Analytical strategies for characterization and validation of functional dairy foods

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Functional foods (FFs) are food products to be consumed as part of a balanced diet. They provide physiological benefits or reduce the risk of chronic disease beyond basic nutritional functions. Functional foods containing probiotics and/or prebiotics have gained much interest in recent years due to their health-promoting capacity.

The main objective of this review is to discuss the analytical strategies that have been used to validate FFs associated with dairy products containing probiotics and/or prebiotics. In these products, the biochemical events, carried out by enzymes of different sources (milk, bacteria, rennet) leading to the transformation of milk to diverse products (e.g., yoghurt and cheese), are glycolysis, proteolysis and lipolysis.

We present the analytical methodologies used to study the microbial probiotic flora and to evaluate the biochemical transformations, the associated functionality in terms of intestinal microbiome and the safety of such FFs. We address the analytical figures of merit. We cover the advantages and the disadvantages of such analytical methodologies and comment on future applications and potential research interest within this field.

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1. Introduction

Nutrient supply through foods has an important role in the health of each person; consciousness of this factor is increasing and consumers’ awareness is noticeable through the increased demand for functional foods (FFs) [1]. Although there is no consensus about the exact definition of FFs, it is accepted that these foods should come from natural sources, and their nutritional characteristics should also be able to provide physiological benefits by improving health and well-being [2] or to reduce the risks of chronic diseases (e.g., cardiovascular diseases, overweight or obesity, dyslipidemia, hypertension, osteoporosis and diabetes) [1,2]. According to Jankovic et al. [3], the main role of FFs is to be able to maintain host health or to help recovery upon temporary illness.

In the worldwide FF market, dairy products are key products and, among those dairy-based products, functional beverages account for an important fraction of this sector [4].

Probiotics and prebiotics may be found in some FFs, namely dairy products. Probiotics are live microorganisms that confer health benefits to the host when administered in adequate amounts [5,6] (e.g., modulation of the immune system, reduction of lactose intolerance, reduction of cholesterol levels, antimicrobial activity, and anti-diarrheal, anti-mutagenic and anti-carcinogenic properties) [6,7]. Additionally, from a safety point of view, probiotic strains cannot be associated with any type of disease or transmission of antibiotic-resistant genes [6].

In order to promote health benefit to the host, probiotic viable cells should be technologically resistant to survive the different processing steps [6,8] and storage conditions, survive the passage through the gastrointestinal tract and adhere to intestinal cells where they will be potentially beneficial [9]. Recommended minimum doses of probiotic viable cells to...
ensure health benefits to the host must be in the range 7–9 log cfu per gram or mL [5] of a product, which must be consumed on a regular basis. The majority of probiotic strains used in functional dairy foods (FDFs) belong to the Lactobacillus and Bifidobacterium genera [9], but other species (e.g., Saccharomyces cerevisiae (boulardi)), Bacillus subtilis, Escherichia coli) are also being marketed with similar purposes [6]. An overview of probiotic dairy products considered as good vehicles for delivering probiotic bacteria to the gastrointestinal tract (e.g., yoghurts and cheese) is given in the review by Granato et al. [10]; beverages are also included. Certain factors (e.g., fat content, concentration and type of protein, non-protein nitrogen, sugars and pH of food) may affect growth and viability of probiotic bacteria. Cheese characteristics (e.g., higher pH, lower titratable acidity, higher buffering capacity, greater fat content, higher nutrient availability, lower oxygen content and a denser matrix) are favorable factors for the survivability of probiotic bacteria [11,12].

Prebiotics are non-digestible oligosaccharides resistant to hydrolysis and absorption in the digestive system [5], reaching the colon almost intact where they promote the proliferation and activity of desirable bacteria in the gut [13] because they are selectively fermented especially by probiotic bacteria. The prebiotics most commonly used are oligosaccharides whose degree of polymerization varies between 2 and 20 monomers [e.g., fructooligosaccharides (FOSs), inulin, galacto-oligosaccharides (GOSs) and xylo-oligosaccharides (XOSs)]. Their benefits have been related to increase of calcium absorption, improvement of bone-mineral content and bone-mineral density, blood-glucose formation, reduction of cholesterol and serum lipids levels, and obesity control [1]. Prebiotic compounds are important for probiotic microorganisms, since they favor their growth and survival in foods and the gastrointestinal tract: food products containing both probiotics and prebiotics are designated as synbiotic [14].

Different analytical strategies have been applied to study probiotic microflora and prebiotic compounds, and to characterize biochemical transformations in FDFs and the associated functionality and safety, including legal requirements to uphold associated claims.

The main goal of this review is to discuss the advantages and the disadvantages of such analytical methodologies, and to comment on future applications and their potential research interest.

2. Analytical strategies

In milk and fermented dairy products, there is a plethora of beneficial compounds [e.g., functional proteins, bioactive peptides, vitamins, antioxidants, oligosaccharides, organic acids, and polyunsaturated fatty acids (e.g., conjugated linoleic acid)] that are important for digestive, gastrointestinal and cardiovascular functions, gastrointestinal microflora, hemodynamics and immunoregulation. There may be a direct probiotic effect (interaction with ingested beneficial microorganisms) or an indirect biogenic effect (action of generated microbial metabolites, including vitamins, proteins, peptides, oligosaccharides, and organic acids) [2]. These products are encouraged and this overview concerns an investigation on the current analytical techniques that are being used to characterize and to validate FDFs (e.g., cheeses, yoghurts, and fermented milks) where probiotics or lactic-acid bacteria (LAB) play a vital role.

Assessment of probiotic bacteria or LAB viability and/or survivability must be assured throughout processing phases and storage until consumption. Therefore, analytical strategies to monitor the viable microflora and their metabolic action require discussion. Additionally, in most fermented dairy products, the biochemical events, performed by enzymes of different sources (milk, bacteria, rennet) leading to the transformation of milk to diverse products (e.g., yoghurt and cheese) are glycolysis, proteolysis, and lipolysis. The analytical methodologies used to evaluate these biochemical transformations have evolved. Nowadays, foodomics, a new discipline that studies food and nutrition via application of advanced “omics” techniques, is changing the analytical approaches and strategies in food-science research; classical methods are being confronted with advanced omics strategies, which include analytical techniques [e.g., mass spectrometry (MS), and nuclear magnetic resonance (NMR) spectrometry] [15,16]. Foodomics covers many fields of research including genomics, transcriptomics, proteomics and/or food metabolomics studies for analysis of biomarkers, and compound profiling for food quality/authenticity purposes and research in food bioactivity and its effect on human health through nutrigenomics and/or nutrigenetics approaches, among others. This review mainly focuses on and discusses the major analytical approaches, confronting classical with advanced analytical techniques that have been applied to study FDFs containing probiotics and/or prebiotics.

2.1. Viability and/or survivability of probiotic bacteria

The presence of viable and metabolic active cells of probiotic bacteria in dairy products is essential in order to assure the beneficial health benefits in the host. According to Gomes et al. [17], the physiological functionality of fermented dairy foods is inherent to the intrinsic biological activity of the microorganisms. However, viability of probiotic organisms can be affected by several factors resulting from processing or even storage conditions (e.g., temperature, pH, oxygen, and antimicrobial substances) and by their resistance throughout the gastrointestinal tract. Generally, the
incorporation of probiotic organisms in diverse food vehicles requires:

1. a selection of probiotic strains that should rely on their technological performance (e.g., genetic stability, growth rate, and acidification rate) besides the safety requirements [17]; and,

2. product and process adaptations in order to promote higher survival enhancing the probiotic functionality [3].

Detection, enumeration and identification of these probiotic strains in dairy products are indeed tools of utmost importance to assure the consumer about the veracity of claims placed on product labels concerning both bacterial species and associated concentration and viability. Undoubtedly, more care and attention is deemed necessary for accurate identification as well as safety and functionality monitoring of probiotic agents [18,19].

