community analysis might be affected significantly by intraspecies operon heterogeneities, as amplification of one bacterial DNA may yield several separate bands, which can then be wrongly interpreted as high microbial diversity. Another drawback is the lack of reproducibility[26]. Finally, DGGE fingerprinting has low sensitivity in the detection of rare members of the community (<1%). The detection limit of PCR-DGGE for the major intestinal bacterial groups is 10^5 cells per mL of faecal sample depending on the DNA extraction method used.

Terminal restriction fragment length polymorphism (T-RFLP)

Another molecular technique that is usually used for comparative microbial community analysis is (T-RFLP). In this technique the marker genes are firstly amplified with fluorescently labelled primers, and then a restriction digestion (typically using 4-base cutters) follows and finally separation and detection using an automated sequencer occurs. Only TRFs are detected and the complexity of the community becomes visual on the electropherogram by their length heterogeneity. A sequencing gel is used by TRFLP electrophoresis due to its high resolution, quantitation, sensitivity, sample throughput and accurate sizing of individual fragments, by the use of size standards. An internal size labelled standard (using different fluorescent dye), allows precise length assignment with single base-pair resolution[13]. The obtained TRFs can be compared to the Ribosomal Database Project and its rapidly expanding sequence database[27], allowing predictions of the organisms present in the analyzed sample.

T-RFLP has been used to study faecal microbiota [28], but also in intestinal samples for the tracking of probiotic *Lactobacillus* strains[29,30]. A novel phylogenetic assignment database for the TRFLP analysis of human faecal microbiota (PADHCM) has been designed enabling the prediction of the terminal-restriction fragments at species level[30]. This will increase the applications of this method in studies of dietary and probiotic effects on the microbiota.

T-RFLP in combination with sequencing has been used in the litter from broiler chickens previously treated with streptogramin growth-promoting antibiotics, prebiotics and probiotics for the identification of their bacterial community composition. The administration of prebiotics and probiotics to flocks led to higher bacterial diversity, while the aging of litter had the opposite effect leading to a decrease[31].

T-RFLP has been also used in rats to explore the colitis (induced by trinitro-benzene sulfonic acid, TNBS) in order to evaluate the impact of the probiotic VSL#3. The results showed that the probiotic VSL#3 protected the rats by the disease may be through the alteration of the composition of the intestinal microbiota[32].

T-RFLP is, however, limited by the choice of primers and the TRF length overlap by phylogenetically distant bacteria[28]. In addition, incomplete or non-specific restriction leads to overestimation of the diversity since the number of fragments increases. Pseudoterminal restriction fragments can also generate overestimation of diversity[33]. Despite these problems, T-RFLP is proved an important molecular technique for the analysis of microbial community, especially in the cases where the throughput and sensitivity are required at high level without the need for direct sequence information[13].

Randomly Amplified Polymorphic DNA (RAPD)

RAPD, is a PCR-based discrimination method. In this method short arbitrary primers anneal to multiple random target sequences of unknown location in the genome of an organism, resulting in patterns of diagnostic value. These sequences are used in PCR reactions with low-stringency annealing conditions resulting in the amplification of randomly sized DNA fragments[22].

RADP is a reliable, sensitive and inexpensive method to distinguish between different strains of LAB and Bifidobacteria and also during fermentation to monitor any changes in the community of LAB[34]. In a study RAPD-PCR was used to detect Lactobacillus species in the human vagina and to evaluate their probiotic persistence[35]. In another study, Schillinger et al. (2003)[36] used group-specific PCR and RAPD-PCR to identify strains of the *L. casei* and *L. acidophilus* groups most commonly used in probiotic yogurts. The identification of lactobacilli was carried out by comparing the RAPD profiles of 20 *Lactobacillus* strains with 11 reference strains of the *L. acidophilus* and *L. casei* group. In another study two random primers (OPL-05 and ArgDei-F) were used in order to subject 149 *Lactobacillus* isolates to RAPD and as a result a considerable degree of genomic diversity in *L. plantarum* isolates was reported[37].

A total of 109 LAB isolated from infant faeces were examined and *Lactobacillus* strains were found to be the most prevalent genus (more specifically *Enterococcus* and *Enterococcus faecalis*).

