

Methods applied in studies about fermented foods

Abstract

Fermented foods have been produced for thousands of years. In recent decades, its production and consumption have increased due to potential beneficial health effects attributed to its intake. This has stimulated the development of scientific studies on food fermentation. In this context, the development of complex methods capable of characterizing the fermenting microbiota and relating it to the desired characteristics and quality has enabled a greater understanding of the role of different microbial groups in the fermentation process. In this article, the main methods used in research on fermented foods are briefly presented, highlighting the most widely used omics strategies, such as metagenomic, metaproteomic and metabolomic analysis.

Keywords: fermented food, sequencing, metagenomics, metaproteomics, metabolomics

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José Guilherme Prado Martin

Microbiology of Fermented Products Laboratory (FERMICRO),
Department of Microbiology, Universidade Federal de Viçosa,
Brazil

Correspondence: José Guilherme Prado Martin, Microbiology
of Fermented Products Laboratory (FERMICRO), Department
of Microbiology, Universidade Federal de Viçosa, Brazil,
Tel +55 31 3612-5070, Email guilherme.martin@ufv.br

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Introduction

Fermented foods have been known to human race since eons and have been produced for consumption for thousands of years. As a matter of fact, it is being considered as the first foods produced by humans. Different types of fermentation—such as lactic (used in the fermentation of vegetables and dairy products), alcoholic (bread and alcoholic beverages) and acetic (vinegar and acetic beverages) – require different raw materials for improving the nutritional and functional properties with an intent of extended shelf life.

In 2021, the International Scientific Association for Probiotics and Prebiotics (ISAPP) proposed a new definition for “fermented foods”: foods produced through enzymatic conversions of food components from desired microbial growth. Therefore, from this induced process, a great diversity of fermented foods are generated, whose production and consumption has increased in recent years, due to potential functional effects attributed to their consumption.

The fermentation assessment, on artisanal or industrial scale, is essential for the final product quality. For this, ordinary methods based on detection of changes in food matrix include determination of pH, titratable or volatile acidity, the content of soluble solids as well as the CO₂ production. In addition, chromatographic methods are used to evaluate the consumption of fermentable carbohydrates, as well as compounds resulting from microbial activity, important for the sensory attributes and related to the food flavour. Furthermore, studies on the microbiome of fermented food include more sophisticated methods based on omics strategies, with emphasis on metagenomic and metabolomic analysis (Figure 1).

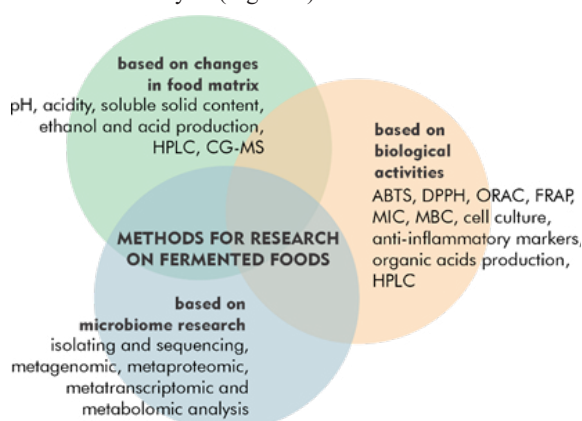


Figure 1 Methods for research on fermented foods.

In this article, the main microbiological methods used in the research of fermented foods are briefly presented, from the traditional to the most recently developed, which emphasis on the omics approach used in studies published in recent years.

Methods for research on food fermentation

Methods for changes in food matrix research

Traditional methods for evaluating the fermentation process are based on the investigation of components of the food matrix consumed or produced during the fermentation process, responsible for physicochemical changes. Among the most commonly used simple methods, the measurement of the hydrogenic potential, expressed in pH values, and the determination of the total titratable or volatile acidity of the product, usually expressed in % acid, depending on the characteristics of the evaluated food matrix, stand out. In addition, the analysis of the soluble solids content using a refractometer can indicate the consumption of fermentable sugars throughout the fermentation process, though the use of chromatographic methods allows for more reliable results, mainly for the evaluation of glucose, fructose or maltose consumption. In addition, ethanol and CO₂ production is also a commonly investigated parameter, especially for carbonated beverages or bakery products. At the same time, the production of compounds related to the flavour formation of the fermented product can also be evaluated through the use of chromatographic methods, especially High-Performance Liquid Chromatography (HPLC) or Gas Chromatography Coupled with Mass Spectrometry (GC-MS), to investigate volatile compounds.