Currently, there are several methods available for detection, enumeration and identification of microorganisms that are also applied to probiotic microorganisms. We discuss these methods in the following sub-sections divided between non-molecular and molecular approaches.

2.1.1. Non-molecular methods. The non-molecular approach to detect, to enumerate and to identify probiotic strains in dairy products includes those methods considered conventional, based on the growth of the microorganism in culture media, and those not requiring this step (Fig. 1). The basis of the conventional methods relies on standard procedures encompassing isolation, counting and identification of the probiotic strains at the genus and species levels based on cell ability to reproduce and to form colonies on selective/differential agar media plates [17]. The monitoring of viability and survival of probiotic bacteria through differential counting is important to assess product quality and to estimate the storage time, but we do not discuss it, since this review is more focused on the most advanced and latest analytical strategies.

Alternative methodologies have been applied to quantify probiotic bacteria in a more accurate way able to reduce the underestimation of viable bacteria obtained by plate-count methods (Fig. 1). Fluorescence methods have been applied in bacterial viability studies [20]; these are able to detect and to differentiate between viable, injured, stressed and dead bacterial cells through fluorescent dyes, which, in turn, can be detected by fluorescence microscopy, fluorometry, flow cytometry, or fluorescence in situ hybridization (FISH). Different fluorescent dyes are used as indicators of dead or alive cells. In order to assess intact and metabolically active cells but not culturable, a nucleic acid dye, TOTO-1, can be used since it will stain only membrane damaged cells [17]. Fluorescent physiological indicators are also available, permitting detection of differences in membrane permeability or enzyme activities [20]. Laniewska-Trokenheim et al. [21] compared fluorescent microscopy (direct epifluorescent filter using carboxyfluorescein diacetate stain) with a plating method where MRS agar was incubated anaerobically at optimal temperature for 48 h for several species of Lactobacillus. Statistically significant higher values of cell counts were obtained by direct fluorescence than by plate counts in MRS agar; the magnitude of the differences were strain dependent and higher for strains of L. acidophilus (P<0.05).

An alternative method is Fourier transform infrared (FT-IR) spectroscopy that has been applied for detection, discrimination, identification and classification of LAB and probiotics and also provides information about cell metabolism [22] from cultures and foods. FT-IR is able to discriminate viable, injured and dead bacteria and is used in the analysis of structural components of bacteria [23]. FT-IR is a method based on the measurement of the modes of vibration of a molecule that is excited by radiation in the infrared region producing a spectrum which represents a “fingerprint” characteristic of any chemical/biochemical substance. The fingerprint results from the stretching, contracting and bending vibrations of molecular bonds or functional groups present in proteins, nucleic acids, lipids, sugars, and lipopolysaccharides, because there is a correlation between IR-band position and chemical structures in the molecule. Since the molecular composition varies from species to species, each bacterium will give rise to a unique, characteristic FT-IR spectrum [23]. Several sampling techniques and spectra recording have been used for microbial characterization: some of the more common are transmittance, diffuse reflectance, attenuated total reflectance and microspectroscopy. Since FT-IR spectroscopy generates large amounts of data, their interpretation requires appropriate multivariate statistical methods – supervised and unsupervised methods [e.g., principal component analysis (PCA) and hierarchical cluster analysis (HCA)].

FT-IR is a relatively fast, simple, sensitive technique, requiring very little sample, and the biological cell remains intact during analysis. It is possible to make qualitative and quantitative analysis and the sample can be in the form of liquid, gas, powder, solid, or film. However, variations in the spectra can be caused by environmental conditions around the FT-IR instrument, so, for the same sample, background scans and multiple scans are required. If the sample is complex, then it can produce overlapping spectra, and so previous separation or purification steps may be needed or may require standardization, rigorous data collection, and expertise in the chemometric analyses of spectra [23].

Vodnar et al. [22] used FT-IR spectroscopy to fingerprint L. plantarum, L. casei, B. infantis and B. breve during fermentation by identifying specific markers located at several wavelengths, especially at 2845 cm\(^{-1}\) and 2929 cm\(^{-1}\).
Figure 1. Non-molecular versus molecular-based approaches for microbial analysis of probiotic bacteria in functional dairy foods targeting assessment of microbial diversity, identification and quantification.
2.1.2. Molecular methods. With the use of molecular methods, the ability to detect and to identify food microbes, including probiotic bacteria, has made tremendous advances in recent years, especially after the introduction of PCR in the 1980s. Several detection techniques have been developed based on PCR, namely denaturing gradient gel electrophoresis (DGGE), real-time PCR (qPCR), terminal restriction fragment length polymorphism (T-RFLP), random amplified polymorphic DNA (RAPD) [17,24]. Identification of probiotic species, including their differentiation in different strains by culture-dependent or culture-independent techniques, the study of microbial community composition, and assessment of its different populations and interactions in food products are some of the main achievements of molecular techniques [17,25]. Procedures involving culture-independent techniques, which are essentially based on the analysis of amplified nucleic acids by PCR present in the sample, overcome the major drawback of the culture-dependent techniques that are unable to detect non-culturable cells [25,26]. The possibility to differentiate or to find new strains of probiotic bacteria, namely of lactobacilli or bifidobacteria, is of utmost importance, since it is known that probiotic capacities/functionality are strain-dependent, and therefore the reliable identification of these strains and of their survival in a food product are indispensable for quality control of FFs [17]. Nowadays, the availability of a large number of bifidobacteria and lactobacilli genome sequences allows further insights into the evolutionary development of these genera using a so-called phylogenomics approach, permitting more insights into gene functions [24]. A detailed list of food microbes including probiotic bacteria (e.g., B. animalis subsp. lactis, B. longum; L. acidophilus, L. brevis, and L. casei) with complete or genome sequencing in progress was published by O’Flaherty and Klænhammer [24].

In recent years, several review papers were published describing the application of molecular techniques to analyze microbial composition in food products, including functional dairy products [17,26–29]; specifically, RAPD, PFGE, DGGE and qPCR reveal great potential to detect, to enumerate and to identify probiotic species at strain level [30–33], which is of utmost important for a relationship of trust between the food industry, the consumer and the regulators. Fig. 1 displays the major steps involved in culture-dependent or culture-independent techniques, whereas Table 1 briefly sets out the main genomics-based methods that can be used to analyze microorganisms, namely probiotic species/strains in FDFs.

The extraction of DNA or RNA is an important step, especially in assessment based on independent culture, because a representative DNA/RNA extraction of total microbial population is needed with high concentration and purity [26]. This step may suffer interferences from the matrix composition of dairy food (i.e. fats, proteins, salts). Commercially-available DNA-extraction kits are able to yield pure DNA [37]. The selection of DNA or RNA extraction relies on the choice to obtain information of entire microbial population not differentiating between alive or dead cells, which is achieved by DNA extraction whereas RNA or DNA staining provides information about live cells.

Following DNA/RNA extraction, the amplification of encoding genes by PCR is a step that directs the outcome of results. Universal primers (e.g., 16S and 23S rRNA), intergenic spacers, functional gene primers or specific primers should be carefully selected to study microbial diversity, functional diversity and/or search of targeted species. According to Justé et al. [25], microbial populations can be characterized considering three types of outcome information: i) diversity; ii) identity; and, iii) quantity.

Different protocols involving molecular methods have been described in the literature, some of them indicated in Fig. 1. Table 2 summarizes some of the studies involving molecular-based protocols that have been recently applied to probiotic bacteria, especially in FDFs.

