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Review

Metabolomics as a tool for the comprehensive understanding of fermented and functional foods with lactic acid bacteria

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ABSTRACT

Metabolomics, also called metabonomics or metabolic profiling, deals with the simultaneous determination and quantitative analysis of intracellular metabolites or low-molecular-mass molecules. The metabolomics field, which has begun a little more than ten years ago thanks to the development of technologies such as nuclear magnetic resonance (NMR) and mass spectrometry (MS), has been successfully applied in different areas of food science. This review deals with the recent achievements of metabolomics in the comprehensive analysis of fermented foods predominated by lactic acid bacteria, the fermentative capacity of these microorganisms and the beneficial effects of functional foods and probiotics.

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1. Introduction

In recent years, development and financing of a large number of huge sequencing projects (such as the Human Genome Project, http://www.ornl.gov/sci/techresources/Human_Genome/home.shtml) have led the rise and improvement of new technologies to obtain large-scale information in the Biology and Bioinformatics fields. The advances achieved in these areas have caused a new paradigm in biological research that proposes a global view of biological processes; this concept is reflected in the emergence of the "The Omics Age".

"Omics" derives from the Latin voice *-omne* that means "everything, totality, whole". This neologism is actually used as a suffix for referring to those disciplines as Genomics, Proteomics, Transcriptomics, and Metabolomics, which study a certain set of molecules in a global way – genes, proteins, transcripts or metabolites, respectively – or even seek to define the relationships among them. "Omics" sciences are based on the analysis of a great volume of information and use bioinformatics for data interpretation. The analyzed data integration generates big contributions to the knowledge of the versatility and functionality of cells and their biotechnological applications.

1.1. What is metabolomics?

Metabolomics is an emerging field within "omics" sciences that began a little more than ten years ago; it is also known as metabolome analysis, metabonomics (Nicholson, Lindon, & Holmes, 1999) or metabolic profiling (Niwa, 1986). Metabolomics deals with the simultaneous determination and quantitative analysis of intracellular metabolites, which have been defined as low-molecular-mass compounds (<1500 Da) that are not genetically encoded and that are 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. (Jewett, Hofmann, & Nielsen, 2006; Wishart, 2008b).

Metabolic footprinting or exometabolomics focuses on what the cell excretes under defined conditions and intends to define a pattern of extracellular metabolites. Although metabolic footprinting gives important information about only a small part of the entire bacterial metabolome, it provides key information that may contribute to the understanding of cell communication mechanisms (Mapelli, Olsson, & Nielsen, 2008).

Metabolomics has been widely applied to many disciplines, including microbiology (Mapelli et al., 2008), human health (Gieger et al., 2008; Hirayama et al., 2009; Psychogios et al., 2011; Sreekumar et al., 2009), diagnostic biomarker discovery (Denery, Nunes, Hixon, Dickerson, & Janda, 2010; Wang et al., in press), food and beverage analysis (Frank, Scholz, Peter, & Engel, 2011; Rochfort, Ezernieks, Bastian, & Downey, 2010), plant physiology (Fiehn et al., 2000), and drug discovery and development (Wishart, 2008a).

Concerning food science, metabolomics has recently been applied for monitoring the quality, processing, safety, and microbiology of both raw materials and final products to improve the consumer's health and confidence (Cevallos-Cevallos, Reyes-De-Corcuera, Etxeberria, Danyluk, & Rodrick, 2009). In this context, Cifuentes (2009) has recently defined Foodomics as a new discipline that studies the Food and Nutrition domains through the application of advanced omics technologies; Nutrigenomics and Nutrigenetics can be considered part of the more general Foodomics term.

Metabolomic analyses have been generally classified as targeted (specific) or untargeted (nonselective or integral) analyses. Targeted analyses focus on a specific group of metabolites that require the identification and quantification of many metabolites within the group. This type of metabolomics is important to evaluate the behavior of a specific group of compounds in a sample under certain conditions; it typically requires a higher level of selective metabolite extraction and purification. In contrast, untargeted or comprehensive metabolomics focuses on the detection of as many groups of metabolites as possible to get patterns or fingerprints without identifying or quantifying a specific compound (Cevallos-Cevallos et al., 2009).

Metabolomics aims to integrate information collected through a series of recent technological findings in metabolite separation, detection, identification and quantification. Metabolite separation and detection have been considered key steps in metabolic profiling. The most popular separation techniques are liquid chromatography (LC) in its high-performance (HPLC) or ultra-performance (UPLC) forms, gas chromatography (GC) and capillary electrophoresis (CE). Among the detection techniques, mass spectrometry (MS), nuclear magnetic resonance (NMR), and near infrared spectrometry (NIR) are the most used.

In Foodomics, most separation analyses have been applied through coupling with various detection techniques; the working principles of these techniques in food analysis are presented below.