L. casei/paracasei CTC1677, CTC1678 and *L. rhamnosus* CTC1679 proved the most suitable as probiotic starter cultures for fermented sausages using RAPD[38]. The main limitation of RAPD is its reproducibility. In general, in order to succeed reproducibility careful controlled conditions are needed. This is important since factors such as DNA template purity and concentration, annealing temperature and primer combinations may influence the reproducibility and discriminatory power of the RAPD fingerprints[21,22,39].

Restriction Fragment Length Polymorphism—RFLP/ Amplified Ribosomal DNA Restriction Analysis—ARDRA

Amplified ribosomal DNA restriction analysis (ARDRA), also known as restriction fragment length polymorphism (RFLP), is a relatively simple PCR-based fingerprinting technique. It is based on the digestion of amplified ribosomal community DNA followed by gel electrophoresis and can be employed in microbial identification. In this technique, PCR-rDNA fragments are digested with base-pair restriction enzymes, which are capable for DNA cleavage at specific sequences, producing fragments from 100 base-pairs to 10 kb in length. Different lengths are the result of different restriction enzymes and are detected on an electrophoresis gel[40].

This technique has been successfully applied in order to identify probiotics in fermented milks[41], such as various species or strains within the *L. acidophilus* complex*, L. casei*, *L. delbrueckii* and its three subspecies (*bulgaricus*, *delbrueckii* and *lactis*), *L. fermentum*, *L. helveticus*, *L. plantarum*, *L. reuteri*, *L. rhamnosus, L. johnsonii* and *L. sakei*. and LAB from kefir[42,46].

ARDRA has been also used for the identification of 57 LAB strains isolated from cassava and maize grains fermentation processes. It was demonstrated that *L. plantarum* had rapid production capability whereas *L. pentosus* exhibited high amylase activity. The factor for the selection of starter cultures was their ability to be tolerant to acidic environment and bile salt[47].

ARDRA was used as a correlation method between lactobacilli and probiotic properties. The attempted classification focused on 11 homofermentative lactobacilli in *L.plantarum* species. These isolates exhibited high resistance to bile salt and strong inhibitory activity against *Salmonella typhimurium* and *E. coli*[48].

The banding pattern and its complexity is the main drawback of this method. Furthermore, due to the complexity of the profiles, it is necessary to use multiple restriction enzymes either separately or in combination in order to obtain the desired resolution[13]. Another drawback is the limited staining sensitivity of gels[39], and therefore this technique is mainly used in communities that are dominated by a few members[49].

Ribotyping

Ribotyping is a variation of RFLP analysis of the genomic DNA, where certain fragments are highlighted by probing in order to obtain less complex patterns that are easier to interpret[50]. In ribotyping, the fingerprint pattern consists of chromosomal DNA fragments derived from the rDNA operon and its adjacent regions that hybridise the rDNA probe.

The probes used in ribotyping vary from partial sequences of the rDNA genes or their spacer regions to the whole rDNA operon[51].

Ribotyping has been proved capable of differentiation between human intestinal lactobacilli and bifidobacteria, at both species and strain level[51]. Using this approach *B. longum*, *B. infantis* and *B. suis* strains were characterized, which demonstrated that the ribotyping differences among these strains are very limited[52]. Moreover, using the intergenic 16S–23S rDNA as a probe, it was possible to divide 26 *L. helveticus* strains into five, nine and ten ribotypes using *Eco*RI, *Pvu*II and *Mlu*I, respectively.

In addition 91 type and reference strains of the *L. casei* group and the *L. acidophilus* group were characterized using an automated ribotyping device (Riboprinter Qualicon, Wilmington, DE, USA)[52].Ribotyping has been used in bacterial cells which were recovered from faecal samples in order to match the strain isolated from faecal sample to the clone administered to the animals-10 calves during the whole farming period- which were daily fed with *Bacillus coagulans* spores[53].

The characteristic of ribotyping is its high discriminatory power. This ability is higher in species and subspecies level compared to strain level[21] and dependent on the specificity of the probe, as well as its size and the restriction enzyme used[54].

