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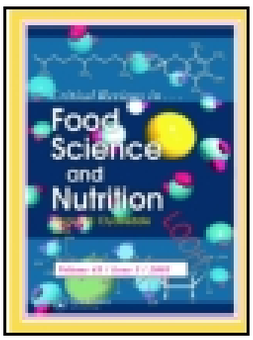

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REVIEW

Challenges and perspectives of quantitative microbiome profiling in food fermentations

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ABSTRACT

Spontaneously fermented foods are consumed and appreciated for thousands of years although they are usually produced with fluctuated productivity and quality, potentially threatening both food safety and food security. To guarantee consistent fermentation productivity and quality, it is essential to control the complex microbiota, the most crucial factor in food fermentations. The prerequisite for the control is to comprehensively understand the structure and function of the microbiota. How to quantify the actual microbiota is of paramount importance. Among various microbial quantitative methods evolved, quantitative microbiome profiling, namely to quantify all microbial taxa by absolute abundance, is the best method to understand the complex microbiota, although it is still at its pioneering stage for food fermentations. Here, we provide an overview of microbial quantitative methods, including the development from conventional methods to the advanced quantitative microbiome profiling, and the application examples of these methods. Moreover, we address potential challenges and perspectives of quantitative microbiome profiling methods, as well as future research needs for the ultimate goal of rational and optimal control of microbiota in spontaneous food fermentations. Our review can serve as reference for the traditional food fermentation sector for stable fermentation productivity, quality and safety.

KEYWORDS

Food fermentation; microbiota; process control; quantitative microbiome profiling

Introduction

Spontaneously fermented foods are an integral part of human life. They include fermented vegetables, meat, dairy, condiments, and alcoholic beverages. They are popular due to prolonged shelf life, enriched flavors, and modified textures as well as high nutritional profiles (Liu et al. 2020a), although they are usually produced with fluctuated productivity and product quality, a potential threat to both food safety and food security (Chen and Zhu 2013). The fluctuation of the productivity and quality is mainly caused by the complexity of microbiota involved in these food fermentations that are spontaneously fermented, i.e., without a standard and defined inoculation and without strict process control. All inoculum enters the culture via air, water, raw materials, operation environment, and residues of previous batches. Regulating the complex microbiota is essential to improve the fermentation productivity and quality (Wu et al. 2021). However, comprehensive and scientific insight is still scarce into the actual and dynamic microbiota structure and function. Without such an insight as the prerequisite, the control of food fermentation seems unfeasible. Therefore, revealing real microbiota structure becomes urgent to provide accurate targets for controllable food fermentations.

To reveal the actual microbiota structure and function, various techniques are developed for microbial quantification. Conventional microbial quantitative methods can be categorized into three groups. The first group is quantitative methods for total microbial load. It includes microscopic observation, plate counting, phospholipid fatty acid analysis, adenosine triphosphate (ATP) analysis, and microbial biomass carbon or nitrogen analysis. Although still routinely widely applied, these methods cannot differentiate microorganisms at the taxonomic level, such as genus or species level. Then, the second group of methods is developed to quantify specific microorganisms. It includes quantitative real-time PCR (qPCR), digital PCR, flow cytometry, fluorescence in situ hybridization, and electrochemical biosensors. Unfortunately, these methods still cannot obtain the full view of all members of the microbiota. To quantify all the microbial taxa, the third group of methods, relative microbiome profiling, is further developed. It includes PCR-denaturing gradient gel electrophoresis and high-throughput sequencing technology. However, these methods can only quantify microbial taxa by relative abundance, the real microbial community dynamics is hidden (Rao et al. 2021). Thus, more accurate and thorough quantitative methods are timely required to reveal the absolute abundance of all microorganisms in food fermentations.

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Recently, advanced microbial quantitative methods, namely quantitative microbiome profiling, are developed to quantify all the microbial taxa by absolute abundance. At present, quantitative microbiome profiling is used to assess actual microbiota profiles and facilitates revealing functional microbiota in other microbial ecosystems such as human gut, marine, and soil ecosystems (Galazzo et al. 2020; Lin et al. 2019; Tkacz, Hortala, and Poole 2018), but rarely used in food fermentations so far. Considering the similarity of microbiota complexity in food fermentations to that in human gut, marine, and soil ecosystems, quantitative microbiome profiling could be explored for the potential application in spontaneous food fermentations. Quantitative microbiome profiling will allow us to understand the real microbial dynamic variation and functional microorganisms in food fermentations, and to control food fermentations with consistent productivity and quality.