1.2. Separation techniques

1.2.1. High-performance liquid chromatography (HPLC)

This commonly used technique 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. However, the simultaneous separation of multiple components is very difficult in a conventional HPLC system. Based on the nature of the stationary phase and the separation process, liquid chromatography can be classified in: a) adsorption chromatography: the stationary phase is an adsorbent (i.e. silica gel) and the separation is based on repeated adsorption–desorption steps. Depending on the relative polarity of the two phases this type of chromatography can be classified as normal and reversed-phase chromatography. In normal phase chromatography, the stationary bed is strongly polar in nature and the mobile phase is nonpolar. Polar samples are retained on the polar surface of the column packing longer than less polar materials. In contrast, in reversed-phase chromatography the stationary bed is nonpolar (hydrophobic) in nature, while the mobile phase is a polar liquid; here the more nonpolar the material is, the longer it will be retained; b) ion-exchange chromatography: the stationary bed has an ionically charged surface of opposite charge to the sample ions. It is exclusively used with ionic or ionizable samples. The stronger the charge of the sample, the longer it will take to elute. The mobile phase is an aqueous buffer, where both pH and ionic strength are used to control the elution time; and c) size exclusion chromatography: the column is filled with material of controlled pore sizes and the sample is screened or filtered according to its solvated molecular size. Larger molecules rapidly elute while smaller molecules penetrate inside the porous of the packing particles and elute later. This technique is also called gel filtration or gel permeation chromatography although, today, the stationary phase is not restricted to a "gel". Detection systems are based on refractive index, ultraviolet light, fluorescence, evaporative light scattering, or mass spectrometry (MS). In high-performance anion exchange chromatography (HPAEC), pulsed amperometry detection or conductivity detection under ion suppression are used. Each chromatographic peak corresponds with a metabolite, which is defined by its interaction with the stationary phase and, hence, its retention time. The peak area allows the comparison of the concentration of a particular metabolite among samples. Fast, highly efficient separations, without requiring rigorous sample pretreatment can be obtained. HPLC, coupled with various detection systems, has been widely used in the last 30 years for the analyses of different compounds in many research fields (Toyo'oka, 2008; Yoshida et al., 2012).

1.2.2. Ultra-performance liquid chromatography (UPLC)

Recent technology advancements in liquid chromatography have been achieved by the introduction of 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 (Wren, 2005). This approach can achieve results similar to those with HPLC, but in much less time. In this respect, Wilson et al. (2005) reported that running times could be reduced 10 times, making UPLC a promising analytical strategy for metabolomic research. UPLC makes use of the same detectors as in conventional HPLC, such as UV–VIS, fluorescence, and MS. The main research fields that use UPLC–MS technology are pharmaceutical analysis and bioanalysis, but it has also been applied in food analyses for determining food components, food additives, and hazardous compounds (Toyo'oka, 2008).

1.2.3. 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; moreover, derivatization may improve the detectability of the derivative. The reactions to produce volatile derivatives can be classified as silylation, acylation, alkylation, and coordination complexation. Also, derivatization methods of samples can be divided into pre- and post-column methods and off-line and on-line methods (McNair & Miller, 1998). An overview of sample preparation techniques by sample type was reported by Snow and Slack (2004). In GC, the temperature of the column is gradually raised using a temperature-programmed oven. The metabolites pass through a capillary column along with a carrier gas, and are separated by interaction with the stationary phase of the column. Common detection systems are based on flame ionization; generally, the metabolite mass is measured by MS (Yoshida et al., 2012).

1.2.4. Capillary electrophoresis (CE)

A major fraction of metabolites are too polar – or ionic – to be retained by columns employed in HPLC. In this sense, CE is a good approach to be used as separation technique. 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. Thus, CE represents a complementary tool to the traditional chromatographic techniques; in many cases, samples that cannot be easily resolved by GC or HPLC can be separated by CE (Montona & Soga, 2007). According to its separation principle, CE can be classified in electrophoretic flow and electroendosmotic flow. In the first process sample ions move under the influence of an applied voltage. The ion undergoes a force that is equal to the product of the net charge and the electric field strength. It is also affected by a drag force that is equal to the product of the translational friction coefficient and the velocity. The electroosmotic flow (EOF) is caused by applying high-voltage to an electrolyte-filled capillary. This flow occurs when the buffer running through the silica capillary has a pH greater than 3; the SiOH groups lose a proton and the capillary wall has a negative charge developing a double layer of cations attracted to it. The inner cation layer is stationary, while the outer layer is free to move along the capillary. The applied electric field causes the free cations to move toward the cathode creating a powerful bulk flow. Currently, the process of CE is a generic term and it can be segmented into many separation techniques depending on the types of capillary and electrolytes used (Sekhon, 2011). The most attractive feature of CE is the use of small amounts of organic solvent and reagents (a few nanoliters), making it particularly convenient for volume-limited samples (Yoshida et al., 2012). This versatile technique enables the separation of a wide range of analytes, from small inorganic ions to large proteins and even intact bacteria (Armstrong, Schulte, Schneiderheinze, & Westenberg, 1999; Bean, Lookhart, & Bietz, 2000; Isoo & Terabe, 2003). In contrast, the small sample volumes used in CE lead to relatively poor concentration sensitivity, explaining the few reports on metabolome analysis involving CE (Yoshida et al., 2012).

1.2.5. Ion mobility spectrometry (IMS)

This is a non-conventional technique where food metabolites are carried in an inert gas flow, ionized, and separated by a drift gas flowing in the opposite direction. IMS has been applied for metabolomic analysis of bacterial metabolites as indicator of microbial growth, cheese and beer production, and food packaging material (Vautz et al., 2006).

1.3. Detection techniques

1.3.1. Nuclear magnetic resonance (NMR) spectroscopy

It is one of the most frequently used analytical tools for fingerprinting and profiling studies. It is quick, non-destructive, and relatively inexpensive, after the initial costs of installation. It can detect all compounds with NMR-measurable nuclei. Both solid- and liquid-state NMR exist. Its principal drawbacks, however, are its poor sensitivity and large sample requirement (Rochfort, 2005; Yang, 2006).

1.3.2. High resolution magic angle spinning (HRMAS) NMR

HRMAS NMR spectroscopy is a rapid and accurate alternative technique, which retains the advantages of both classical solid- and liquid-state NMR (Sitter, Bathen, Tessem, & Gribbestad, 2009), allowing the direct examination of the whole food product without component extraction (Shintu & Caldarelli, 2006).