As safety and functionality of probiotics are strain-dependent, the results published by Coereut et al. [27] demonstrated the need to control probiotic bacteria present in commercial FFs at not only the level of species and their viable cells but also the strain level (Table 2) to ensure product quality and protect consumer. This concern was also the main driving force in Collado et al. studies [18], where different molecular techniques were tested to evaluate the presence of B. animalis subsp. lactis in commercial Spanish fermented milks. Multiplex PCR and RAPD were revealed as the most suitable, rapid and precise for identification until strain level, being able to be used routinely in quality control procedures as confirmed by follow-up study [18].

García-Cayuela et al. [32] used a procedure based on the combined use of propidium monoazide (PMA) with quantitative qPCR for the specific detection and enumeration of viable bacteria of four species of LAB and of B. lactis mixed culture in fermented milk products.

According to Justé et al. [25], the use of molecular techniques does not have to eliminate the classical approaches to study microorganisms; instead, the complementarities between both approaches will enable better understanding of the cell itself and overcome technological limitations of non-molecular methods (e.g., those involving the growth of cells in agar media).

2.2. Metabolic assessment

Nowadays, the analytical strategies to characterize and to control the quality and the safety of food products are tools that have been implemented in routine laboratory procedures and food research. These analytical strategies comprise a tool for food-product differentiation, validation and authentication, since all food constituents
Table 1. An overview of the main molecular techniques used by dependent or independent culture approach to study microbial communities in dairy food for which an example of specific application is given

<table>
<thead>
<tr>
<th>Method</th>
<th>Brief description of technique</th>
<th>Major achievement</th>
<th>Advantages/Limitations</th>
<th>Sample</th>
<th>Ref.</th>
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<tbody>
<tr>
<td>Amplified ribosomal DNA restriction analysis (ARDRA)</td>
<td>Digestion of amplified ribosomal DNA followed by gel electrophoresis being all fragments detected.</td>
<td>Microbial identification and microbial communities and dynamics analysis.</td>
<td>A: Relatively simple PCR-based fingerprinting technique. L: Due to a complexity of the profiles, multiple restriction enzymes need to be used to attain higher resolution; Limited staining sensitivity of gels; Best results in low diversity communities.</td>
<td>Probiotic fermented milks</td>
<td>[19]</td>
</tr>
<tr>
<td>Random amplified polymorphic DNA (RAPD)</td>
<td>Use of short and low stringency hybridization to randomly amplify DNA fragments originating a fingerprinting pattern.</td>
<td>Identification of LAB including probiotic bacteria.</td>
<td>A: Reliable method to distinguish species in dairy products and to monitor changes in the LAB community during fermentation. L: Careful controlled conditions are needed to assure reproducibility.</td>
<td>Probiotic fermented milks and cheeses</td>
<td>[31]</td>
</tr>
<tr>
<td>Denaturing and temperature gradient gel electrophoresis (DGGE/TGGE)</td>
<td>Electrophoretic separation of small PCR-amplified DNA fragments on acrylamide gel by denaturing chemical gradient (DGGE) using urea or formamide or by temperature gradient (TGGE) with a constant denaturants concentration.</td>
<td>Microbial diversity analysis and microbial communities and dynamics analysis.</td>
<td>A: Affordability and outcome data relative easy to interpret. L: PCR small fragments can difficult reliable identification; Possible co-migration of different fragments with identical electrophoretic mobility; Lack of reproducibility that can be enhanced by use of internal standard; Low sensitivity due to gel staining that can be improved by use of fluorescent labeled primers.</td>
<td>Probiotic fermented milks</td>
<td>[30]</td>
</tr>
<tr>
<td>Single-strand conformation polymorphism (SSCP)</td>
<td>Electrophoretic separation of PCR-amplified products based on conformational differences of folded single-stranded products obtained by denaturation on non-denaturing acrylamide gel being able to separate fragments with similar molecular weight.</td>
<td>Microbial diversity analysis.</td>
<td>A: Similar limitations and advantages as DGGE/TGGE but with more PCR amplification since no clamped primers are required. L: Formation of several stable conformations out of one single stranded DNA fragment resulting in possible multiple bands.</td>
<td>No studies found in functional dairy food; Livarot cheese</td>
<td>[34]</td>
</tr>
<tr>
<td>Terminal restriction fragment length polymorphism (T-RFLP)</td>
<td>Marker genes are amplified with fluorescent labeled primer followed by restriction digestion and separation and detection. Since only labeled terminal restriction fragments are detected on electropherogram, their length heterogeneity is an indication of a complex microbial community.</td>
<td>Comparative microbial community analysis.</td>
<td>A: Precise length assignment with single-base pair resolution by use of internal size standard which by comparison to sequence database of Ribosomal Database Project allows predictions of the microorganisms present in the sample. L: Multiple restriction enzymes are needed to improve specificity and reliability; Primers and labeling dyes should be selected carefully.</td>
<td>No studies found in functional dairy food; Yoghurt and hard cheeses</td>
<td>[35]</td>
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may be meticulously identified and qualified, and verification of compliance with nutritional labeling of FFs, validating their claims regarding composition in beneficial compounds.

Reviews on analytical methods for evaluation of food identity, in general [39], and for dairy products, in particular [40], show physicochemical analysis, liquid chromatography (LC) and gas chromatography (GC) techniques with different detectors as some of the traditional approaches used for the determination of the quality and/or authenticity of dairy products based on chemical characteristics. While measurement of the traditional nutrients (i.e., proteins, fat, carbohydrates, and minerals) relies on basic chemical techniques, the identification of more specific components (e.g., individual phenols, bioactive peptides, and polyunsaturated fatty acids) demands complex processes involving extraction, detection and quantification.

Due to the complexity of food matrices, the use of advanced analytical techniques able to generate a chemical fingerprint of a product is required, especially for FDFs in order to identify and to quantify the beneficial compounds resulting from biochemical reactions, assessing their bioactivity and bioaccessibility/bioavailability throughout the food-product life-cycle. MS, NMR spectroscopy, capillary electrophoresis (CE) and high-performance LC (HPLC), coupled with several detectors, are some of the advanced techniques to study food matrices [15,41]. These techniques are also used in combination, designated “hyphenated” techniques, to analyze multiple components (i.e., HPLC-NMR, HPLC-MS, GC-MS, and CE-MS) in food analysis [15,41].

The analytical technique to be employed depends on the target compounds and the matrix, where physicochemical properties (e.g., polarity, size, and volatility) will be determinant for the choice of:

1. sample-preparation procedures;
2. separation mechanism and technique (GC, HPLC, and CE); and,
3. type of detector [ultraviolet spectroscopy (UV), refractive-index detector (RID), fluorescence spectroscopy detector (FLD), flame ionization detector (FID), and MS] [41].

Fig. 2 gives an overview of the most commonly used analytical techniques and the alternative advanced technologies to analyze parameters able to reflect glycolysis, proteolysis and lipolysis in traditional dairy foods and/or in FDFs, which we discuss in the following subsections.