Methods for research of compounds with biological activities

In addition to methods used to evaluate the fermentation process described in the previous section, there are also methods for identifying and quantifying compounds with biological effects produced from microbial activity related to potential health benefits, like antioxidant, antimicrobial and anti-inflammatory. It is noteworthy that although the results obtained from such methods indicate biological activity of biological compounds present in fermented foods, *in vivo* studies are necessary to prove their real effects in humans. Traditional methods for evaluating antioxidant activity include the scavenging activity using 2,2 diphenyl 1, picrylhydrazyl (DPPH), superoxide and hydroxyl free radical assay (ABTS), Oxygen Radical Absorbance Capacity (ORAC) and Feric Reducing Antioxidant Power (FRAP), widely used in several studies on fermented foods.¹⁻⁴ Methods for

evaluating antimicrobial activity generally include the determination of minimum inhibitory concentrations (MIC) in 96-well microplates containing different types of target microorganisms⁵ or using agar spot test.⁶ Studies focused on disclosing anti-inflammatory activity generally used in cell culture assays⁷ or commercial kits which may vary according to the type of inflammatory markers to be evaluated, such as for example TNF- α , IL-6, and IL-1 β .⁸

Regarding animal model studies, these are generally used to evaluate the effects of consuming fermented foods on weight gain control, diabetes prevention, and immunomodulation, among others. The anti-obesity effect of kimchi added to citrus concentrate (*jeju*) was evaluated from the assessment of serum and liver lipid concentrations in mice with high-fat diet-induced obesity;⁹ Body weight gain, feed efficiency rate and tissue weight were also evaluated by the researchers. The study showed that the consumption of kimchi plus *jeju* resulted in an anti-obesity effect in the evaluated animals. The effect of consuming brown rice and rice bran fermented with *Aspergillus oryzae* (a filamentous fungus commonly used in the production of soy sauce and sake) prevented diabetes in mice.¹⁰ The immunomodulatory activity of fermented figs was evaluated in mice treated with cyclophosphamide.¹¹ The consumption of fermented extracts reduced the lesions in immune organs, promoted the production of cytokines related to the immune system, improved the histopathological state and modulated the intestinal microbiota of the evaluated animals.

Furthermore, several methods have been used for detection of compounds harmful to health in fermented foods. Biogenic amines are low-molecular-weight nitrogenous organic bases that can be produced by microbial activity during food fermentation. Their amounts in fermented foods require attention due to its toxic effects.¹² Methods for its determination include colorimetric sensor analysis, biosensors and enzymatic determination, besides thin layer chromatography, CG-MS, HPLC, ion-exchange chromatography and capillary electrophoresis.¹³ Ethyl carbamate, a known carcinogen, can often be found in Korean fermented foods. A method to investigate this compound was developed and found satisfactory results after using column chromatography with dichloromethane in different volumes, depending on the type of food evaluated.¹⁴ The authors highlight the importance of the method developed for the monitoring and evaluation of ethyl carbamate concentrations in foods by Korean official regulatory agencies.

Methods for researches on microbiome

In comparison to methods based on food matrix changes, studies involving microbiology of food fermentation are significantly more complex. It can range from traditional methods of evaluating biomass production, to culture-dependent methods (such as those used in isolation studies and microbial identification by sequencing) or culture-

independent methods, such as metagenomic studies using total DNA extracted from the food of interest. In recent years, relevant insights into microbial dynamics in fermented foods have been provided from metagenomic and metatranscriptomic sequencing, in addition to the integration of metaproteomic methods with metabolomic analysis.¹⁵ Briefly, while metagenomics aims to identify the composition of a microbial community under different environmental or culture conditions, metatranscriptomics aims to identify which genes are being collectively expressed under such conditions; on the other hand, metabolomics is used to determine which products are being generated by the microbiota under the conditions evaluated.¹⁶ This topic is focused on strategies for microbiome studies, mainly using methods developed or improved in recent years.