1.3.3. 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 (Montona & Soga, 2007; Yoshida et al., 2012). There are several direct MS analysis systems, the most commonly being Direct infusion mass spectrometry (DIMS), Matrix-assisted laser desorption ionization mass spectrometry (MALDI-MS), and desorption electrospray ionization (DESI). Whereas MS based on a triple quadrupole is optimal for quantitative analyses, accurate qualitative analyses are performed with MS based on, for instance, time-of-flight analysis. Recently, selective ion flow tube mass spectrometry (SIFT-MS) has been introduced for food analysis (Agila & Barringer, 2012; Noseda et al., 2010, 2012).

1.3.4. Fourier transform infrared spectroscopy (FTIR)

In the early 1990s, infrared (IR) spectroscopy was introduced as a tool that allowed classifying, characterizing, and identifying microorganisms (Helm, Labischinski, Schallehn, & Naumann, 1991; Naumann, Helm, & Labischinski, 1991). The application of Fourier transformation in IR spectroscopy resulted in the development of the FTIR technique, which was rapidly introduced into the group of metabolomic procedures. Later, FTIR has been proposed as a metabolomic fingerprinting tool (Fiehn, 2001; Johnson, Broadhurst, Goodacre, & Smith, 2003) for rapid and non-destructive analysis of the quality and composition of a large number of different products. For instance, the use of FTIR for milk analysis allows more accurate measurement of the major (fat, protein and lactose) and minor (urea, fatty acids, ketones, calcium and phosphor) milk components (Heuer et al., 2001; Soyeurt et al., 2009). Samples, either liquid or solid, do not require any pre-treatment (extraction and derivation). Solid samples represent a great feature when performing on-line measurements (Ikeda, Kanaya, Yonetani, Kobayashi, & Fukusaki, 2007).

1.4. Hyphenated techniques

Currently, the main analytical techniques used in metabolomics are hyphenated techniques such as GC, HPLC, and CE coupled to MS (GC–MS, HPLC–MS, and CE–MS, respectively). GC–MS provides a high resolution and reproducibility; innovative techniques have been developed to make metabolic fingerprints by GC–MS (Pongsuwan et al., 2007). Alternatively, HPLC–MS is the most commonly hyphenated method used in metabolome analysis, displaying a high sensitivity compared with GC–MS and CE–MS, and it is a useful tool for non-targeted metabolome analysis (Yoshida et al., 2012). CE–MS represents a viable platform for metabolomic studies; the poor sensitivity for low concentrations, often cited as a disadvantage of CE when using absorbance-related detectors, does not represent a problem if using MS for detection. Alternatively, NMR, FTIR and DIMS have been applied for metabolic fingerprinting, thanks to their high performance and minimal sample preparation (Dunn & Ellis, 2005; van der Greef, Stroobant, & van der Heijden, 2004). However, detection limits for NMR and FTIR are higher than for MS-based techniques, limiting the application range only to those metabolites that are present in high concentrations (Koek, Jellema, van der Greef, Tas, & Hankemeier, 2011).

It is worth mentioning that none of the individual methods is capable of detecting all the metabolites of a sample, so a combination of different techniques is required to ensure that the obtained results are complementary. Therefore, hyphenated techniques are generally preferred in metabolomics, since simultaneous quantification and identification of as many as possible metabolites makes it feasible getting close to determine the entire metabolome.

1.5. Data analysis

Chemometrics is an essential tool in analyzing differences among metabolomes, enabling the identification of metabolites relevant for a specific condition. To investigate whether one group is different from another, the alignment of the data is usually achieved by the use of multivariate data analysis (MVDA) techniques, e.g., principal component analysis (PCA), partial least squares-discriminant analysis (PLS-DA), or principal component regression (PCR). When working with multivariate data, severe multicollinearity usually exists as the number of variables commonly exceeds the number of observations. For instance, both NMR and MS techniques produce data with thousands of variables while the number of samples is usually limited to fewer than 100. To overcome dimensionality problems bilinear factor models are usually designed. In this respect, the basic bilinear factor model is PCA, which decomposes the data into score vectors and loading vectors, that when taken outer products and summed, will recreate the original data. The most interesting phenomena can be observed in the first few components while the majority of components are regarded as uninteresting or noise. When an informative response (sample-specific information) is available, the family of partial least square can do a more efficient and interpretable decomposition than PCA. With a single continuous response PLS regression (PLSR) maximizes the covariance between the explanatory variables and the response. When PLS is used with a discrete response to form PLS discriminant analysis (PLS-DA) ,maximization is done on the estimated between groups covariance matrix (Liland, 2011). Applications of PCA and PLS-DA in metabolomics are given later on. Currently, PCA is the most common tool used as data mining method in Foodomics (McGhie & Rowan, 2012; Ochi, Naito, Iwatsuki, Bamba, & Fukusaki, 2012).

2. Metabolomics in fermented foods

The study of metabolite profiling in fermented foods is used to observe metabolite changes during fermentation and the possibility to predict, among others, the sensory and nutritional quality of the fermented final product. Here, a few examples of fermented foods where metabolomics has been applied so far are presented.

2.1. Fermented soy foods

Several metabolomics studies were carried out mainly for fermented soy foods such as *meju* (Kang et al., 2011), *doenjang* (Namgung et al., 2010), *cheonggukjang* (Park et al., 2010) and fermented soymilk (Yang et al., 2009), in which food components such as proteins, amino acids, organic acids, and sugars have been analyzed (Table 1).