Pulse Field Gel Electrophoresis (PFGE)

In PFGE large DNA fragments derived from restriction digests with rare-cutting enzymes can be separated throughout the run with increasing pulse times and the resulting fingerprint profiles can be further explored in culture identification[42]. The DNA fingerprint that is generated depends on the restriction enzyme used, and mainly on its specificity, and on the bacterial genome's sequence. These features make it characteristic for a particular bacterial species or strain. This fingerprint represents the complete genome and thus can detect specific changes within a particular strain over time (DNA deletion, insertions or rearrangements)[21]. PFGE, compared to other fingerprinting techniques, may be more time consuming due to the long pulse times. However, the generated fingerprint profile of PFGE represents the whole genome and therefore PFGE has superior discriminatory power compared to ribotyping[22].

PFGE has been used successfully for the differentiation of probiotics in milk samples and cheeses[34], such as *B. longum* and *B. animalis*, *Lb. casei* and *Lb. rhamnosus*[55], *Lb. acidophilus complex*[56], *Lb. helveticus* and *Lb. Johnsonii*[45], *L. delbrueckii* and its three subspecies (*bulgaricus*, *delbrueckii* and *lactis*), *L. fermentum*, *L. helveticus*, *L. plantarum* and *L. sakei.*[43,57]. Furthermore, PFGE is an useful technique for monitoring the changes in the populations of predominant lactobacilli and bifidobacteria of human origin[58].

In another research study, PFGE was used to characterize Lactobacillus strains that were isolated from feta cheese and newborn infants' gastrointestinal tract and it was found that these strains possess certain cell surface traits such as hydrophobicity, autoaggregation and high adhesive capacity[59].

PFGE has been of great significance in strain typing for *L.acidophilus complex, L.casei, L. delbrueckii, L.fermentum , L.s helveticus, L.plantarum, L.rhamnosus and L.sakei*[57,43]. An additional field in which this method has contributed is the discrimination within strains of LAB species and even the placement of isolates in specific Lactobacillus species[22].

Hybridisation techniques and use of oligonucleotide probes

Probing techniques are based on the hybridisation of synthetically designed oligonucleotides to specific target sequences in bacterial DNA. The probes that are used in such techniques are linked to a label (usually radioactive or fluorescent) so that the target becomes visual after its hybridisation. The probe's specificity is dependent mainly on the target sequence; however other factors such as the stringency of the hybridisation and washing conditions are also important. Such probes have been used in many assays like colony, dotblot and in situ hybridisations[50].

Direct hybridisation of an oligonucleotide probe to the target microbial nucleic acid (dot blot hybridisation) is a straightforward method to detect

specific nucleic acid sequences or genes. Cultured microbes or other sample are used for the extraction of DNA or RNA. The extracted DNA or RNA is fixed to a membrane, from nylon or nitrocellulose that is positively charged and finally is hybridised with an oligonucleotide or a fragment of DNA that has a radioactive, chemiluminescent or digoxigenin label. This echnique may also be quantified in order to measure the amount of specific target rRNA/rDNA in a mixture relative to the total amount of rRNA/rDNA[51].

Over the last decade, hybridizations with rRNA-targeted probes have been proved very valuable tools to understand the structure and spatiotemporal dynamics of complex microbial communities. The design of nucleic acid probes can be made in such a way in order to target taxonomic groups at different levels of specificity (from species to domain) by means of variable evolutionary conservation of the rRNA molecules[21]. Some of the 16S rDNA probes used to detect and identify LAB and *Bifidobacteria sp.* are listed in Satokari et al. (2003), Amor et al. (2007) and Venture et al. (2004) papers.

Oligonucleotide probes have the potential to be used as reliable and rapid diagnostic tools since they are complementary to regions of 16S or 23S rRNA and have been successfully used for the identification of LAB[21]. However, the human gut shows a very complex microflora and therefore makes its study very difficult by using only primers that are specific at the species level[52]. Nowadays, probes have been found for all *Bifidobacterium* species of the human intestine. The same applies for lactobacilli of human intestine apart from species like *L. vaginalis, L. mucosae,* and *L. buchneri*[51]. Finally, dot plot hybridisation has its own limitation. This method requires probe design and validation, but in general both techniques are very important for the analysis of gut microbiota[21,51,52].