Culture-dependent methods are generally used in studies to evaluate the fermentation process in which the prospect of microorganisms for the purpose of biotechnological application is also aimed. This type of study allows, for example, the development of cultures for use in the dairy industry, the production of microbial enzymes for use in the medical and/or pharmaceutical industry, or even the development of biomaterials for different purposes. Amplicon analysis is related to use of the amplification of conserved DNA markers – especially the bacterial 16S rRNA gene and the ITS region for filamentous fungi and yeast – from the study of genomic DNA with primers for PCR analysis. The alignment of DNA sequences and their comparison with reference databases allow the taxonomic identification of the isolates, useful for the construction of culture collections; this method can be used, for example, for the identification of microbial isolates obtained from fermented foods for the production of bacterial or fungal enzymes for use in the food, textile, medical and pharmaceutical industries, among others.

Culture-independent methods have been increasingly used in research on fermented foods (Table 1), as they do not require isolation steps that can be relatively time-consuming and costly; in addition, many microbial species are not cultivable or live in a viable non-culturable state, which would make it impossible to identify them through cultivation-dependent techniques. In this sense, metagenomic studies allow the direct identification of the DNA of the microbial community present in the food, after the construction of genomic libraries. Metagenomic methods also allow the identification of microorganisms that are not abundant in foods, which would be difficult to isolate in traditional culture media. Furthermore, they allow the study of functional genes involved in the interaction between microbial groups and their importance in the structure of the microbial community involved in the fermentation process, with direct impacts on the sensory and functional quality of foods. However, the use of bioinformatics tools is necessary, requiring high qualification and extensive knowledge for data processing and interpretation of the results found.

Table 1 Studies published last 5 years using omics approaches for fermented food microbiome

Fermented food	Place	Method
Artisanal cheese	Brazil	High-throughput sequencing ¹⁷
Dajiang-meju (soypaste)	China	Metaproteomic analyses ¹⁸
Fermented fish	China	Metaproteomic analyses ¹⁹
Fermented milk	China	Metagenomic analysis ²⁰
Fermented vegetables	China	Metagenomic sequencing and Metagenomic assembled genomes ²¹
Kimchi	Korea	Metataxonomic and metabolomic analysis ²²

Table Continued...

Fermented food	Place	Method
Kimchi	Korea	Metabolomic analysis ²³
Kombucha	Ireland	Metabolomic analysis ²⁴
Milk kefir	Turkey	High-throughput sequencing ²⁵
Noni juice (<i>Morinda citrifolia</i> L.)	China	Metatranscriptomic analysis ²⁶
Pickled vegetables	Africa/Asia	Shotgun metagenomic sequencing ²⁷
Serofluid dish	China	Metatranscriptomic analysis ²⁸
Sourdough bread	Brazil	Metagenomic analysis ²⁹
Sourdough bread	China	High-throughput sequencing ³⁰
Tej (honey wine)	India	High-throughput sequencing ³¹
Toddy (palm wine)	India	Metagenomic analysis ³²
Yucha (cooked rice and fresh food fermented)	China	Metagenomic sequencing and Metagenomic assembled genomes ²¹
Yu-lu (fish sauce)	China	Metagenomic analyses ³³

Using metagenomic analyses, the microbiomes of 23 samples of different types of fermented milks produced in China, such as *koumiss*, *nunu*, kefir and yogurt, were evaluated.²⁰ The study revealed a significant difference in the composition of the microbial community of the evaluated products, especially in relation to the profiles of dominant species, including 4 new species detected belonging to the *Lactobacillus*, *Streptococcus*, *Acetobacter* and *Rothia* genera. The influence of temperature on bacterial dynamics during the fermentation of sourdough bread produced in Brazil was analysed by metagenomics.²⁹ The study revealed that the temperature of 21°C favored the development of an exclusively lactic microbiota on the dough, therefore beneficial for the process, unlike the fermented dough at 30°C which favored the development of atypical groups such as *Pseudomonas* and Enterobacteriaceae. The bacterial diversity and metabolic profile of *toddy*, a palm wine popular in India, were studied.³² The metagenomic analysis identified the dominance of *Acetobacter*, *Lactobacillus*, *Candidatus phytolasma* and *Gluconobacter*, highlighting the first two genera as the most predominant in the Indian *toddy*.