2.2.1. Glycolysis. In the majority of dairy products (e.g., fermented milks or cheeses), glycolysis is mainly characterized by degradation of lactose. Depending on LAB or probiotic bacteria and their fermentation pathways, lactose is degraded, originating small organic acids (e.g., lactic acid, propionic acid, citric acid, and acetic acid).
Table 2. A short description of protocols based on molecular methods for detection, enumeration and identification of potential probiotic *lactobacilli* and *bifidobacteria* bacteria in dairy foods and their main results in studies published between 2004 and 2010

<table>
<thead>
<tr>
<th>Study</th>
<th>Procedure description</th>
<th>Main results</th>
<th>Ref.</th>
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<td>Determination of <em>lactobacilli</em> counts and species probiotic fermented milks and cheeses</td>
<td>1. DNA extraction in culture dependent technique (growth in MRS broth) by phenol-chloroform method; 2. Genus specific PCR, followed by multiplex PCR to group lactobacilli and species-specific PCR to identify lactobacilli species; 3. RAPD to differentiate potential replicates; 4. Isolates typed by PFGE.</td>
<td>– <em>Lactobacilli</em> genus and species (<em>L. casei</em>, <em>L. zeae</em>, <em>L. rhamnosus</em>, <em>L. delbrueckii</em> subsp. <em>bulgaricus</em>) were identified;  – The auditors found that many products were incorrectly labeled both in terms of probiotic species and their viable counts in the products.</td>
<td>[31]</td>
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<tr>
<td>Evaluation of <em>B. animalis</em> subsp. <em>lactis</em> in commercial fermented milk</td>
<td>1. DNA extraction in culture dependent technique (growth in BFM agar) by phenol-chloroform method; 2. Genus specific PCR and FISH using 16S rRNA probe specific of bifidobacteria followed by ARDRA-PCR using Lm26 and Lm3 primers to identify all known strains of <em>Bifidobacterium</em> and distinguish them from other bacteria genera; 3. Multiplex PCR by application of oligonucleotide pair Bflact2-Bflact5 for fragment amplification of DNA from <em>B. animalis</em> subsp. <em>lactis</em>; 4. RAPD-PCR analysis in genomic DNA with Bif primer and fragments were separated by NuSieve agarose gel electrophoresis; 5. AFLP analysis in genomic DNA digested with restriction enzyme HindIII and restriction fragments were ligated with ADH1 and ADH2 adapters; amplified fragments were separated by agarose gel electrophoresis; 6. ARDRA, RAPS and amplified ribosomal length polymorphism (AFLP) stained gels were processed by software package.</td>
<td>– <em>Bifidobacterium</em> genus was identified by both Genus specific PCR and FISH;  – Multiplex PCR was able to identify <em>B. animalis</em> subsp. <em>lactis</em> in all fermented samples. A rapid, easy and low cost technique useful for rapid monitoring of this strain in dairy products;  – According to ARDRA results, all the isolated strains belong to <em>B. animalis</em> subsp. <em>lactis</em>;  – RAPD-PCR results demonstrated that the strains were not all the same although very similar among them;  – AFLP results were similar as those obtained by RAPD-PCR.</td>
<td>[18]</td>
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<tr>
<td>Enumeration and identification of mixed cultures in fermented milks</td>
<td>1. DNA extraction from fermented milk (culture independent technique); 2. Species specific primers PCR for <em>Streptococcus thermophilus</em>, <em>L. delbrueckii</em> subsp. <em>bulgaricus</em>, <em>L. acidophilus</em>, <em>L. paracasei</em> subsp. <em>paracasei</em> and <em>B. lactis</em>; 3. DGGE was performed with a DCode system.</td>
<td>– The species-specific PCR primers were suitable for identification each specie by culture independent analysis in the fermented milk using the PCR-DGGE methods;  – The combination of species-specific PCR and DGGE analysis demonstrate great potential for identification purposes and for evaluation of the accuracy in the species-labeling in commercial fermented milk by an independent culture technique.</td>
<td>[30]</td>
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<tr>
<td>Enumeration and identification of mixed cultures in fermented milks</td>
<td>1. PMA treatment of cultures of pure strains (culture dependent technique) in order to be distinguishable viable cells from irreversibly damaged cells; 2. DNA extraction by DNA kit; 3. Quantitative qPCR using species-specific primers PCR for <em>Streptococcus thermophilus</em>, <em>L. delbrueckii</em> subsp. <em>bulgaricus</em>, <em>L. acidophilus</em>, <em>L. paracasei</em> subsp. <em>paracasei</em> and <em>B. lactis</em>; the quantification by RTi-PARPCR was performed by a multicolor qPCR detection system cycler.</td>
<td>– Loss of viability of the species through 28 days of storage at 4°C was able to be quantified by PMA-quantitative qPCR;  – Enumeration of viable bacteria was able in 3 h;  – The use of species-specific primers in the PMA-quantitative qPCR assay allowed the identification and enumeration of the viable bacteria in complex mixture present in fermented milk products.</td>
<td>[32]</td>
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Other sugars, also present in milk, are used as fermentation substrates, namely galactose, glucose and sucrose.

The classical approach used to study glycolysis and fermentative pathways involves HPLC coupled to detectors (e.g., UV/VIS, RID or FID) to monitor organic acids, sugars (Table 3), diacetyl, acetoin and the first intermediates of amino-acid degradation. Rodrigues et al. [42] monitored glycolysis in probiotic and synbiotic dairy matrices inoculated with different probiotic bacteria throughout 7 days, analyzing the variation of organic acids (i.e. lactic, acetic, formic and citric acids) by HPLC-UV and sugars (i.e. lactose, fructose and glucose) by HPLC-RID. The limit of detection (LOD) for HPLC/UV obtained by Rodrigues et al. [42] in the assessment of glycolysis in probiotic and synbiotic curdled milk is reported in Table 4. This was the only figure of merit (FOM) found among examples listed in Table 3 with HPLC-UV and HPLC-RID; this may be explained by the fact that the authors [42] used a classical approach where techniques have been previously validated, so FOMs will naturally be scarcer. Nevertheless, it is important to remember that FOMs are not only used for validation of a new methodology (sensitivity, selectivity, LOD which are related to the methods and analyte) but should also be used for validation of the final results (traceability, uncertainty, and representativity), since, being quantifiable, they may indicate the quality of the process [51]. Despite the several advantages pointed out for HPLC-UV, including being considered a straightforward, robust and reproducible technique, other approaches (e.g., CE) are indicated as also suitable for analysis of small organic compounds variations {e.g., lactic-acid enantiomers were determined in milk and yoghurt samples by CE with contactless conductivity detection [52]}.

Certain oligosaccharides (i.e. GOS, FOS and XOS) and inulin are considered prebiotic agents due to their capacity to stimulate microbial growth and metabolism of several lactobacilli and bifidobacteria. They have been studied by diverse analytical approaches, ranging from classical to advanced. Ignatova et al. [53], in their study on the effect of oligosaccharides on the growth of L. delbrueckii subsp. bulgaricus, monitored the variation of fermentation metabolites (e.g., n-lactic acid, acetic acid and ethanol) enzymatically through commercially-available kits, whereas the sugars and the oligosaccharides in the fermentation broth were analyzed by HPLC-RID.

High-performance anion-exchange chromatography coupled with pulsed amperometric detection (HPAEC-PAD) comprised an alternative tool to evaluate FOS and inulin, namely the changes in their chain-length distribution by bifidobacteria, as demonstrated by Corradini et al. [54], who reported that HPAEC-PAD provided important information about the potential use of the oligosaccharides and inulin as prebiotic agents. A review
of the principles and applications of HPAEC-PAD to evaluate carbohydrates of food interest was recently published by Corradini et al. [55].

Alternative approaches to characterize molecular weight and structure of potential prebiotic oligosaccharides have been performed using electrospray ionization (ESI) MS and $^1$H-NMR [56]. Bengal gram-husk and wheat-bran oligosaccharides obtained by enzymatic hydrolysis and separated by gel permeation chromatography were collected in fractions, which were submitted to $^1$H-NMR. Proton-NMR spectra of oligosaccharides indicated chemical shifts in the region of $\delta$ 3.00–5.00, characteristic of $\alpha$-linked arabinofuranose and $\beta$-linked xylopyranoside residues. Further analysis by ESI-MS was able to identify tri, penta, hexa and tetra saccharides [56].