The method was performed in order to elucidate the correlation of probiotics and their clinical benefits. These benefits are associated with the prevention and treatment of acute and antibiotic-associated diarrhoea, the treatment of allergies and intestinal, liver and metabolic diseases. This positive impact in human health originates from the regulation of intestinal permeability, the improvement of gut immune barrier function and the balance between pro- and anti-inflammatory cytokines[60].

Clone libraries

Bands can be excised from gels, cloned and sequenced in order to achieve identification of (differential) signals obtained with the common community profiling techniques for gel-based approaches such as DGGE, TGGE, SSCP, ARDRA and RISA[13]. Alternatively, the PCR-amplified sequences can be directly cloned and sequenced and species of individual community members can be identified. In practice, PCR-amplified 16S rDNA (or rRNA) fragments are cloned, sequenced and compared to the available sequences found in rRNA databases such as GenBank, EMBL and Ribosomal Database Project (RDP)[51]. The diversity of the intestinal microbiota has been vastly underestimated as revealed by the sequencing of 16S rRNA gene libraries of human intestinal microbiota, generated by PCR amplification of the 16S rRNA gene of DNA from human faeces and mucosa-associated bacteria[18].

After sequencing of 284 clones an estimated 85% coverage of one person's faecal 16S rDNA diversity was obtained. The clones were classified into 82 molecular species, the majority of which were found to be novel [51]. Vaughan et al. (2005) reported that according to the database analysis more than 80% of the 16S rRNA sequences retrieved from the human intestinal tract represent uncultured bacteria. They also supported a combination of sequence analysis of 16S rRNA gene libraries and FISH approaches targeting the 16S rRNA. They showed that the most abundant bacterial groups in the human intestine belong to the phyla of the *Firmicutes* (including the large class of *Clostridia* and the LAB), *Bacteroidetes*, *Actinobacteri*a (including *Colinsella* and *Bifidobacterium spp.*) and *Proteobacteria*[18] at the order of numerical importance.

The 16S rRNA libraries of adult faecal samples proved that *L. ruminis* is the predominant Lactobacillus intestinal species. However, many other species were also detected including *L. crispatus*, *L. gasseri*, *L. plantarum*, *L. acidophilus*, *L. delbrueckii*, *L. casei*, *L. paracasei* and *Leuconostoc argentinum* as well as food-associated bacteria such

as *L. sakei*, *L. curvatus*, *Leuconostoc mesenteroides* and *Pediococcus pentosaceus*[18].

However, approaches based on cloning are rather tedious and not optimal for analysis of large numbers of samples. Like all PCR-dependent methods, construction of clone libraries may include bias, possibly leading to falsification of the library structure. A major drawback of their use is that sometimes a few thousand clones should be analyzed to cover the phylogenetic richness hidden in a prokaryotic gene library depending also on the matrix[13]. The approach is rather laborious and hence, appropriate only for the screening of a limited number of samples although direct sequencing can always provide significant new information about the microbial diversity in the GI tract[51].

DNA arrays

The development of DNA-chips, also called DNA microarrays is an important further step in hybridisation methods. Identification can be accomplished with designed diagnostic arrays within a matter of hours without prior cultivation. Oligonucleotides targeting a set of sequences, usually the 16S rRNA gene, are formed within detection-type arrays[21]. Using this technique, specific detector oligonucleotides are immobilized on a solid support, hybridize with homologous-labelled target amplicons and then can be detected. Typically, microarrays contain hundreds of oligonucleotide probes, usually based on the 16S rRNA gene. These probes are specific for different strains or species or genera of microorganisms that are detected in a single assay[18]. This strategy has proved to be successful for microbial identification, even when species can only be discriminated by a single-nucleotide polymorphism[13].

There are many different forms of arrays to which the probes can be attached including low to medium density arrays such as macroarrays, and glass microarrays, and very high density microarrays (>10^4 probes typically 25 mer per chip)[61]. Currently, two types of arrays have been developed, low-density microarrays (developed on a nylon membrane) and high-density microarrays (on a glass slide) which may contain up to hundreds or millions of detector oligonucleotides[13]. DNA arrays can be divided into three categories based on the genes targeted by the array. The first category encompasses the most common DNA arrays, the phylogenetic arrays, based on a diagnostic marker such as the 16S rRNA gene and used for microbial identification. A second category comprises functional gene arrays, designed for the detection of key functional genes in a specific environment. The third category consists of metagenomic arrays which contain DNA fragments produced directly from environmental DNA and can be applied with no prior sequence knowledge.