Meju is a brick of dried fermented soybeans that belongs to the traditional Korean cuisine. While not consumed on its own, it serves as the basis of several Korean condiments, such as *doenjang*, *gochujang* or *kanjang* (soy sauce). Kang et al. (2011) analyzed the metabolites produced during *meju* fermentation that contribute to the nutritional qualities of *doenjang* and *kanjang*. For this purpose, *meju* was prepared from soybean blocks that were naturally dried for 1 day and fermented in rice straw for two months. Microorganisms such as *Bacillus* sp., *Aspergillus* sp., and *Mucor* sp. are usually involved in *meju* fermentation. *Meju* samples corresponding to different fermentation periods were analyzed by UPLC-quadrupole-time of flight mass spectrometry (UPLC-Q-TOF MS) and data were statistically processed by PLS-DA. Changes in metabolites such as amino acids, small peptides, nucleosides, urea cycle intermediates, and organic acids, which are responsible for the unique taste and nutritional quality of fermented soy foods, were clearly altered throughout fermentation. The authors noted that proteins were degraded as the fermentation proceeded, generating relevant amino acids and peptides related to the taste and nutritional quality of the product. The concentration of glutamic acid, the metabolite responsible for the characteristic *umami* flavor, reached a maximum at the end (60 days) of the fermentation. Also, the concentrations of the amino acids threonine and proline contributing to the sweet taste; and phenylalanine, tyrosine, leucine, isoleucine, valine, and methionine contributing to the bitter taste, increased after 60 days of fermentation compared with non-fermented soy. Regarding the organic acids, only citric acid and pipecolic acid were detected, whereby the former declined throughout the fermentation while the latter increased. In this study, twenty-two metabolites were determined as the major compounds contributing to the discrimination of *meju* samples. The authors found that those metabolites were positively correlated with the nutritional and sensory qualities of *meju* and that they could be used as biomarkers for monitoring the fermentation. Moreover, they concluded that monitoring metabolite changes during the *meju* fermentation might be relevant for the preparation of other related foods such as *doengjang* and *kangjang*.

Doenjang, a fermented soybean paste among the Korean traditional fermented foods, is prepared from *meju*, which can be consumed as a condiment in raw-paste form with vegetables, as flavored seasoning, or even as a dipping condiment. It is traditionally prepared by spontaneous fermentation of *meju*, involving bacteria and fungi, such as *Bacillus subtilis*, *Rhizopus*, *Mucor*, and *Aspergillus* species, which are naturally present. Namgung et al. (2010) studied metabolite changes during *doenjang* fermentation by applying GC–MS and PCA for metabolite determination and data processing, respectively. For the elaboration of *doenjang*, *meju* was exposed to sunlight during 3 months, giving rise to a spontaneous fermentation; then, the pulp was passed to pottery jars containing 26% brine for another two months. The authors noted that the concentrations of the amino acids glycine, alanine, serine, and threonine, responsible for the sweet taste, and glutamic acid and asparagine, responsible for the *umami* taste, increased considerably between 140 and 160 days of fermentation, glutamic acid being an amino acid of major occurrence. The concentration of the amino acids responsible for the bitter taste (leucine and isoleucine) progressively increased up to 100 days of fermentation. Organic acids depended on the existent microbiota; while the concentrations of lactic acid, succinic acid, mandelic acid, propionic acid, and glutaric acid increased rapidly with

Table 1
Metabolomics applied to different fermented foods. Analyses of metabolites and detection/separation techniques.

Fermented foods			Metabolomic analysis			References
	Microorganisms	Time of fermentation	Technique	Amino acids*	Organic acids*	
Meju	*Bacillus* sp., *Aspergillus* sp., *Mucor* sp.	Two weeks	UPLC-Q-TOF MS and PLS-DA	Glutamic acid Isoleucine Leucine Methionine Phenylalanine Tyrosine Proline Threonine Valine	Citric acid Pipecolic acid	Kang et al. (2011)
Doenjang	*Bacillus subtilis*, *Rizhopus*, *Mucor*, *Aspergillus* sp.	Three months	GC–MS and PCA	Alanine Glutamic acid Asparagine Glycine Isoleucine Leucine Serine Threonine	Citric acid Glutaric acid Lactic acid Mandelic acid Propionic acid Succinic acid	Namgung et al. (2010)
Cheonggukjang	*Bacillus subtilis* and *Bacillus* sp.	Two or three days	GC–MS and PCA	Alanine Asparagine Aspartic acid GABA Glutamic acid Glycine Leucine Lysine Phenylalanine Proline Threonine Tryptophan Tyrosine	2-Hydroxyglutaric acid Citric acid Fumaric acid Galactaric acid Gluconic acid Itaconic acid Lactic acid Malic acid Malonic acid Oxalic acid Succinic acid Tartaric acid	Park et al. (2010) and Baek et al. (2010)
Fermented soymilk	Bifidobacteria and Streptococci	24 h	H NMR and PCA	Phenylalanine	Citric acid Lactic acid Malic acid Oxalacetic acid Succinic acid	Yang et al. (2009)
Gouda cheese Cheddar cheese Parmigiano Reggiano	Lactic acid bacteria	Ripening time 60–180 days 180 days Up to 30 months	GC/TOF-MS and PCA	Asparagine Ethionine GABA Glutamic acid Glycine Isoleucine Leucine Lysine Ornithine Proline Pyroglutamic acid Tryptophan Tyrosine Valine	Aspartic acid Lactic acid Succinic acid	Ochi et al. (2012)

Amino acid* and organic acid* analyzed.
UPLC-Q-TOF MS: ultra-performance liquid chromatography-quadrupole-time of flight mass spectrometry.
PLS-DA: partial least squares-discriminant analysis.
GC–MS: gas chromatography–mass spectrometry.
H NMR: proton-nuclear magnetic resonance.
GC/TOF-MS: gas chromatography/time of flight-mass spectrometry.
PCA: principal component analysis.

the time of fermentation; the concentration of citric acid gradually decreased upon fermentation. In this study, the production of glucosamine and the concentration increase in polyols such as xylitol, inositol and mannitol during fermentation was evidenced. On the whole, *doenjang* samples corresponding to different fermentation periods were differentiated by analyzing their non-volatile metabolites. Thus, sugar derivatives were considered as main contributors to discriminate samples during early steps of fermentation, while the amino acids were indicators of the later fermentation stages. The major metabolites involved in *doenjang* sample differentiation during fermentation were leucine, isoleucine, aminoadipic acid, lysine, malic acid, oxalic acid and glucosamine.