In order to gain knowledge about metabolism of XOS by B. animalis subsp. lactis BB-12, Gilad et al. [57] combined the proteomic and transcriptomic approaches, involving DNA microarrays, qPCR and two-dimensional difference gel electrophoresis (2D-DIGE) analysis of strain samples grown in cultures with XOS or glucose. The concentration of XOS with different degrees of polymerization measured by HPAEC-PAD decreased during growth of B. animalis subsp. lactis BB-12. Microarray analysis showed that 9–10 genes encoded proteins involved in XOS catabolism, including degrading and metabolizing enzymes, transport enzymes and a regulatory enzyme. Comparative proteome analysis using 2D-DIGE was able to differentiate 28 protein spots, which were submitted to matrix-assisted laser desorption ionization – time-of-flight (MALDI-TOF)-MS analysis, which identified 25 proteins; carbohydrate-metabolizing proteins, including XOS-metabolizing proteins and enzymes of the biliš shunt, accounted for the majority of the proteins identified. This analytical approach was able to show that B. animalis subsp. lactis BB-12 possessed metabolic pathways to utilize XOS as a carbon source and also to enable the authors to propose a transcriptomics/proteomics-based model for XOS catabolism.

Metabolic profiling is a powerful tool to probe beneficial probiotic mechanisms in both the food and the human host, as we discuss below.

2.2.2. Proteolysis. The degradation of milk proteins, designated proteolysis, is an important biochemical event that determines textural and flavor characteristics of dairy products. In cheeses, this event starts with milk coagulation and continues during ripening [58], whereas, in fermented milks, this event is not as evident. It is known that hydrolysis of milk proteins can release bioactive peptides with specific biological activities (e.g., antihypertensive, antimicrobial, opioid, antioxidant, immunomodulant, antithrombotic or mineral binding) [59,60] that can be found in fermented milks, yoghurt, cheese and in dairy by-products (e.g., whey) [61].

Proteolysis is catalyzed by enzymes derived from the residual coagulant (chymosin or pepsin) if cheese is
<table>
<thead>
<tr>
<th>BE</th>
<th>Sample</th>
<th>Composition</th>
<th>Methodology/analyzed parameters</th>
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<th>Ref.</th>
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<td>Glycolysis</td>
<td>Sweet whey cheese stored 21 d at 7°C</td>
<td>Whey with 10% ovine milk precipitated at 95°C</td>
<td>Analysis of organic acids (lactic acid, acetic acid) by HPLC-UV; Analysis of sugars (lactose, sucrose, fructose, glucose) by HPLC-RID.</td>
<td>Statistically significant increase of lactic acid over storage time (P &lt; 0.05) but no statistical differences between sweet whey cheese types. Concomitant degradation of lactose over time</td>
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<td>L. paracasei L26</td>
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<td>Sweet whey cheeses with sugar, aloe vera, chocolate fiber and powder, strawberry jam</td>
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<td>Probiotic and synbiotic curdled milk matrices ripened over 60 d at 12°C</td>
<td>Cow's milk curdled with animal rennet</td>
<td>Analysis of organic acids (lactic acid, acetic acid, formic acid, citric acid) by HPLC-UV; Analysis of sugars (lactose, sucrose, fructose) by HPLC-RID.</td>
<td>Lactic acid increase correlated with lactose consumption in both probiotic and synbiotic matrices. Higher increase of acetic acid in probiotic matrix with B. lactis B94. Fos:Inulin did not affect overall post-acidification</td>
<td>[42]</td>
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<td>L. casei-01; L. acidophilus La5; B. lactis B94</td>
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<td>50:50 mix FOS:Inulin</td>
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<td>Starter cultures: S. thermophilus/ L. del-brueckii spp. bulgaricus with or without probiotic bacteria (L. acidophilus, B. lactis, B. longum)</td>
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<td>Starter cultures: S. thermophilus/ L. del-brueckii spp. bulgaricus</td>
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<td>B. longum BL05, L. acidophilus La14</td>
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<td>Proteolysis</td>
<td>Traditional and probiotic yoghurt stored for 26 d at 4°C</td>
<td>Oxine milk fermented at 42°C until pH = 4.7</td>
<td>Nitrogen fractions*: WSN and N-TCA quantified by Kjeldahl method; Soluble peptide profiles by RPLC-UV/Vis; Isolated peptides by RPLC were characterized by pulsed liquid-phase protein-peptide sequencer</td>
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<td>[46]</td>
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<td>Starter cultures: S. thermophilus/ L. del-brueckii spp. bulgaricus</td>
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<td>Probiotic adjunct cultures: L. paracasei subsp. paracasei DC412</td>
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<td>Cow's milk curdled with chymosin</td>
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<td>Starter cultures: S. thermophilus</td>
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<td>Adjunct cultures with probiotic potential: L. casei 190, L. plantarum 191, L. rhamnosus 173/177</td>
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<td>Cremoso soft cheese with potential probiotic lactobacilli ripened over 60 d at 50±0.5°C</td>
<td>Cow's milk curdled with chymosin</td>
<td>Nitrogen fractions: WSN, N-TCA and N-PTA quantified by Kjeldahl method; Protein degradation in insoluble fraction at pH = 4.6 by UREA-PAGE; Soluble peptide profiles by RPLC-UV/Vis; FAA by HPLC-UV/Vis</td>
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<td>Semi-hard cheese (Pategrás Argentino cheese) with probiotic strains ripened over 60 d at 12°C.</td>
<td>– Cow’s milk curdled with chymosin&lt;br&gt;– Starter cultures: *S. thermophilus&lt;br&gt;– Adjunct cultures: <em>L. paracasei</em> subsp. <em>paracasei</em>, <em>L. acidophilus</em> and <em>B. lactis</em> used as single strain or three-strain mixed culture</td>
<td>Nitrogen fractions: WSN, N-TCA and N-PTA quantified by Kjeldahl method; Protein degradation in insoluble fraction at pH = 4.6 by UREA-PAGE; Soluble peptide profiles by RPLC-UV/Vis; FAA by HPLC-UV/Vis.</td>
<td><em>L. acidophilus</em> had a significant influence on increase of small nitrogen compounds, FAA and peptide profiles. Synergistic effects observed for the three-strain mixed culture.</td>
<td>[47]</td>
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<td>Probiotic Pecorino cheeses ripened over 30 d</td>
<td>– Ovine milk curdled with lamb rennet&lt;br&gt;– Starter cultures: <em>S. thermophilus</em>/<em>L. delbrueckii</em> spp. <em>bulgaricus</em> with or without probiotic bacteria (<em>L. acidophilus, B. lactis, B. longum</em>)</td>
<td>Nitrogen fractions: WSN quantified by Kjeldahl method; Protein degradation in insoluble fraction at pH = 4.6 by UREA-PAGE; Soluble peptide profiles by RPLC-UV/Vis; Total and individual AA determined in WSN by EZ:fast physiological AA CG/FID.</td>
<td>Probiotic cheeses displayed higher percentages of αS1-casein fraction up to 15 d. Higher accumulation of FFAs in cheeses with <em>L. acidophilus</em> and with a mix of bifidobacteria</td>
<td>[43]</td>
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<td>Lipolysis Probiotic Pecorino cheeses ripened over 60 d</td>
<td>– Ovine milk curdled with lamb rennet&lt;br&gt;– Starter cultures: <em>S. thermophilus</em>/<em>L. delbrueckii</em> spp. <em>bulgaricus</em> with or without probiotic bacteria (<em>L. acidophilus, B. lactis, B. longum</em>)</td>
<td>Extracted underivatized FFA determined by CGC-FID; Extracted and methylated CLA’s determined by HPLC with diode array detector</td>
<td>Total FFA was higher in ovine milk cream containing probiotic strains. Increase of cheese FFA over the first 30 d of ripening period. Cheese with <em>L. acidophilus</em> with higher amounts of CLA1 and CLA2.</td>
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<td>Probiotic Pecorino cheeses ripened over 30 d</td>
<td>– Ovine milk curdled with lamb rennet&lt;br&gt;– Starter cultures: <em>S. thermophilus</em>/<em>L. delbrueckii</em> spp. <em>bulgaricus</em> with or without probiotic bacteria (<em>L. acidophilus, B. lactis, B. longum</em>)</td>
<td>Extracted and derivatized FFA were determined by CGC-FID.</td>
<td>Higher content of FAA, CLA1, CLA2 and CLA3 in probiotic cheeses especially containing <em>L. acidophilus</em>.</td>
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<td>Probiotic and synbiotic cheeses ripened over 60 d at 12°C</td>
<td>– Cow’s milk curdled by animal rennet&lt;br&gt;– <em>L. casei</em>-01; <em>B. lactis</em> B94&lt;br&gt;– FOS and 50:50 mix FOS:Inulin</td>
<td>Extracted and methylated FFA and CLAs determined by GC-MS.</td>
<td>Increases of total FFA and total CLA over ripening period in probiotic and synbiotic cheeses. Synbiotic cheeses containing <em>B. lactis</em> B94 and 50:50 FOS:Inulin with higher content of CLA especially of CLA1 and CLA3. Fermented organic milks: Lower levels of saturated FA, higher levels of monounsaturated FA and of CLA.</td>
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<td>Probiotic fermented milk</td>
<td>– Fermentation at 42°C until pH = 4.7 of conventional and organic cow’s milk&lt;br&gt;– <em>B. animalis</em> subsp. <em>lactis</em> BB12, BL04, B94, HN019</td>
<td>Extracted and methylated FFA and CLAs determined by GC-FID.</td>
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<td>[49]</td>
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*Biochemical event