Therefore, we introduce and critically evaluate available conventional microbial quantitative methods and advanced quantitative microbiome profiling methods, as shown in Figure 1. Meanwhile, we provide application examples of conventional microbial quantitative methods and the potential application of quantitative microbiome profiling in food fermentations. Finally, we address challenges and perspectives of quantitative microbiome profiling methods, as well as future research needs.

Conventional microbial quantitative methods

As we described earlier and shown in Figure 1, conventional microbial quantitative methods can be categorized into three groups: (1) quantitative methods for total microbial load, (2) quantitative methods targeting specific microorganisms, and (3) relative microbiome profiling methods. Here, we address these conventional methods, including their advantages and disadvantages in quantifying microbiota in food fermentations (Table 1).

Quantitative methods for total microbial load

Quantitative methods for total microbial load were first developed in the 17th century and have evolved ever since, starting from direct counting such as microscopic observation and plate counting to methods based on analyzing microbial substances such as phospholipid fatty acid, ATP, and microbial biomass carbon or nitrogen (Figure 1). These methods have significantly contributed to the microbial quantification in food fermentations.

Microscopic observation and plate counting are two most widely used quantitative methods for food fermented samples (Bracquart 1981; Nickelson, Hosch, and Wyatt 1975). However, microscopic observation cannot differentiate live, inactive, or dead cells (Anderson, Pollock, and Brower 1965). For plate counting, a vast majority of microorganisms still cannot be cultured by plate culture (Lewis et al. 2021). Therefore, the methods of microscopic observation and plate counting cannot obtain the composition and structure of whole microbiota in food fermentations. The substance or components of microbial cells can also be used to quantify the total microbial load in food fermentations. In particular, these methods compensate the drawbacks of plate counting for uncultured microorganisms. For example, phospholipid fatty acid, a main cell component, can be used to estimate total microbial load (Veum, Lorenz, and Kremer 2019).

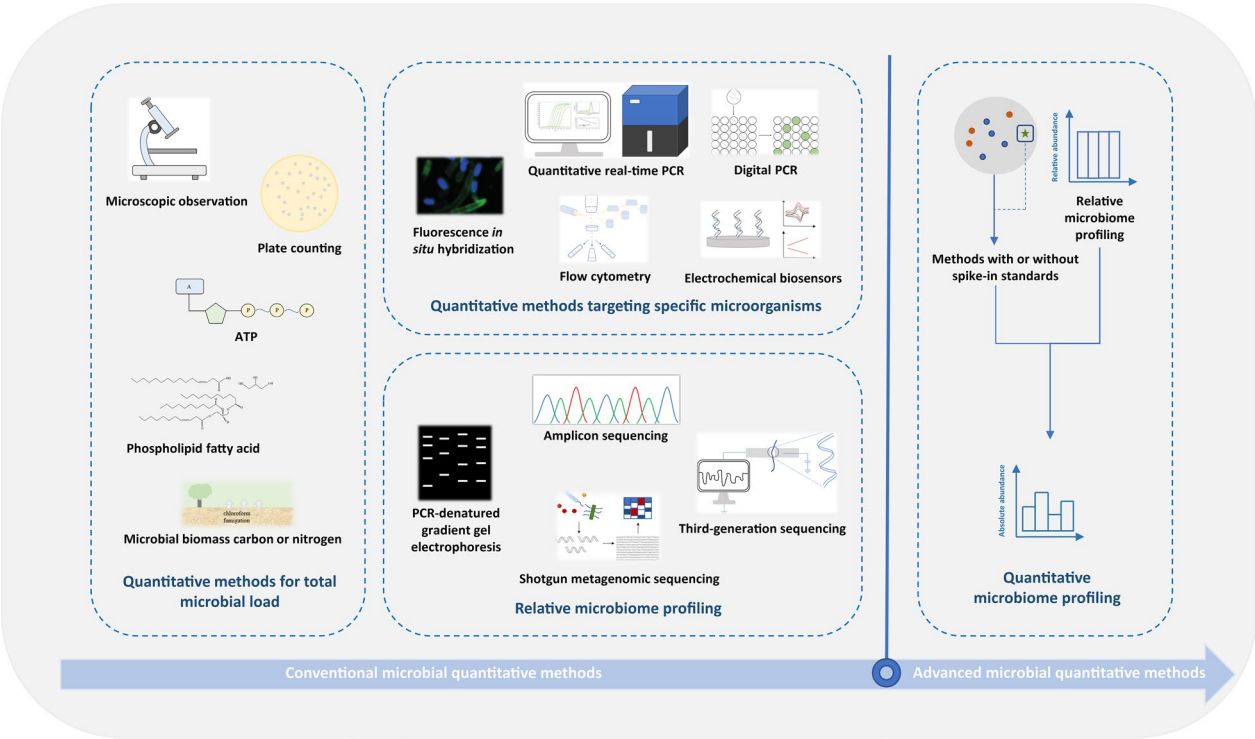


Figure 1. Development of microbial quantitative methods.