A major advantage of phylogenetic or functional gene arrays is the unlimiting expanding capacity to detect specific microorganisms or genes of interest. Its most important drawback is the need for specification of the target organisms or genes and the lack of identification of taxa for which no oligonucleotides are developed yet[13]. This is valid for a very complex microbial ecosystem such as that of the human gastrointestinal tract[21]. Some applications of *DNA array technology include their use in the human intestinal microbiota and also in Lactobacilli* and *Bifidobacterium* species detection[18,39,50].

Quantitative Real time PCR (qRT-PCR)

Real-time PCR is a DNA-based technique monitoring the amplification of the target DNA in real time by monitoring fluorescence. qRT-PCR can be used to quantify bacteria from various samples including milk, faeces, food and water, and can be employed in processing, detection and pathogens confirmation in multiple samples at any time [22]. In addition, real-time PCR allows accurate template quantification over a wide dynamic range (>10^7-fold). Typically, DNA amplification is continuously monitored based on the emission of fluorescence. The initial concentration of target DNA is linked to a precise threshold cycle, defined as the cycle number at which fluorescence increases above the background level. Ultimately, the target DNA is quantified using a calibration curve that relates threshold cycles to exact concentrations of template DNA[13]. Several techniques have been introduced following the advent of real-time PCR such as primers with fluorescent dyes, dual probes and intercalating dyes such as SYBR Green I. qRT-PCR is a superior technique for quantification of nucleic acids. However, a major drawback of the method is that the total amount of PCR reactions in a single tube is restricted to a handful of targets due to the

limited number of different fluorescent dyes and the nature of the energizing light source that can be used in real-time instruments[62]. Moreover, it has limitations in the quantitative community analyses of complex matrices. Real-time quantitative PCR with SYBR Green I has been reported to be 10-fold less sensitive than a corresponding TaqMan assay due to the formation of non-specific products in reactions starting with small amounts of template DNA[63]. However, SYBR Green I is advantageous in situations where a diverse target population is to be detected with PCR. Hence, the development of quantitative multiplex assays that can effectively screen large numbers of targets in a given sample represents one of the present challenges in molecular biology[13]. Numerous real-time PCR based assays are in the process of development for the major groups within the faecal microbiota of humans, as well as lactobacilli and *Bifidobacterium* species[18,64]. Moreover, a qRT-PCR approach has been developed with hybridization probes that were designed according to the differences among the 16S rRNA genes of *L. casei*, *L. paracasei* and *L. rhamnosus.* A melting curve analysis of the hybridization probe was employed for their differentiation. This approach identified *L. paracasei* and *L. rhamnosus* correctly but did not separate *L. paracasei* from *L. casei* due to the existence of same 16S rRNA sequence in both species[22]. Finally, qRT-PCR has also been employed in the molecular quantification of lactic acid bacteria in fermented milk products[65], meat and fermented sausages[66] and dairy microbiology[67].

Exploring probiotic bacteria profile by ''omic'' approaches

In the last decade, omics approaches have affected at a great extent the way of studying the biology of microorganisms, including probiotics. Omics methodologies comprise high-throughput techniques directed to understand the cell metabolism as one integrated system, rather than independent parts, by using information about different molecules' relationships[68]. To date, probiotics have been selected on the basis of ecology and phenotypic characteristics that ensure safety, robust manufacturing, storage stability, and survival through gastrointestinal transit[69]. As far as probiotic research is concerned, scientific research has moved towards fully integrated approaches compiling information at various molecular levels i.e. DNA, RNA, proteins and metabolites rather than exploring single gene/protein functions and phenotypes[70].

The rational selection of candidate probiotics for specific health targets can be achieved through the understanding of the biology of microbe–host interactions in GI track70. Various methods have been developed for the functional characterization and quantification of gut microbiota gene products. The large-scale study of genes, transcripts or proteins is carried out by 'Omics' technology. Last but not least, genomics has become a rapid and cost effective way for the characterisation of probiotics and new developments in sequencing technology will further reduce both time and cost[71].