WSN – Water-soluble nitrogen at pH = 4.6; N-TCA – Nitrogen soluble in 12% trichloroacetic acid; N-PTA – Nitrogen soluble in 2.5% phosphotungstic acid.

considered, milk (plasmin) and the starter/non-starter bacterial/probiotic microflora [58,62], so a general pattern of milk proteolysis is recognized – initial degradation of \( \alpha_1 \)-casein by bacteria and/or rennet enzymes or by acids – whereas \( \alpha_2 \)-casein, \( \kappa \)-casein and \( \beta \)-casein are degraded by plasmin-originating polypeptides/peptides. If production or storage conditions favor further enzymatic activities, the residual rennet and/or bacterial endo-cellular or exo-cellular proteinases and peptidases will continuously generate smaller peptides and free amino acids (FAAs), some of which may be precursors of the flavor compounds (e.g., alcohols, aldehydes, ketones and acids) [62].

In order to assure product quality, characterization and control of the proteolysis level is important, since peptide and protein content can affect both functional and biological properties of functional and/or traditional dairy products. In general, the classical approaches to assess and to quantify proteolysis in dairy products, namely in cheeses, have mainly been based on:

1. Fractionation procedures in order to obtain nitrogen fractions soluble in different extractants with different contents in polypeptides, peptides of different sizes and/or FAAs, which are quantified by the Kjeldahl method, giving information about proteolysis indexes [12,42,63];

2. Analysis of casein (\( \alpha_1 \), \( \alpha_2 \), \( \beta \) or \( \kappa \)) degradation by urea-polyacrylamide gel electrophoresis (PAGE), whereby use of casein standards and image treatment of the gel quantification of casein degradation is performed [46];

3. Peptide profiles by reversed-phase LC (RPLC)-UV–Vis and FAAs by HPLC-UV/Vis [46,63].

This classical approach takes time, consumes reagents and is very laborious, but it is still in use in proteolytic studies of FDFs – some examples are described in Table 3. According to De Simone et al. [61], the use of classical analytical methodologies to characterize milk-derived peptides is very challenging, due to the complexity of the milk-protein fraction and the high relative abundance of peptides.

Alternative approaches to avoid methods that are time-consuming, expensive and involving extensive chemical use have been studied, namely the use of FT-IR to monitor amino acids, organic acids and ripening changes in cheese [64] and of NIR spectroscopy for amino acids during cheese ripening [62] and peptides in cheeses with different ripening times [58]. No studies using this approach were found for FDFs, namely probiotic or symbiotic cheeses. Subramanian et al. [64] observed that FT-IR spectroscopy has the potential to monitor 20 amino acids and three organic acids simultaneously, besides age characterization during cheese ripening, in 20 min while requiring less than 1 mL of solvent per sample.

NMR is also considered a valuable tool to study structural and compositional aspects of food chemistry and food analysis. \(^1\)H nucleus is the most exploited, but \(^1\)C and \(^{31}\)P have also been applied in food matrices. Metabolic profiling of fermented foods and potential probiotic or symbiotic cheeses by \(^1\)H-NMR have demonstrated the potential of this technique to study metabolic processes in
fermented foods [65] and probiotic and/or synbiotic dairy matrices [63]. In the study by Choi et al. [65], the major peaks of $^1$H-NMR spectra assigned to amino acids (isoleucine/leucine), organic acids (lactate, acetic acid, citric acid), choline and sugars (fructose, glucose, sucrose) analyzed by PCA were able to discriminate the different samples with different periods of fermentation.

Although the term proteome refers to the proteins expressed by a genome at a particular point in time, and proteomic tools have been shown as important to screen for proteins expressed by microorganisms in several fermented foods, these have also been applied to milk-protein analysis to study proteolysis [66]. According to several authors [66–68], proteomic approaches by the use of high-resolution two dimensional gel electrophoresis (2D-GE), mono- or multi-dimensional LC coupled with MS, MALDI-TOF-MS and ESI-MS can provide information on:

1. proteins, including minor proteins in complex mixtures;
2. peptides produced in food matrices, some of them exhibiting functional or bioactive properties, and the proteases responsible for their release in situ;
3. reference proteomic maps to detect strain-strain variation, including that of probiotic lactobacilli [69] and bifidobacteria [70], elucidating the mechanisms of in vitro and in vivo adaptation to environmental stresses or predominant metabolic pathways that are active.

According to Mamone et al. [68], MS is the core technology to characterize food proteins and peptides, being able to perform qualitative and quantitative analysis, helping to increase knowledge about their nature, structure, functional properties and impact on human health.

The peptidome can be defined as the whole peptide pool present in food products or raw materials or obtained during food processing or storage, namely those produced by proteolysis [67]. Gagnaire et al. [67] suggest that food peptidomics can provide information about product authenticity, origin, functional and/or biological activities, allergenicity and sensory properties. Panchaud et al. [71] describe how to analyze food bioactives and their health effect, and discuss the technological challenges related to small, medium and large bioactive peptides based on MS techniques. Integrated analytical strategies for the characterization of bioactive peptides from food (milk, yoghurt, cheese) are described by Mamone et al. [68], including a food bioactive peptide-analysis workflow for in vitro or in vivo analysis, where the main steps involve:

1. protein/peptide separation by techniques such as 2D-GE, RPLC, or CE;
2. analysis of protein and peptide fractions by MS; and
3. structural characterization by ESI-MS, MALDI-TOF-MS, and the potential of in silico analysis for predicting possible bioactive sequences.

De Simone et al. [61] used this combined approach for whey-peptide extracts from Mozzarella di Bufala Campana cheese; peptide fractions obtained from ultrafiltration with a 3-kDa cut-off membrane were characterized by MALDI-TOF-MS analysis, whereas peptide extracts fractionated by RP-HPLC were characterized by ESI-MS (off-line or on-line with RP-HPLC for fractionation). They identified two peptides derived from β-casein (β-CN F57–68 and F60–68), precursors of agonist opioid β-casomorphin 7 and β-casomorphin 5, which appear to inhibit cell proliferation of particular interest to target some cancers.

To complement proteomics studies focused on identifying proteins and peptides expressed by microorganisms, quantitative proteomic analysis based on MS has been developed – a topic also developed in Gagnaire et al. [67]. Indeed, MS-based approaches involving isotopic labeling for protein or peptide quantification purposes have been proposed as a way to overcome difficulties normally encountered when trying to quantify proteins in gels [72].