Table 1. Comparison of microbial quantitative methods.

Microbial quantitative methods	Principles	Advantages	Disadvantages	References
Quantitative methods for total microbial load				
Microscopic observation	Magnifying and counting microorganisms with an optical or electron microscope	Direct and rapid	Inability to differentiate live, inactive, or dead cells	(Bakken 1985)
Plate counting	Counting viable cells on an agar plate	Relative convenient	Time-consuming; high variability; inability to quantify uncultured microorganisms	(Lewis et al. 2021)
Phospholipid fatty acid analysis	Quantifying phospholipid fatty acid contents	Ability to quantify living and uncultured microorganisms	Error-prone due to the diversity and instability of fatty acids	(He et al. 2016)
ATP analysis	Measuring the luminescence from the reaction between dissolved ATP and Luciferin-Luciferase complex	Ability to estimate viable biomass	Low sensitivity; error-prone due to the concentration variation among microorganisms	(Hammes et al. 2008)
Microbial biomass carbon or nitrogen analysis	Calculating different values of carbon or nitrogen contents between chloroform fumigated and non-fumigated samples	Easy	Inapplicability for low contents of microbial biomass	(Gong et al. 2021)
Summary	–	Preliminary understanding of microbial abundance	Unsatisfactory microbial taxonomic resolution	–
Quantitative methods targeting specific microorganisms				
Quantitative real-time PCR	Obtaining the copy number of target gene by standard curve between Ct value and gene copy number	Easy; relatively high accuracy	Interference of dead cells; amplification biases	(Green and Field 2012)
Digital PCR	Quantifying amplicons in droplets	High sensitivity	High cost; relative long reaction time	(Quan, Sauzade, and Brouzes 2018)
Fluorescence in situ hybridization	Determining the fluorescence intensity by hybridizing the probe with DNA or RNA sequence in cells	Visible; rapid	Rapid decrease of fluorescence signals; low signal intensity from insufficient hybridization of probe; high sensitivity to background noises	(Liu et al. 2018)
Flow cytometry	Counting microbial cells with a flow cytometer	High speed; high precision and accuracy	Limited to the liquid samples; high sensitivity to background noises	(Vieira-Silva et al. 2019)
Electrochemical biosensors	Quantifying available electrical signals from a biological recognition	Rapid; great potential in quantifying specific microorganisms online	Influenced by physicochemical factors	(Ahmed et al. 2014)
Summary	–	Ability to quantify specific microorganisms	Inability to quantify all microbial taxa at the same time	–
Relative microbiome profiling				
PCR-denaturing gradient gel electrophoresis	Roughly calculating the brightness and width of electrophoresis bands as relative abundance of microorganisms	Visible; easy; rapid	Low accuracy; low richness of the microbiota	(Muyzer and Smalla 1998)
High-throughput sequencing	Calculating the ratio of sequencing reads of each microbial taxon to total reads	Comprehensively quantifying all microbial taxa by relative abundance	Relatively low microbial taxonomic resolution	(Rao et al. 2021)
Summary	–	Ability to quantify microbiota structure by relative abundance	Inability to quantify all microbial taxa by absolute abundance; relatively low microbial taxonomic resolution	–
Quantitative microbiome profiling				
Quantitative microbiome profiling with spike-in standards	Converted from the abundance of spike-in standards and the relative abundance of each microbial taxon	Comprehensively quantifying all microbial taxa by absolute abundance	Affected by categories, purities, concentrations, and the addition order of spike-in standards	(Tkacz, Hortala, and Poole 2018)
Quantitative microbiome profiling without spike-in standards	Total microbiota: multiplying the total microbiota abundance by relative abundance of each microbial taxon Indigenous internal standards: converted from the abundance of indigenous internal standards and the relative abundance of each microbial taxon	Comprehensively quantifying all microbial taxa by absolute abundance	Affected by quantification biases of total microbiota; affected by categories, concentrations, and quantification biases of indigenous internal standards	(Du, Wu, and Xu 2020a; Yao et al. 2022)
Summary	–	Ability to quantify microbiota structure by absolute abundance	Complicated operating steps; affected by quantification biases of total microbiota or internal standards; relatively low microbial taxonomic resolution	–

However, different microorganisms contain diverse compositions of fatty acids under different environmental stresses in food fermentations (Ding et al. 2015; Hill et al. 2000), and this may lead to inaccurate total microbial load. ATP is an effective indicator of cell viability (Hammes et al. 2010), and can be used to estimate viable biomass (Learbuch, Smidt, and van der Wielen 2021; Novitsky 1987). However, ATP measurement cannot differentiate intracellular ATP from extracellular ATP (Hammes et al. 2008), and the conversion of ATP concentrations to microbial cell concentrations is error-prone due to the different ATP concentrations in various microbial cells (Learbuch, Smidt, and van der Wielen 2021). Microbial abundance can also be characterized by the amount of microbial biomass carbon or nitrogen that

is usually determined by chloroform fumigation method. This method cannot differentiate microbial taxa, and is inapplicable for low content of microbial biomass carbon or nitrogen (Gong et al. 2021).