Metatranscriptomics is an approach that aims to study the total transcripts (transcriptome) of a microbial community. Therefore, the method provides a snapshot of the expression of genes in the microbial community at a given time under specific conditions, from the capture of total mRNA.¹⁶ It is reverse transcribed to obtain the cDNA, which is then sequenced, revealing the total expressed genes that may be associated with metabolic changes related to the formation of sensory characteristics in fermented foods.¹⁵ With the development and cost reduction of next-generation sequencing (NSG) techniques, this tool has been increasingly used in the study of the metabolic functions of the microbiota involved in fermentation processes.³⁵ The technique has already been used, for example, to determine the microbial community and key metabolic functions involved in the fermentation of noni juice (*Morinda citrifolia* L.)²⁶ and in the evaluation of the role of fungi on carbohydrate and amino acid metabolism during the fermentation of vegetables for the production of *serofluid* dish, a typical fermented product from northwest China.²⁸

Metabolomics consists of the identification and quantification of all metabolites produced by microbial metabolism. Metabolomic analyses can be targeted (specific) or non-targeted (non-selective). The first ones are directed to a specific group of metabolites to evaluate their behaviour in a given condition; the second are used to obtain

patterns or fingerprints from the study of the largest possible number of groups of metabolites generated by the microbial community.³⁶ In comparison to metagenomics and metatranscriptomics, which depend on sequencing steps, metabolomic studies also require tools for the identification and quantification of metabolites. It combines techniques of chromatography (liquid or gas) and detection methods, such as MS or nuclear resonance (NMR).¹⁶ Metabolomics can be used together with other omics strategies in complex studies capable of providing relevant insights about the importance of the microbial community in food fermentation.

The kinetics of kombucha fermentation was evaluated by metabolomic analysis;²⁴ it revealed a close relationship between the fermenting microbiota (especially *Komagataeibacter*, *Gluconacetobacter*, *Gluconobacter*, *Brettanomyces* and *Schizosaccharomyces*) and the production of bioactive compounds in the fermented beverage (for example organic acids and phenolic compounds), with antioxidant and anti-inflammatory effects *in vitro*. On the other hand, the effect of different salt concentrations on kimchi fermentation was investigated.²³ The metabolomic study revealed that the speed of fermentation varied according to the concentration of salt used; in addition, the profile of metabolites in the product after 50 days of fermentation was significantly different among the formulations evaluated. Higher concentrations of lactic acid, acetic acid and xylitol were observed in kimchi with 5% salt; moreover, greater microbial diversity was detected in this product compared to those with a lower salt content.

Finally, metaproteomic analysis is used for the detection and quantification of low molecular weight compounds – including peptides, amino acids, organic acids and amines – as biomarkers of safety and quality in fermented foods. Such information also provides important details related to potential biological activities, allergenic and sensorial properties resulting from the fermentation process. This type of study requires an extraction of total proteins from the food, which usually involves cellular disruption by physical means, followed by dissolution in buffers, purification steps by precipitation, and protein lysis. Subsequently, the separation and purification steps can be carried out by methods with or without the use of gels; finally, the identification of compounds is usually carried out using MS.³⁴ Therefore, metaproteomic provides a better understanding of the proteins produced during fermentation, the functional components of the microbial populations involved in the fermentation process,

allowing the genotype to be directly related to the phenotype observed in the food matrix. However, compared to the omics discussed above, metaproteomics remains a promising strategy, but it is still underexplored in research with fermented foods.¹⁵ Some examples of studies with metaproteomics in fermented foods include the investigation of proteins expressed by the microbiota involved in the fermentation of *dajiang-meju*, an ingredient used for the production of Chinese soybean paste,¹⁸ and the detection of important proteins for the sensory attributes of fermented fish (*Siniperca chuatsi*) produced in China.¹⁹

Final remarks

The evaluation of the fermentation process can be performed using different methods. The study of the microbiome of fermented foods through omics approaches has gained prominence in recent years, as it provides a better understanding of the impacts of microbial activity on product quality, as well as on its functionality. Besides the methods described above, the relevance of *in silico* studies should also be highlighted; it can be applied to prospect microorganisms for underexplored biotechnological uses.³⁷ Furthermore, considering the functionality of fermented foods, clinical studies are necessary to ensure the real health benefits that can in fact be attributed to the regular intake of this type of product; in addition to *in vitro* and animal model studies,³⁸ researches with human beings will contribute to a better understanding of the real benefits of food fermentation and its impacts on well-being and quality of life.³⁹

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None.

Conflicts of interest

Authors declare that there is no conflict of interest.

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