Jardin et al. [72] applied quantitative proteomic analysis to bacterial proteins in Swiss-type cheese extracts with different ripening times based on the analysis of standardized protein cheese samples by:

1. 2D-GE for qualitative analysis;
2. nano-LC coupled on-line with ESI-QTOF-MS analysis of samples submitted to trypsinolysis and labeled with iTRAQ tag, one per ripening time. 30 proteins of bacterial (carbon-metabolism enzymes, stress proteins) and milk origin could be identified and quantified and, depending on the protein, a 2.5–20 times increase in quantity was registered within 7–69 d of ripening.

According to Jardin et al. [72], this dynamic approach can contribute to a better insight into in situ starter metabolism.

The use of the proteomic and peptidomic approach in the near future will certainly permit more insight into FDFs, namely with respect to bioactive peptides produced by probiotic bacteria.

2.2.3. Lipolysis. Lipolysis in dairy foods is also a biochemical event resulting from enzymes (esterases/lipases) originating from the milk, coagulation agents and microbial flora (starters/non-starters/probiotic bacteria) that cleave the ester linkage between free fatty acids (FFAs) and glycerol in the triacylglycerides. From their activity, FFAs are released, and, in turn, comprise precursors of certain compounds (e.g., methyl ketones, esters, secondary alcohols, lactones and aldehydes), which may influence the flavor or the aroma of dairy products. Analysis of the lipidic composition and the FFA profile in milk and dairy products has therefore been a topic of interest for decades, especially analysis of FFAs of
potential interest for human health produced by probiotic bacteria, e.g.:  
(1) mono and polyunsaturated FFAs;  
(2) conjugated linoleic acids (CLAs), to which antiadipogenic, anticarcinogenic, antiatherogenic, antidiabetogenic and anti-inflammatory properties have been attributed and found in FDFs [14,42,50];  
(3) other conjugated FFAs (e.g., conjugated γ-linolenic acid (CLNA), and conjugated stearidonic acid produced by probiotic bacteria, which are currently becoming of interest because of their biological properties (e.g., anti-inflammatory, immunomodulatory, anti-obese and anti-carcinogenic effects) [73,74]).  

The classical approach to evaluate lipolysis through study of the main changes in FFA and CLA profiles in functional dairy products or produced by probiotic bacteria (bifidobacteria) is mainly based on certain methodologies (e.g., GC-FID, GC-MS or Ag+-HPLC-UV) [14,48,63,75]. Table 3 displays studies of lipolysis assessment in FDFs containing probiotics and/or prebiotics.  

In Carrasco-Pancorbo et al. [76], there can be found a description of lipid-extraction procedures from different samples and discussion about:  
(1) chromatography procedures [e.g., GC-FID, high-performance thin-layer chromatography (HPTLC) commonly coupled with densitometric quantification, HPLC and Ag+-HPLC with various detection methods (UV, RID, electrochemical detection (ECD)) and the multi-dimensional chromatographic systems (GC-GC, LC-LC, LC-GC and two-dimensional supercritical fluid chromatography);  
(2) electrophoretic methods based on CE, where some of the most used for lipid determination include capillary-zone electrophoresis (CZE) or capillary electrochromatography (CEC).  

A review of analysis of bioactive fatty acids (CLAs, γ-linoleic acid, stearidonic acid) based on recent advances in GC and HPLC techniques, including sample-preparation procedures, was published by Ruis-Rodrigues et al. [77].  

An alternative methodology based on an optical fiber (OF) to screen the effect of probiotic bacteria on CLA in curdled milk was proposed by Silva et al. [8]. The OF-based methodology was validated by comparison with GC-MS and showed comparable linearity, accuracy and LODs, which were 1.92–2.56 mg for CLA methyl ester and oleic acid methyl ester, respectively (Table 4). It is important to highlight that, for this study, since the validation of a new method was the issue, other FOMs (e.g., sensitivity, uncertainty and analytical error) were determined, besides LOD and linear range.  

More recently, NMR has also become a universal method for lipid analysis, providing information about structures, and qualitative and quantitative analysis [76]: 3H- and 13C-NMR are indicated for elucidation of molecular structures of purified lipids whereas 31P-NMR is more suitable for analysis of phospholipid mixtures.  

Microbial production of CLNA by γ-linolenic acid with L. plantarum AKU 1009a, a potential probiotic bacterium, was studied by Kishino et al. [73], who found that this strain is able to produce a mix of two CLNAs. These isomers (cis-6, cis-9, trans-11 and cis-6, trans-9, trans-11 octadecatrienoic acid) were purified by HPLC and confirmed by MS and NMR analysis.

3. Advanced omics approaches and microbiome  

Increasing awareness of the intestinal microbiota (also designated as intestinal microbiome) has promoted considerable efforts to define their complex role better in host physiology and to discover the underlying mechanisms [78]. Recall that the gastrointestinal (GI) microbiome comprises about 10^{14} bacteria that are mainly located in the large intestine. These have been associated with multiple functions, including energy homeostasis, bioavailability of nutrients, prevention of mucosal infections, maintenance of an intact intestinal barrier, and regulation of the mucosal immune system by acting as an important source of stimulators [78,79]. Any interruption of these functions may be closely related to alterations or maladaptations of the GI microbiome and can consequently impair many homeostatic and physiological signals, resulting in a number of disease states, including allergy, inflammatory bowel disease (IBD) and obesity [80].  

Diet and food-associated bacteria (that play a role in food-fermentation processes, as previously described) may interact with the gut microbiome and impact on host response [24], although the associated molecular mechanisms are still unknown. In order to understand these effects better, and to contribute to the validation of FDF functionality, for example, there is a need to characterize not only the phylogenetic profile of human microbial communities but also the functional capacity of their members. Approaches toward this goal have included direct bacterial culture, 16S rRNA sequencing, shotgun metagenomic sequencing, PCR probing for specific genes, and chemical profiling of microbial metabolites [80]. Results have shed light on developmental changes in the composition of the GI microbiota during infancy and childhood [81], the influence of diet on composition of the intestinal microbiota [82] and alterations in the bacterial phyleotypes and altered microbial profiles in humans with IBD [80].  

Omics technology combined with potent software systems, through high-throughput sequencing of 16S rRNA, may constitute a powerful tool to distinguish between species and to assess bacterial diversity in different communities and environmental niches within a short period of time, an advantage as far as analytical
strategies are concerned [24]. For example, the 16S rRNA PhyloChip is a high-density micro-array based on this technology, which has the ability to sample and to detect species that represent as little as 0.01% of the bacterial community, allowing high-resolution profiling of approximately 8500 bacterial taxa (defined as groups of organisms that share at least 97% 16S rRNA sequence identity) in a single experiment. It has been successfully used to demonstrate the positive effect of *L. casei* subsp. *rhamnosus* GG (LGG) supplementation on the infant gut microbiome [83]. The positive effect of LGG on children with high risk of atopic disease was due to not only its presence in high numbers but also the impact of these high numbers on the modulation of the bacterial community structure. Confirmation of the variation in relative abundance observed by the microarray in infant-stool samples was confirmed by independent quantitative qPCR analysis (*n* = 11) – an important step, if one considers that the 16S rRNA gene has a confined representativeness of the microbial genome (represents only a small region), so inference of phylogenies from one gene alone has greater misclassification possibilities. Regression analysis demonstrated a correlation between the two independent molecular methods (*r* = 0.63; *P* < 0.05), upholding the versatile application capacity of this array. Such an approach has also enabled identification of new candidates as beneficial microbes that, after further study and analysis, may be potential probiotic bacteria. For example, the probiotic *Faecalibacterium prausnitzii*, which was identified by high-throughput sequencing of the 16S rRNA gene of the mucosa-associated microbiota of Crohn’s patients was shown to prompt strong anti-inflammatory responses in *in vitro* co-culture models and to protect against trinitrobenzene sulphonic acid-induced colitis in mice: such results may ultimately lead to the use of this species in healthy humans or IBD patients [84].