In conclusion, quantitative methods for total microbial load described above open our views on the microbial quantity, although these methods cannot differentiate microbial taxonomy. The unsatisfactory taxonomic resolution of these methods hinders their application in quantifying microbiota at genus level or species level in food fermentations. Therefore, quantitative methods targeting specific microorganisms are developed to quantify specific microorganisms involved in food fermentations.

Quantitative methods targeting specific microorganisms

Here, we will introduce the second group of conventional microbial methods, namely, quantitative methods targeting specific microorganisms in food fermentations, as shown in Figure 1. These methods generally require the identification of specific microorganisms by specific nucleotide sequences.

PCR technique is one of the most efficient methods for specific microbial quantification (Quan, Sauzade, and Brouzes 2018). qPCR technique is widely used to quantify specific microorganisms with specific primers in food fermentations. Various specific primers (Table 2), as well as primer databases (Du et al. 2022), can be referenced for quantification of specific microorganisms in food fermentations. However, the accuracy of qPCR analysis should be improved. The contamination of humic acid and ethanol should be avoided in DNA extraction, to reduce their inhibitions on polymerase activity (Green and Field 2012). Moreover, the interference of dead cells should also be reduced. For example, propidium monoazide can be used to inhibit amplification of dead cells during qPCR (Seinige et al. 2014). Digital PCR is an ultrasensitive method for counting cells owing to the single-molecule sensitivity of PCR (Shen et al. 2010). Compared with qPCR, digital PCR is independent on a standard curve (Quan, Sauzade, and Brouzes 2018). It can lessen biases due to the potential uneven amplification of 16S rRNA (Bogatyrev, Rolando, and Ismagilov 2020). Although large-scale application of digital PCR is still limited due to its high cost and relatively long reaction time, it is expected to be extensively applied to quantify microorganisms in food fermentations in the future (Quan, Sauzade, and Brouzes 2018).

Fluorescence in situ hybridization and flow cytometry can also be applied to quantify species-specific microorganisms in complex microbiota (Davies 2012; Kim et al. 2020; Young, Jackson, and Wyeth 2020). The disadvantages of fluorescence in situ hybridization include the difficult hybridization of low abundant microorganisms with probes (Moter and Gobel 2000), the quick decrease of emitted signals during photoexcitation (Liu et al. 2018), and background noises in samples (Johannes et al. 2010). These disadvantages would lead to false results of microbial quantification in food fermentations. Flow cytometry shows advantages for liquid food fermentation samples. However, false positive fluorescence signals are easily generated in the presence of other solid substances (background noises) in samples (Vieira-Silva et al. 2019), and seriously interfere with quantification results.

Electrochemical biosensors can also be used to quantify specific microorganisms. They contain a bioreceptor, and a transducer transferring a biological recognition into an available electrical signal (Velusamy et al. 2010). The signals can be used to quantify targeting specific microorganisms (Deshmukh et al. 2016). Environmental factors, including pH, temperature, or mass variation, can influence transduction process (Ahmed et al. 2014). However, this method shows great potential in quantifying specific microorganisms online in food fermentations in the future.

Above discussed quantitative methods are widely applied to quantify the abundance of specific microorganisms. However, they are influenced by various disadvantages, such as amplification biases of qPCR, and background noises of fluorescence in situ hybridization and flow cytometry. The biggest problem is that these methods cannot quantify all members in the microbiota at the same time. It is still an obstacle to analyze all microbial taxa at higher taxonomic resolution such as species level involved in food fermentations at once.

Relative microbiome profiling

Relative microbiome profiling methods quantify all the microbial taxa in the community by relative abundance. We introduce here the relative microbiome profiling methods, including PCR-denaturing gradient gel electrophoresis and high-throughput sequencing (Figure 1).

PCR-denaturing gradient gel electrophoresis is used to profile the compositions of the microbiota (Muyzer, Waal, and Uitterlinden 1993). This technique can quickly quantify dominant microorganisms (Wei et al. 2021). However, contents of microorganisms can only be roughly estimated by the brightness and width of bands in the gel map. In addition, the number of DNA fragments separated by PCR-denaturing gradient gel electrophoresis is relatively small (Muyzer and Smalla 1998), a large amount of microorganisms cannot be identified by this method (Xiong et al. 2020).