Cheonggukjang is also a Korean traditional fermented soybean paste, but unlike *doenjang*, it is made by a spontaneous fermentation (at 42 °C for 2 or 3 days) of cooked soybeans, involving whole soy and ground and with *B. subtilis* as the predominant microorganism. Recently, Park et al. (2010) studied changes in a selected number of pre-defined metabolites during this food preparation by applying GC–MS and PCA. Twenty amino acids, twelve organic acids and nine fatty acids were determined as targeted metabolites in *cheonggukjang*. In general, the concentrations of amino acids decreased at the beginning of the fermentation, while they increased at the end. Interestingly, the concentrations of the amino acids tyrosine, phenylalanine, alanine, glutamic acid, threonine, and aspartic acid declined after 50 h of fermentation, while γ-aminobutyric acid (GABA), tryptophan and asparagine levels decreased during fermentation. Fatty acid levels generally increased along with the fermentation period, while the concentrations

of the majority of the organic acids declined with the exception of tartaric acid. This study revealed that tryptophan, citric acid, β-alanine, itaconic acid, 2-hydroxy-glutaric acid, GABA, leucine, malic acid and tartaric acid were the major components that differentiated the various *cheonggukjang* samples upon fermentation.

Baek et al. (2010) studied the metabolomic profiling of this soybean pasta of different fermentation times and inoculated with different strains of *Bacillus*, such as *Bacillus amyloliquefaciens* CH86-1, *Bacillus licheniformis* 58 and *B. licheniformis* 67, by using GC-TOF–MS, PCA and PLS-DA. A total of twenty amino acids, ten sugars, five sugar alcohols and seven organic acids were identified in the different samples, the metabolite patterns being distinctive according to the fermentation period. Significant differences in targeted metabolite contents were detected depending on the inoculated strain; for instance, consumption of sugars was higher with *B. licheniformis* 58 and 67, which had higher hydrolytic activities than *B. amyloliquefaciens* CH86-1. *Cheonggukjang* fermented for 72 h was discriminated by its amino acid contents in particular glycine, glutamic acid, leucine, proline, phenylalanine and lysine.

Yang et al. (2009) conducted metabolomic profiling of fermented soymilk, which was prepared with an inoculums of *Bifidobacterium* and *Streptococcus* strains and fermented at 30 °C for 24 h. The metabolites were studied using H NMR spectroscopy followed by PCA. The authors noted a reduction in sugars and a simultaneous increase in the concentrations of lactic acid and succinic acid along the fermentation, due to the action of the inoculated microbial strains. The concentrations of malic acid, citric acid and oxaloacetic acid, raffinose and stachyose, sucrose and phenylalanine diminished during fermentation. *Lactococcus lactis* converted fumarate acid into succinic acid, and malic acid into lactic acid.

2.2. Cheeses

Metabolomics has also been employed to create sensory predictive modeling for both cheeses (Ochi et al., 2012) (Table 1) and wines (Table 2) (Rochfort et al., 2010). Characterization of the sensory attributes of these foods and beverages is traditionally performed with tasting assays, for which people should be trained to be able to classify and describe the different flavors involved in cheeses and wines. Alternative techniques are searched and the study of metabolites could be one of them.

Ochi et al. (2012) used GC-TOF–MS and PCA to analyze the components of different cheeses such as Cheddar, Gouda, and Parmigiano Reggiano. Cheddar cheese is a relatively hard pressed cheese with acidic flavor that is made with pasteurized cow's milk, Gouda cheese is a semi-firm cheese with soft and creamy flavor that becomes stronger and more consistent with ripening, while Parmigiano Reggiano cheese is a hard cheese with PDO (protected designation of origin) status (protection is by a specified law reserved exclusively to the cheese produced in the Italian area of Parma, Reggio Emilia, Modena, Bologna and Mantova). The authors analyzed samples from these three cheeses produced in different countries and with different manufacturing processes and dates. The peak profiles obtained for Parmigiano Reggiano cheese showed a marked difference with respect to the other two varieties; while although manufacturing and ripening processes of Cheddar and Gouda cheeses are different, the final metabolite profiles were not much influenced and displayed similar peaks. PLS regression models for six sensory attributes such as “rich flavor”, “sour flavor”, “bitter flavor”, “salty flavor”, “creamy flavor”, and “milky flavor” were used in an attempt to characterize the cheeses. However, by using GC-TOF–MS only a secure model for “rich flavor” and “sour flavor” was reached; the results indicated that the hydrophilic low-molecular-mass-components that allowed differentiation of these two flavors were aspartic acid, leucine, methionine, tyrosine, pyroglutamic acid, glycine, glutamic acid, valine, lysine, isoleucine, and asparagine, which positively contributed to the “rich flavor”, while lactose and tryptophan contributed negatively. The prediction of “sour flavor” was influenced by 4-aminobutyric acid, lactic acid, and ornithine as positive contributors, and lactose, succinic acid and proline as negative ones. Thus, the analyses of hydrophilic and low-molecular-mass-metabolites allowed the prediction of specific sensory characteristics related with cheese ripening. Interestingly, the metabolomics approach is an effective way to verify the authenticity of food products (i.e., PDO cheeses), as it identifies a “molecular fingerprint” that accurately represents the food product and discriminates it from different or fraudulent varieties (Lindon, Nicholson, & Holmes, 2007).