(Meta)genomics

Metagenomics, is defined as "the application of modern genomics in the study of communities of microbial organisms directly in their natural environments, bypassing the need for isolation and lab cultivation of individual species". This has given great insight into the composition and functioning of microbial communities in terrestrial and marine environments[72].

A much greater diversity has been revealed by the study of entire microbial communities using metagenomic approaches and has helped to determine the community structure of several previously unknown ecosystems. Over 3 million genes from the intestinal microbiome have been sequenced so far and the existence of different human enterotypes has been identified. In the probiotics field these aberrations constitute clear targets for the future development of products directed to counteract them[73].

Metagenomic studies have indicated that the normal microbiota may be better defined at functional than at phylogenetic level[73]. Applying genomics to bifidobacteria has contributed to a better understanding of stress responses, bacterial phylogeny and ecological adaptation and genetic variability through comparative genomic studies of sequenced genomes[74]. Twenty two complete genomes of bifidobacterial strains are currently available, with another fifteen still to be completed[75]. Representative strains of only six species of

bifidobacteria among the currently recognized 37 species have been sequenced to completion. There are 36 complete genomes available, representative of 19 species, and a large number of still ongoing sequencing projects from the currently recognized 154 species of *Lactobacillus*[75]. Some functional characteristics of *Lactobacillus* and *Bifidobacterium* strains revealed using metagenomics are highlighted in the extensive review of Sánchez et al. (2013).

Metagenomics is undoubtedly a powerful analytical strategy. However, it requires very complex computation, data-storage and handling procedures, and more sophisticated algorithms in order to facilitate improvement of sequence assemblies in complex microbial communities[39]. The sequencing of genomes from probiotic strains and commensal microorganisms has been achieved through metagenomics development at a low price[39]. This increasing knowledge of genomics of probiotic and intestinal bacteria allows the understanding of their role and effect in human health and their role in bacterial community and human physiology[75].

(Meta) proteomics

Proteomics (Metaproteomics, also referred to as whole community proteomics) analyzes all proteins produced simultaneously by a cell, or a tissue, under specific conditions. It might also analyze precise mechanisms such as quorum-sensing[76]. In the case of probiotics, proteomics is also an effective method for the characterisation of different strains77.

The official definition of probiotics reports on the beneficial effect exerted by living cells, necessitating the survival of the ingested probiotic bacteria in the GI track. Bile salts concentration in the small intestine is quite high and acts as a detergent on bacterial membranes. A potential probiotic strain must adapt to this environment. The proteomic analysis referring to the response of lactobacilli, bifidobacteria and propionibacteria to bile salts and in the analysis of surface-exposed proteins, has shown upregulation of proteins involved in amino acid, carbohydrate, and nucleotide metabolism. Moreover, general stress responses have been reported in redox control indicating a global metabolic adaptation in response to the toxic compounds[78].

In this perspective, 2-DE has been employed to characterise response to different gastrointestinal stresses and experimental conditions including response and adaptation to bile salts and acid pH in *B. longum*, *B. animalis, Lactobacillus casei, Lactobacillus reuteri, Lactobacillus rhamnosus GG, plantarum* 299V and *L. delbrueckii subsp. lactis* 200*, L. lactis, Bifidobacterium longum* NCC2705 and *Propionibacterium freudenreichii*[78,75]. 2-DE has also been used to study the response of probiotic bacteria to technological stresses, such as the response of *B. longum* BBMN68 to oxygen-stress conditions or the response of *L. reuteri* to selenium for nutraceutical use[79]. Finally, proteomic evidence in *Bifidobacterium* is aligned with an increased production of exopolysaccharides (EPS) in bile tolerant strains. This supports the hypothesis that EPS rotective coating may allow the bacterium to better withstand bile salts and stomach acid[80].

Proteomics contributes to the study of the mechanistic aspects of probiotics underlying their ability to change their lifestyle and analyses their responses to stress in order to better adapt to GI track. The limitations of this methodology include the difficulty of automation and hence its time-consumption, expensiveness and labor intensiveness. 2D-MS also has a low dynamic range, and the gel-to-gel variability depends largely on staining and visualization techniques[74]. 2-DE has failed in the detection of high and low molecular mass proteins, low abundant proteins and proteins with very high isoelectric points[75]. These technical limitations have restricted the analysis to abundant proteins involved in certain informational (translation, stress response) or metabolic pathways (glycolysis, nucleotide biosynthesis)[78].