Recently, advances in high-throughput sequencing, known as next-generation sequencing, enable us to study the composition and dynamics of complex microbiota (Knight et al. 2018). High-throughput sequencing includes high-throughput amplicon sequencing and shotgun metagenomic sequencing. High-throughput amplicon sequencing of marker genes provides a wealth of information about microbiota structure (Jeong et al. 2021). However, amplicon sequencing may misinterpret the microbiota structure, because sequencing short fragments, such as V3–V4 region of 16S rRNA gene, cannot always present the accurate taxonomic result. Shotgun metagenomic sequencing can improve microbial taxonomic resolution (Yulandi et al. 2020). Additionally, the third-generation sequencing for the full-length gene is

Table 2. Primers for qPCR reactions of quantification in food fermentations.

Fermented food types	Microorganisms	Target genes	Primer names	Sequences (5′-3′)	References
Fermented vegetable	*Weissella*	16S rRNA	wei472f	GAGTAACTGTTCAGTGTGTGACGG	(Liang et al. 2016b)
			wei662r	TCATCCAGTTTCCAAAGCCAT	
	Saccharomyces	35S rRNA	$F_{35S\ rRNA1}$	GCCCAGTGCTCTGAATGTC	
			$R_{35S\ rRNA1}$	GCTCAACAGGGTCTTCTTTCC	
	Escherichia	*uidC*	F_{uidC1}	GGTGCTCGTGCTGATATGAGTA	(Xiong et al. 2019)
			R_{uidC1}	GCCCAATAACCTAATGCTCCTTC	
	Lactobacillus plantarum	*tal*	F_{tal1}	AACATTTCGCGGAACTTGGTT	
			R_{tal1}	ATCATCTCTTCGGCCTTGGT	
Fermented meat	*Penicillium nordicum*	*otapks*PN	F-pkstr	CGAAGATGTCTCCACGGAAT	(Rodríguez et al. 2014)
			R-pkstr	TTGCGAGTGTCTTTGGTCAG	
	Penicillium nordicum	*otapks*	otapksF3	CGCCGCTGCGGTTACT	(Bernáldez et al. 2018)
			otapksR3	GGTAACAATCAACGCTCCCTCTT	
	Listeria monocytogenes	*gap*	gapF	ACCAGTGTAAGCGTGAA	(Mataragas et al. 2015; Tasara and Stephan 2007)
			gapR	TCACAGCGCAAGACAAA	
	Cladosporium oxysporum	*β-tubulin* gene	BPS-F	CAACGAGGTGTGAAAATCCGA	(Lozano-Ojalvo et al. 2015)
			BPS-R	AGGCCTGTGATGGGATGTGA	
	Weissella viridescens	*recN*	WvrecNF	CGCAAACACAACAAGCCTAT	(Gómez-Rojo et al. 2015)
			WvrecNR	TGTTGAGCAAGTTCCAAAGC	
	Staphylococcus spp.	*tufA*	TstaG422	GGCCGTGTTGAACGTGGTCAAATCA	(Fonseca et al. 2013; Martineau et al. 2001)
			Tstag765	TIACCATTTCAGTACCTTCTGGTAA	
	Staphylococcus equorum	*sod*A	SdAEqF	GTGGAGGACACTTAAACCATTC	(Blaiotta et al. 2004)
			SdAEqR	CAATTTACCATCGTTTACAACTAG	
	Penicillium urticae	*idh*	F-idhtrb	GGCATCCATCATCGT	(Bernáldez et al. 2013)
			R-idhtrb	CTGTTCCTCCACCCA	