Recently, Mazzei and Piccolo (2012) have applied HRMAS-NMR metabolomics to assess the quality and traceability of Mozzarella di Bufala Campana (MBC), a PDO cheese made from buffalo's milk belonging to the Campania Region of Italy. The obtained spectra, selectively simplified with two NMR pulse sequences and combined with multivariate analyses, enabled significant metabolic differentiation between MBC samples from different manufacturing sites in the Campania Region. Only four variables, assigned to galactose, lactose, acetic acid and glycerol, contributed to distinguish between samples. To evaluate MBC aging, two-day old MBC samples were compared with fresh cheeses; an increase in the concentratins of isobutyl alcohol, lactic acid and acetic acid was detected in the former ones. These components are by-products of mozzarella biodegradation and represent specific elements for characterizing aged MBC metabolome. Also, Shintu and Caldarelli (2005) succeeded in the characterization of Parmigiano Reggiano cheese, according to its ripening age, by applying HRMAS NMR combined with PCA and Discriminant Analysis (DA). The authors (2006) also showed that application of HRMAS NMR together with chemometric methods was effective in assessing aging and traceability of Emmental cheese.

Table 2
Metabolomics applied to wines and fermented cocoa beans. Analyses of metabolites and detection/separation techniques.

Fermented foods	Metabolomic analysis			References
	Microorganisms involved	Techniques	Metabolites*	
Wines: Cabernet Sauvignon and Shiraz Control, exposed highly exposed and shaded	Yeasts	NMR and PCA	Proline Fructose Glucose Methanol Succinate Acetate Aliphatic amino acids Ethanol Glycerol Malic acid Tannin	Rochfort et al. (2010)
Fermented cocoa bean	Yeasts, lactic acid bacteria, acetic acid bacteria	HPAEC and HPLC	Polyphenols Theobromine Caffeine Epicatechin Catechin Ethanol Lactic acid Acetic acid Citric acid	Camu et al. (2008)

Metabolites* analyzed.
NMR: nuclear magnetic resonance.
HPAEC: high-performance anion exchange chromatography.
HPLC: high-performance liquid chromatography.
PCA: principal component analysis.

2.3. Wines

Wine is an alcoholic beverage made from grapes through alcoholic fermentation of their must or juice. Fermentation occurs by the metabolic action of yeasts that transform the sugars of the fruit into ethyl alcohol and carbon dioxide. However, wine characteristics are the

sum of a set of environmental factors that include climate, latitude, altitude, daylight hours, etc. Red wines can be classified by the varieties of grape fermented into, for example, Cabernet Sauvignon, Malbec, Merlot, Syrah, Pinot Noir, Tempranillo, Bonarda, Sangiovese, etc. Rochfort et al. (2010) analyzed different wine samples to predict their sensory characteristics through metabolomics. For this purpose, NMR and PCA for data analysis were used to study two varieties of wines, Cabernet Sauvignon and Shiraz, with different light exposure levels and classified as Control (standard practice vineyard), Exposed (achieved with a foliage wire 600 mm above the top cordon), Highly Exposed (using a wire leaf plucking in the fruit zone with foliage), and a Shaded treatment (using 70% shade-cloth). The results showed a clear differentiation between wines of the grape varieties Cabernet Sauvignon and Shiraz and among the different degrees of sunlight exposure. The differentiation between varieties was mainly due to the higher concentrations of proline in the Cabernet Sauvignon wines compared to Shiraz, while the later one presented higher concentrations of sugars (fructose and glucose), methanol, succinate, acetate and some aliphatic amino acids. Regarding the sunlight exposure, it was possible to discriminate the Shaded from the Control treatment, the differences being similar in both varieties. Both wines showed less concentration of ethanol and higher concentrations of glycerol, acetate and malic acid, as well as more sugar and amino acids. The Shaded wines were characterized by a lower astringency, less body and a slight increase in sour taste (Joscelyne, Downey, Mazza, & Bastian, 2007). The metabolic study of Rochfort et al. (2010) corroborated with the higher concentrations of acids and a lower tannin concentration, responsible for the sourness flavor and astringency, respectively, compared to the control. Alternatively, the Exposed samples were partially distinguishable from the Control samples. As a result, the metabolite profiling made a differentiation between wine varieties possible, even among different sunlight exposure treatments, and allowed to predict the sensory qualities of wines by using a NMR tool.

The described reports represent the scarce studies available on metabolomics of fermented foods that were undertaken to evaluate their nutritional quality and to generate predictive models of sensory characteristics as noted in wines and cheeses. Although this methodology is much easier and cheaper than the traditional ones used for the characterization of sensory attributes, much work still remains to be done.