(Meta) transcriptomics

According to Wang et al. (2009), the transcriptome can be defined as the complete set of transcripts in a cell and their quantity in a specific developmental stage or physiological condition. Transcriptomics aims in (i) making a catalogue of all species of transcripts (mRNAs, noncoding RNAs and small RNAs) (ii) the determination of the transcriptional structure of genes and (iii) the quantification of the changing expression levels of each transcript during development and under different conditions. Nowadays, the most frequently

employed (meta)transcriptomics techniques are based on the reverse-transcriptase polymerase chain reaction (RT-PCR), DNA microarrays, RNA-seq and cDNA-AFLP.

RT-PCRT has been extended to monitor RNA transcripts by first converting target RNA sequences into complementary DNA (cDNA) molecules by the reverse transcriptase enzyme, which copies the RNA sequence into a base-pair complementary DNA product [82]. The multiplicity of transcriptomics analysis has exploded with the advent of DNA microarrays or DNA chips[82].

Metatranscriptomics relies on RNA isolated directly from complex microbial populations arising from high-throughput sequencing[83]. RNA-sequencing (RNA-Seq) is a major technique employed in metatranscriptomics analysis. RNA-Seq transcriptomics replaces the hybridization of nucleotide probes with sequencing individual cDNAs produced from the target RNA. Emerging methods for these fully quantitative transcriptomic analyses might overcome the limitations of microarray technology and there is a lot of debate whether sequencing approaches might replace microarrays in the near future[84]. cDNA amplified fragment length polymorphism (cDNA-AFLP) is an alternative method for metatranscriptomic analysis. This technique involves total isolation of RNA from gut samples, enrichment for mRNA and then synthesis of the double-stranded cDNA, digestion, followed by ligation of adaptors. Compared with microarray-based approaches, cDNA-AFLP has the advantage of study of any unknown genome or set of genomes without any prior sequence knowledge. Moreover, the cDNA-AFLP allows the detection of genes expressed quite low[85].

Extensive studies have focused on whole-genome transcriptional responses to gastrointestinal conditions such as the presence of bile salts, growth in different oligosaccharides, milk from different sources. They have revealed a set of molecular strategies that probiotics might use to overcome GI track conditions[75]. For example, a transcriptomic analysis revealed the presence of a bile inducible transporter in several bifidobacteria, which following a further characterization showed a bile tolerance effect[75].

O'Flaherty and Klaenhammer (2010) reported that transcriptomics have shown a complex communication between bacteria (commensal and probiotic) and the GI track and play a significant role in probiotic functionality as well as in the analysis of specific proteins. The two researchers underline the case of *L. acidophilus.* Using genetic tools they confirmed important probiotic traits such as cell surface factors including S layer proteins, bile tolerance, acid stress, bacteriocin transport and oxalate degradation. In another study by Herve´-Jiminez et al. (2009) some new insights on the interactions between probiotic bacteria in yoghurt (the production of H_2O_2 by *L. bulgaricus* modifies iron metabolism in *S. Thermophilus*) have been revealed by transcriptomic and proteomic analysis.

In the field of LAB, an inducible response to the membrane-disrupting effects of bile has been suggested by transcriptional responses to bile in *L. plantarum* and identification of several proteins putatively present in the cell membrane or cell wall[88]. Moreover, characterisation of some of the health-promoting effects of probiotics, such as the induction of *Bacteroides thetaiotaomicron* genes involved in carbohydrate metabolism when cocultured with *L. casei* could be achieved by transcriptional analyses of both probiotic and commensal species[88].

The development of RNA sequencing technologies bypassing some of the limitations of microarrays (requires genome sequence; depends on the annotation of open reading frames for a classical microarray not for a tiling array; only a "snapshot" view of transcription; difficult to obtain sufficient RNA from in vivo samples; good analytical tools are needed; role of identified genes needs to be confirmed by downstream analyses) [89] forms a step forward in transcriptomic analysis. These technologies identify transcripts de novo, and for that reason are independent of the presence of probes for particular transcripts on the array. The recent validation of next generation sequencing technologies in comparison to microarray platforms has arisen from the comparison of transcriptomic profiles from well known bacteria cultured under well defined conditions. This validation supports its utility in analysing and interpreting information from complex samples[75].