Fermented dairy	*Lactobacillus delbrueckii subsp. Bulgaricus*	–[a]	F	TCAATCAAGACCCACAAAACTTTC	(Pega et al. 2018)
			R	GGAACCACCTCTCTCTAGCTGTAG	
	Lactobacillus acidophilus	*pyrG* and *recA*	La-F	GCAGGCTACCTTTACAACAC	(Ren et al. 2017)
			La-R	TCCCTAAACAAATACCCAAG	
	Lactobacillus delbrueckii		Ld-F	CCGACCCAGCAGTCAGTTTC	
			Ld-R	TCTGGTCAACGCCTTGTTCC	
	Lactobacillus paracasei		Lc-F	CGGAAGATATGAAGAAGAAA	
			Lc-R	AGTGGTATGGGTCAAATGCT	
	Lactobacillus sakei		Ls-F	GGTATCACAGATGCCACAAC	
			Ls-R	CAGCAAATACTAAGCCCTTG	
	Lactobacillus fermentum		Lf-F	ACGGTTCATTGACAACGACT	
			Lf-R	TTCATCTGGCGAATTGCTTC	
	Lactobacillus plantarum		Lp-F	AAAATCATGCGTGCGGGTAC	
			Lp-R	ATGTTGCGTTGGCTTCGTCT	
	Lactobacillus helveticus		Lh-F	TGACCGATCCGATCACTCTT	
			Lh-R	CCAGGTGGTCGTGCTCTTAA	
	Bifidobacterium spp.		Bi-F	CGGTACGGCAATCGCGATAT	
			Bi-R	TTGCGTTGATCACAGATTCA	
	Lactococcus lactis	–[a]	F	CATCGTTGATGAATACATCCCAACT	(Pega et al. 2017)
			R	CGACTGGAAGAAGGAGTGGTTT	
Fermented alcohol beverages	*Lactobacillus acetotolerans*	*transcriptional regulator* gene	Place F	AAAAAGCAGAGTGGAGAAAATACT	(Du, Wu, and Xu 2020a)
			Place R	CAATAAAAAGAGCAACAGCA	
	Lactobacillus jinshani	16S rRNA	PljinF	CGCACTCCCGTAGATGATTTTGA	(Du, Wu, and Xu 2020b)
			PljinR	TCACTACCAAGCCATTTCCTAC	
	Pediococcus pentosaceus	*recA*	PedPen3 F	CTATTGACTTGGTCGTTATTGATTCC	(Stevenson et al. 2006)
			PedPen3 R	CCCCCATCTCTCCATCAATTT	
	Weissella paramesenteroides	*yjzC*	PwarF	CTAGAGGCGGCGAAGTCAGT	(Du, Wu, and Xu 2020a)
			PwarR	CTATTCGCGTCGCCAACCAT	
	Bacillus coagulans	*comK*	P1	CTCACGGAAGAGCAAGCTTG	(Yan et al. 2018)
			P2	GTTTCTGAAATGTATGCACG	
	Acetobacter aceti	16S rRNA	F	CGGAATGACTGGGCGTAAAG	(Zhang et al. 2020)
			R	CAGTAATGAGCCAGGTTGCC	
	yeasts	26S rRNA	YEASTF	GAGTCGAGTTGTTTGGGAATGC	(Soares-Santos, Pardo, and Ferrer 2018)
			YEASTR	TCTCTTTCCAAAGTTCTTTTCATCTTT	
	Brettanomyces bruxellensis	LSU rRNA[b]	DBRUXF	GGATGGGTGCACCTGGTTTACA	
			DBRUXR	GAAGGGCCACATTCACGAACCCCG	
	Saccharomyces cerevisiae	ITS2-5.8S rRNA	CESP-F	ATCGAATTTTTGAACGCACATTG	
			SCER-R	CGCAGAGAAACCTCTCTTTGGA	
	Zygosaccharomyces bailii	26S rRNA	ZBF1	CATGGTGTTTTGCGCC	
			ZBR1	CGTCCGCCACGAAGTGGTAGA	
	Saccharomycopsis fibuligera	ITS-5.8S rRNA and *β-tubulin* gene	Sfi-F	ACTCTTTGTGGGATTCTAT	(Lv et al. 2017a)
			Sfi-R	TGTTCGCTATCGGTCT	
	Monascus purpureus		Mp-F	GTGTTATTCCCGCATCAA	
			Mp-R	CATCTGGTCCTCAACTTCC	
	Rhizopus oryzae		Ro-F	GTAGCAAAGTGCGATAA	
			Ro-R	AGCAAGCCAGACAGAA	