2.4. Cocoa bean fermentation

Cocoa beans are used as raw material for the production of chocolate, the flavor of which depends on certain metabolites or aroma precursors present in the cocoa beans and those developed mainly during cocoa bean fermentation and drying. Chocolate flavor is a very complex mixture of more than 550 compounds. It has been found that undesirable bitter and astringent tastes decrease during fermentation, while desirable fruity, floral and cocoa flavors develop during fermentation and drying; making these two processes the key ones in chocolate flavor development. During fermentation, the role of microorganisms is limited to removal of the pulp that surrounds the beans and the production of essential metabolites. This happens through pectin depolymerization by yeasts and anaerobic yeast fermentation of sugars to ethanol, microaerophilic LAB fermentation of sugars and citric acid to lactic acid, acetic acid and mannitol, as well as aerobic exothermic oxidation of ethanol into acetic acid by acetic acid bacteria. Camu et al. (2008) studied the formation of targeted metabolites during cocoa bean fermentation and drying to unravel the influence of microbial activities on chocolate flavor. Spontaneous cocoa bean fermentations of wet beans (heap method) on two small farms in two different seasons (main- and mid-crop) were carried out. Variations in concentrations of polyphenols, theobromine and caffeine were determined during fermentation and drying, using high-performance anion exchange chromatography (HPAEC) and HPLC. During fermentation, the polyphenol and alkaloid concentrations of cocoa beans were heap- and crop-dependent, epicatechin and theobromine concentrations decreasing during fermentation. The total polyphenol content was reduced throughout fermentation by approximately 10–50% during the main-crop, while almost no reduction in total polyphenol content was found upon fermentation during the mid-crop. At the end of the fermentation, ethanol, lactic acid, acetic acid and citric acid were found, their formation being dependent on the heap. The concentrations of these compounds were different in pulp and in beans; the concentration of ethanol and acetic acid was slightly higher in the pulp than in the beans, while the concentration of lactic acid was considerably higher in the pulp. When fermented cocoa beans were dried, the polyphenol content further decreased and approximately 50% of epicatechin and 60% of catechin were lost. Metabolic analysis carried out by Camu et al. (2008) allowed differentiating metabolites present in fermented cocoa beans obtained from different crop periods, and those present in the beans and pulp, that will later be reflected in the chocolate flavor. Understanding metabolite occurrence and their development during fermentation will enable controlling the fermentation process to achieve a high quality end product.

3. Metabolomics in fermented functional foods

Functional foods contain compounds or ingredients that may help reduce the risk of disease or promote better health beyond providing adequate nutritional effects. Functional foods, which include probiotics, prebiotics and synbiotics, have a significant and growing global market, especially in Europe, Australia and Japan (Stanton, Ross, Fitzgerlad, & van Sinderen, 2005). In this review, the effect of probiotics in the human gut analyzed through metabolomics is discussed.

3.1. Probiotics

Probiotics have been defined as “live microorganisms that when being administered in an appropriate dose, they confer a benefit of health to the receiver” (FAO/WHO 2001). Probiotic bacteria have been studied for their impact on the metabolism of food molecules in the intestine such as lactose digestion and cholesterol metabolism. They are also known to contribute to protein and ammonia metabolism and to alter the composition of the gut microbiota (Rabot, Rafter, Rijkers, Watzl, & Antoine, 2010). The impact of probiotic strains on the composition and functioning of the human gut and their influence on the resident microbiota is not entirely understood. The difficulty lands on the quantity and diversity of human microbiota, as our gut contains 100 trillion microbes belonging to nine different divisions, among which the most relevant ones are Firmicutes and Bacteroidetes. Moreover, the microbial composition of the intestine varies among individuals. An additional complexity arises with the fact that some bacteria are residents in the gut while others have a transitory effect when passing through with food and water (Sonnenburg, Chen, & Gordon, 2006). These facts show the importance of creating a simplified model for understanding the effect of the microbial communities on the health of the host and the effect of probiotic administration.

The effect of probiotics on the human gut has been studied by different approaches such as Genomics, Transcriptomics, Proteomics, and more recently, Metabolomics. By applying Metabolomics, the multi-parametric metabolic response of an organism to biological stimuli can be analyzed (Martin et al., 2007). Thus, this methodology may elucidate if the metabolites released or degraded by probiotic strains have an effect on cytokine expression and may also give information about changes in beneficial metabolite concentrations in different organs when probiotics are present in the gut.

Techniques such as UPLC, HRMAS-NMR, LC–MS, etc., coupled to complex statistical data analysis allows measuring multiple metabolite concentrations, which enables the elucidation of the effects of probiotics on the host and on the metabolism of other microorganisms present in the gut.

Thanks to the use of GC–MS analysis, De Preter et al. (2010) could show a dose-dependent saccharolytic fermentation of the prebiotic oligofructose-enriched inulin (OF-IN) by means of an in vitro fecal model. A total of 107 different volatile organic compounds were identified; the concentrations of acids, esters and some aldehydes, significantly increased with increasing doses of OF-IN, while the concentrations of sulfur compounds and phenolic compounds, which are the result of toxic protein fermentation, decreased.

Several reports using animal models in which germ-free mice were used as host have been released (Martin et al., 2010; Matsumoto et al., 2012; Sonnenburg et al., 2006). These methods allow direct colonization of the experimental subject with probiotic strains or establishing different microbiota, simulating the adult or infant human gut, and supplying different probiotics and/or pathogenic strains. Martin et al. (2007) confirmed the effect of some probiotic strains on the host, analyzing different parts of germ-free mouse intestines by HRMAS NMR. The authors found that the administration of *Lactobacillus paracasei* NCC2461 could increase the lactate concentration in the jejunum, suggesting nutrient processing by this strain. Also, the concentrations of choline, glycerophosphorylcholine (GPC) and acetate was lowered in the jejunum and ileum; these components are key intermediates of lipid metabolism, indicating that this LAB strain could modulate the intestinal fat metabolism. Moreover, the authors showed that the concentrations of taurine and creatine, which regulate intestine hyper-contractility, were diminished in the presence of *L. paracasei* NCC2461 and that the concentrations of glutathione (a potent antioxidant) and its precursors were lowered, indicating that this probiotic strain could regulate enterocyte glutathione metabolism, which could be related to gastrointestinal cancer. Similar studies were done by the same authors (Martin et al., 2008) using germ-free mice colonized by an infant microbiota and the probiotic strains *L. paracasei* NCC2461 and *L. rhamnosus* NCC4007. In this work, metabolites were analyzed in gut, plasma, urine and fecal extracts by HRMAS-NMR. The results showed that these probiotic strains could alter hepatic lipid metabolism, diminish plasma lipoprotein level and stimulate glycolysis. The presence of probiotics influenced also amino acid catabolism and the concentrations of methylamines and short-chain fatty acids.