(Meta)bolomics

The metabolome represents the final omic level in a biological system due to its reflection of changes in phenotype and function[90] and is used to study the function of gut microbiota through survey of their metabolic profiles as well as host metabolic profiles[85] whereas the genome, transcriptome and proteome can be seen as mediums in the flow of gene expression.

Metabolomics focuses on the simultaneous determination and quantitative analysis of intracellular metabolites (low-molecular-mass compounds, <1500 Da, not genetically encoded and produced and modified by the metabolism of living organisms i.e. microbes). These compounds include endogenous and exogenous small molecules such as peptides, amino acids, nucleic acids, carbohydrates, organic acids, vitamins, polyphenols, alkaloids and minerals[91]. From the "omics" perspective, metabolomics allows a more comprehensive understanding of living organisms[92].

The analytical strategies for metabolomics can be broadly divided into "non-targeted" and ''targeted methods". The targeted methods focus on a specific group of intended metabolites with the requirement of identification and quantification of many metabolites within the group such as bile acids, or metabolites of central carbon metabolism[93]. The non-targeted approaches aim to cover the metabolome as broadly as possible while allowing the quantification of the metabolites at least differentially[94]. The most popular separation techniques are high-performance liquid chromatography (HPLC) or ultra-performance (UPLC) form, gas chromatography (GC), capillary electrophoresis (CE) and ion mobility spectometry (IMS) (Table 4). Among the detection techniques, mass spectrometry (MS), nuclear magnetic resonance (NMR), high resolution magic angle spinning (HRMAS) NMR and Fourier transform infrared spectroscopy (FTIR) are the most employed (Table 5)[90,91]. It should be mentioned that no single analytical methodology or platform can detect, quantify, and identify all metabolites in a certain sample, hence a combination of different techniques is required to ensure that the obtained results are complementary[90,95].

Recent research in the field of metabolomics has demonstrated the large impact of the gut microbiome on mammalian blood metabolites, suggesting a major interplay between bacterial and mammalian metabolism and a deep understanding of the mode of action of specific probiotics[96]. Regarding the effect of gut microbiota on human metabolism, metabolomic techniques strengthen preexisting evidence that fermented food, in particular bovine milk, is a key vector for the delivery of bacteria, and metabolites derived from the fermentation process to the gut in order to modulate human health[97]. Another study that indicates the role of gut microflora in health, was conducted by Martin et al. (2009) where it was reported that feeding pro-, pre- or synbiotics could induce microbial changes in mice and that microbial activity was directly correlated with dietary calorie recovery, fat absorption and lipid metabolism.

The application of metabolomics involves the analysis of the multi-factorial metabolic response of an organism to biological stimuli. Thus, this methodology may elucidate the mechanism by which the metabolites released or degraded by probiotic strains have an effect on cytokine expression. It may also give information about changes in beneficial metabolite concentrations in different organs when probiotics are present in the gut[91]. Martin et al. (2009) confirmed the effect of some probiotic strains on the host, analysing different parts of germ-free mouse intestines by HRMAS NMR. In another research (Martin et al. 2008), metabolites were analysed in gut, plasma, urine and fecal extracts by HRMAS-NMR. The results showed that *L. paracasei* NCC2461 and *L. rhamnosus* NCC4007 probiotic strains could alter hepatic lipid metabolism and stimulate glycolysis. The presence of these probiotics strains also affected amino acid catabolism and the concentrations of methylamines and short-chain fatty acids.

The application of metabolomics to the metabolic profiling of faeces offers the potential to investigate gut microflora metabolism and its interaction with host metabolism. Detailed metabolic profiling of faeces samples from 39 healthy subjects at three different time points has shown inter- and intra-individual variability[100]. Finally, metabolomics is increasingly used in clinical applications with

regard to disease etiology, diagnostic stratification and potentially mechanism of action of therapeutical and nutraceutical solutions. It explores relationships between environmental factors and health and highlights the role of probiotic supplementation and the effect of probiotics in disease prevention and inflammatory bowel disorders[101].

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