[a]Not mentioned in the reference.
[b]Large subunit domain of the rRNA gene.

gradually developed, to quantify the microbiota taxa effectively by relative abundance at a higher taxonomic resolution (Yang et al. 2021). Unfortunately, relative microbiome profiling is a primary estimation of the microbiota and may lead to misinterpretation of the microbiota structure and function (Rao et al. 2021).

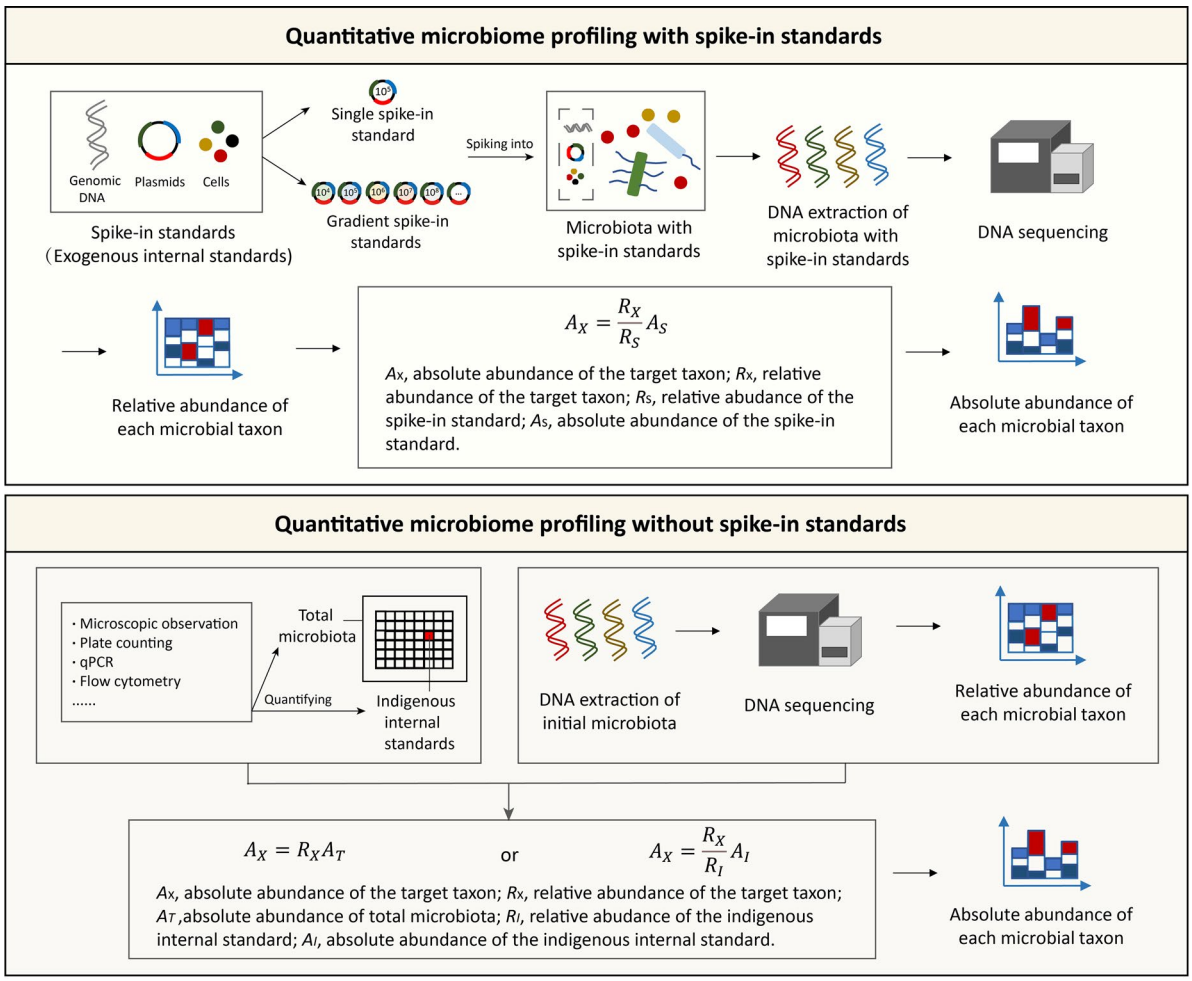
In conclusion, although these conventional microbial quantitative methods can be widely used in microbial quantification, they are far from satisfactory to reveal the real microbiota structure and function in food fermentations, that limits the ability to regulate microbiota and further control food fermentations to improve the fermentation productivity and quality.

Quantitative microbiome profiling

In contrast to conventional methods, quantitative microbiome profiling, as an advanced microbial quantitative method (Figure 1), quantifies all microbial taxa with absolute abundance. The quantification can be achieved by combining high-throughput sequencing results with the abundance of spike-in standards, total microbiota or indigenous internal standards. Figure 2 shows the workflow of quantitative microbiome profiling. Here, we will introduce them in detail.

Quantitative microbiome profiling with spike-in standards

Spike-in standards, also known as exogenous internal standards, are widely used for quantitative microbiome profiling (Figure 2). Spike-in standard mainly includes genomic DNA of exogenous microorganisms (Smets et al. 2016), synthetic DNA (mainly plasmids containing bacterial 16S rRNA, eukaryotic 18S rRNA, or fungi ITS sequences) (Tkacz, Hortala, and Poole 2018; Tourlousse et al. 2017), and exogenous microbial cells (bacteria, fungi, and archaea) (Rao et al. 2021; Stämmler et al. 2016; Yang et al. 2018). These spike-in standards with known amount can be added to fermented samples, then the total DNA is extracted and used for high-throughput amplicon sequencing. No matter which spike-in standard is added, the absolute quantification abundance of all microbial taxa can be converted according to formula (1) (Smets et al. 2016):



Note: (1) Spike-in standards, known as exogenous internal standards, are spiked into initial microbiota, they are not present in the initial microbiota. (2) Indigenous internal standards are specific microorganisms with a suitable concentration in the initial microbiota.