Martin et al. (2010) studied also the effect of administering a symbiotic, a prebiotic (non-digestible food ingredient that stimulates the growth and/or activity of bacteria in the digestive system) together with a probiotic strain, on a humanized baby intestinal microbiota metabolome. This symbiotic combination was directly related to the increased growth of bifidobacteria and lactobacilli; these bacterial variations were related to lower concentrations of unassigned fatty acids, higher concentrations of oligosaccharides and greater increase of acetate, compared to mice that did not receive the synbiotic. Also, modulation of protein metabolism was observed as reduced concentrations of glutamate, ornithine, glycine and valine and increased concentrations of 5-aminovalerate were found.

In another study, Wikoff et al. (2009) analyzed the effect of the gut microbiome on blood metabolites of germ-free and conventional mice by using LC–MS, confirming a significant cooperation between bacterial and mammalian metabolism. The authors showed that microorganisms in the gut could influence the amino acid metabolism as well as the synthesis of the antioxidant indol-3-propionic acid.

Matsumoto et al. (2012) analyzed the mouse metabolome using CE–TOFMS; differences between the metabolomic profile of germ-free mice and ex-germ-free mice colonized with bacteria from the feces of conventional mice were described. The authors showed that prostaglandin E2, an interleukin (IL)-10-independent innate immune suppressor, was present only in ex-germ free mice, indicating that the intestinal microbiota contains activation factors for innate immunity, similar to inflammation.

The effect of probiotics using human subjects was also analyzed by the metabolomic approach. In this respect, the lipidomic profile and its influence on the inflammatory variables (C-reactive protein, interferon-α and IL-6) in serum of healthy adults receiving *L. rhamnosus* GG (LGG) was studied using UPLC coupled to MS by Kekkonen et al. (2008). The administration of the LGG strain showed a decrease in lysophosphatidylcholines (LysoGPCho) levels. This lipid affects many cell functions such as survival, migration and secretion. It is also involved in oxidative metabolism, angiogenesis and carcinogenesis. LysoGPCho is an atherogenic lipid that has been associated with inflammation, endothelial dysfunction and coronary atherosclerosis. This lipid induces the secretion of several inflammatory cytokines in human peripheral mononuclear cells. A direct regression between LysoGPCho production and IL-6 release was found, explaining the anti-inflammatory effect of LGG. Also, Kekkonen et al. (2008) observed a decrease in the concentrations of sphingomyelins in plasma samples of individuals treated with LGG, as this sphingolipid functions also as regulator of inflammation response. The decrease of the concentrations of these lipids could partially explain the effect of LGG on ulcerative colitis and normalized gut permeability.

Another useful model for studying the effect of probiotics on human health is by using subjects suffering from irritable bowel syndrome (IBS). This disease is a common disorder affecting 5–10% of the population; its symptoms include abdominal pain, irregular bowel movements, constipation and/or diarrhea (Madsen, 2011). Hong et al. (2011) analyzed by HRMAS-NMR serum and feces of individuals suffering from this disease before and after being treated for 2 months with fermented milks containing lactobacilli and bifidobacteria strains. After probiotic administration, the alterations in blood levels of glucose, tyrosine and lactate were normalized. Alternatively, Ponnusamy, Choi, Kim, Lee, and Lee (2011) showed differences between the metabolites present in the intestines of individuals suffering from IBS and healthy adults and made a correlation among the metabolites found and the microorganisms present in the gut. Feces of individuals suffering from IBS had higher concentrations of hydroxyphenyl acetate and hydroxyphenyl propionate, which are produced from phenylalanine by *Clostridium* sp., and showed increased concentrations of aminobutyric acid, which is correlated with the presence of *Faecalibactreium prausnitzii*. A correlation between the concentrations of alanine, glutamic acid and pyroglutamic acid and the presence of lactobacilli was found; however, the cell counts of these bacteria in the intestines was higher in subjects suffering from IBS. On the contrary, the cell counts of *Bifidobacterium* strains were higher in healthy adults.

Metabolomics is an interesting tool for studying the effect of probiotics on the host health. This “omics” together with the study of the host's Proteomics and Genomics can provide new information related to the effects of probiotics on consumer's health. Moreover, metabolomics may give information on the effect of administrating a probiotic strain on the metabolic behavior of the resident microbiota of healthy subjects and of those suffering from different diseases such as IBS.

4. Conclusions

Metabolomics, an emerging field within the “omics” sciences, deals with the simultaneous determination and quantitative analysis of intracellular metabolites that are produced and modified by the metabolism of living organisms. Several hyphenated techniques combined with multivariate analyses have been applied for accurate and discriminative metabolite determination of samples. Metabolomics have been successfully used in food science to evaluate the molecular fingerprints of fermented foods, such as soy foods, cheeses, and wines. This approach allowed assessing food quality and maturity,

as well as traceability and authenticity of fermented products such as PDO cheeses. Moreover, metabolomics has been useful in evaluating the beneficial effect of probiotics in certain human diseases such as IBS. Although recently applied, metabolomics became a promising approach to rapidly evaluate as many as possible metabolites to determine globally the quality, traceability and safety of fermented food products.

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