Further limitations to the 16S rRNA sequencing approach (e.g., nucleotide composition bias within the 16S rRNA gene which can lead to incorrect phylogenetic characterizations) may be overcome by metagenomics using shotgun sequencing [24,85]. This technology sequences the total community DNA directly from a sample, providing information about both the phylogenetic profile and the functional genes. Undoubtedly, 16S rRNA sequencing is much cheaper if only community profiling is required (yet limitations should be maintained present), however metagenomic profiles are essential for understanding the functions encoded in those genomes.

The use of MALDI-TOF-MS Biotype (MTB) has also been used to detect, to identify and to characterize food products, including probiotic ones [86] and human microbiota [87]. According to Emami et al. [88], MTB supported by reliable, accessible bacterial databases becomes a useful method when the molecular analysis (e.g., 16S RNA gene analysis) is unable to differentiate bacterial isolates. 92% specificity was obtained by MALDI-TOF-MS in comparison to molecular analysis (sequencing of *tuf* and 16S rDNA genes) in probiotic food and yoghurts [86].

The large MetaHit consortium of European and Chinese researchers developed a new approach, termed quantitative metagenomics, in order to characterize gut microbial communities. A central element was the use of the Illumina sequencing platform to create a reference catalogue composed of 3.3 million, non-redundant intestinal microbial genes [89], on which a high number of short sequences generated from total stool DNA of an individual are mapped, which enables determination of the presence and the abundance of each catalogue gene harbored by that individual. Use of this approach has led to detection of three robust gut enterotypes, characterized by different bacterial communities, to which humans belong, spanned across geography, age, gender, and disease [90]. These enterotypes will allow stratification of individuals and assessment of the microbial communities associated with health and disease; several authors are of the opinion that these stratifications may be helpful in predicting individual responses to therapeutics [90] and eventually to FFs containing probiotics and/or prebiotics.

As understanding of microbial variation and corresponding genetic parameters deepens, the information generated may be applied to restructure the gut microbial communities and their associated functions, thus contributing to improvement of human health; specific probiotic strains may provide missing microbial components with proved beneficial functions for the human host, whereas prebiotics may increase the proliferation of probiotics or other beneficial bacteria, in order to maximize sustainable alterations in the human microbiome [79]. In this context, natural probiotic strains may be useful in FFs.

In terms of food science, microarrays have contributed significantly to knowledge of food microbes [e.g., transcriptomics of food microbes during growth in stressful environments and various foods (e.g., milk, cheese, and yoghurt)] [24]. To study the impact of three lactic-acid bacteria strains, with proven clinical functions, gene-expression studies from the duodenum of humans were performed and results demonstrated that administration of *L. plantarum* can stimulate immunomodulatory responses [91].

Metagenomics is without doubt a powerful analytical strategy even though it does require very complex computation, data-storage and handling procedures, and more sophisticated algorithms to help improve sequence assemblies in complex microbial communities (e.g., the gut). An important aspect to consider in omics technology is to link specific functional capacities to specific microorganisms, so that vector recommendation
(food, supplement) or strain selection can be better targeted. Although metagenomics provides information about functional gene content in the gut, it is not known whether the genes are actively expressed and have any functional role in a given sample. In order to obtain information about active functions, it is necessary to look at the expression profile (metatranscriptomics) or the protein products (metaproteomics). These two technologies are still technically demanding and have only recently begun to be applied for the study of microbial communities in the gut [92]. Metabonomics is also an important approach to couple to metagenomics if the functional capacity of the gut is to be assessed. Metabonomics can be described as the computational analysis of spectral metabolic data (obtained by MS and NMR spectroscopy platforms) in order to determine the time-specific metabolic changes in a complex system, thus providing an important overview of the metabolic state of the host [80].

The many advances in omics strategies are promising for the validation of FDFs discussed herein, including biomarker discovery and health-promoting strategies. Support for this statement is provided by recent omics studies that have demonstrated the relevance of diet on modulating gut microbiota. For example, the characterization of fecal samples of 98 individuals revealed clustering of fecal communities into enterotypes strongly correlated with long-term diets, with Bacteroides associated with diets high in protein and animal fat versus Prevotella associated with carbohydrate-rich diets [93]. In another perspective, Gordon and colleagues showed in a microbial profiling study that the ratio of Firmicutes/Bacteroides is higher in obese than in lean individuals; this trend was associated with a higher capacity for polysaccharide fermentation [94].

This new omics approach has opened many avenues of understanding, and, as more studies are published, the symbiosis between gut microbiome and host will become clearer. The impact of diet on the currently known microbial communities needs to be further explored and properly designed FFs may find in these strategies important answers to uphold their beneficial role. These initiatives will continue to benefit the study of food microbes, among which probiotics are naturally included.

4. Conclusions and future trends

Nutrition plays an important role in promoting health and well-being. FDFs may help in this role, yet their functionality needs to be characterized and maintained. Several technologies are available toward this goal and go from conventional physicochemical and chromatographic/spectroscopic techniques to more advanced new-generation methodologies, which rely highly on molecular methods allied to MS and NMR analysis. Independently of their degree of complexity, the overall objective is to enable characterization of food-matrix composition, to validate functionality associated with bioactive compounds present, to increase knowledge on added value of food matrix and to identify all potential applications.

In FDF, probiotics and prebiotics are among the most common ingredients employed. Their technological role and specific contributions in the FF need to be assessed by many the analytical strategies available to characterize:

1. species/strains of the microflora, their viability/survivability and their metabolic action;
2. the biochemical events – glycolysis, proteolysis and lipolysis.

Among these methods, there are those that have undoubtedly accelerated knowledge and understanding of the complexities of food matrices, in particular, dairy matrices, and have simultaneously contributed to the validation of aspects important for human nutrition and health. Despite its recent character, omics technology has greatly contributed to this endeavor, at both bacterial and molecular levels. For example, by probing the proteome, peptidome and lipidome in food, specific markers can be identified that substantiate the specific role of the probiotic or the prebiotic – namely by production of specific bioactive peptides or polyunsaturated acids (e.g., CLA or CLNA). The use of the proteomic and peptidomic approach in the near future will certainly give more insight into FDFs, namely with respect to bioactive peptides produced by probiotic bacteria. Independently of the parameter being analyzed, analytical strategies need to be sound in order to guarantee effectiveness, sensitivity, reproducibility and traceability. FOMs are parameters that may indicate the extent of the quality of these strategies, and their assessment is required to ensure the quality of results, even more so during characterization and validation of FDFs. The availability of FOMs concerning the final results obtained by quality-control and quality-assurance procedures should be strongly recommended as far as FDFs are concerned, since the FOM is a measure that may guarantee concordance between product composition and functionality and wording on the associated label.

As the analytical approaches are further improved and applied to food microorganisms and food matrices, not only will quality and nutritional value be assured but also we expect that a better understanding of their beneficial aspects on gut microbiota and human health will be achieved. That will naturally impact on the food industry, the scientific community and above all the consumer.

Metabolic profiling is recognized as a powerful tool to probe probiotic beneficial mechanisms further in both food and human host. The new omics approach has enabled new knowledge on gut microbiome, and, as
more studies are published, the symbiosis between gut microbiome and host will become clearer. The impact of diet on the currently known microbial communities needs to be further explored, and properly designed FFs may find in these strategies important answers to uphold their beneficial role. These initiatives will continue to benefit the study of food microbes, among which probiotics are naturally included.

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