Figure 2. The workflow of quantitative microbiome profiling.

$$A_X = \frac{R_X}{R_S} A_S \quad (1)$$

Where A_X is the absolute abundance of the target taxon (initially unknown), R_X is the relative abundance of the target taxon, R_S is the relative abundance of the single spike-in standard, and A_S is the absolute abundance of the single spike-in standard (initially known).

Spike-in standards should not be present in the fermented samples, otherwise, they will interfere with the real absolute abundance of all microbial taxa (Barlow, Bogatyrev, and Ismagilov 2020). As a result, the synthetic DNA would be a better choice. The order of adding spike-in standards can also affect quantification results. Tkacz, Hortala, and Poole (2018) suggested that the most accurate results are acquired by adding spike-in standards to the sample before DNA extraction. In addition, single spike-in standard (only one concentration) may lead to the inaccurate quantitative microbiome profiling result (Wang et al. 2021a), because diverse microbial taxa cover an extended range of concentrations in the sample (Vandeputte et al. 2017). Therefore, gradient spike-in standards are later developed to improve the accuracy of quantitative microbiome profiling. For example, 9 spike-in standards with at least 4 concentrations (10^3, 10^4, 10^5, and 10^6 of copies) are spiked into one sample (Jiang et al. 2019). Additionally, gradient spike-in standard concentration groups are also simultaneously added to Chinese liquor fermentation samples, that can quantify microbiota with different orders of magnitude (Wang et al. 2021a).

In conclusion, quantitative microbiome profiling can be realized by single or gradient spike-in standards efficiently. However, this method is required to check the inexistence of added spike-in standards in fermented samples. It is also required to perform control assays to check the influence of spike-in standards on the microbiota structure. Moreover, we need to optimize the range of spike-in standard concentrations because of a wide range of concentrations in diverse microbial taxa. These requirements increased the complexity of quantitative microbiome profiling method (Du, Wu, and Xu 2020a). Therefore, it is important to develop more accurate and efficient methods without spike-in standards to obtain quantitative microbiome profiling results in food fermentations.

Quantitative microbiome profiling without spike-in standards

Alternative quantitative microbiome profiling methods are then developed to get rid of spike-in standards, namely by quantifying total microbiota or indigenous internal standard. We can determine the abundance of total microbiota or indigenous internal standard by the first or second groups of conventional microbial quantitative methods. Then, quantitative microbiome profiling can also be converted from relative microbiome profiling and the abundance of total microbiota or indigenous internal standard.

For the method based on the abundance of total microbiota, quantitative microbiome profiling can be obtained by multiplying the total microbial abundance by relative abundance of each corresponding taxon (Figure 2), according to formula (2) (Yao et al. 2022):

$$A_X = R_X A_T \quad (2)$$

Where A_X is the absolute abundance of the target taxon, R_X is the relative abundance of the target taxon, and A_T is the absolute abundance of total microbiota. However, biases like universal primers of qPCR assays affect the accuracy of total microbial abundance. Therefore, quantitative microbiome profiling analysis based on total microbial abundance still has deficiencies and needs to be improved.

Currently, indigenous internal standards are increasingly adopted to quantify all microbial taxa in food fermentations. Indigenous internal standards include specific microorganisms with a suitable concentration in the microbiota (Wu et al. 2020). The abundance of indigenous internal standards can also be quantified by conventional microbial quantitative methods such as qPCR, or flow cytometry. The absolute quantification abundance of all microbial taxa can be directly obtained by calculating the absolute abundance of indigenous internal standards and the relative abundance of each taxon according to formula (3) (Du, Wu, and Xu 2020a):

$$A_X = \frac{R_X}{R_I} A_I \quad (3)$$

Where A_X is the absolute abundance of the target taxon, R_X is the relative abundance of the target taxon, R_I is the relative abundance of the indigenous internal standard, and A_I is the absolute abundance of the indigenous internal standard. Fewer procedures are needed in quantitative microbiome profiling based on an indigenous internal standard (Du, Wu, and Xu 2020a). Recently, Du, Wu, and Xu (2020a) screened indigenous internal standards including *Lactobacillus jinshani* and *Lactobacillus acetotolerans* to standardize the high-throughput amplicon sequencing result. This method avoids checking the added concentration and control experiments of spike-in standards. However, similar to quantifying total microbiota, the accuracy is also influenced by biases from qPCR assays or background noises from flow cytometry assays. Therefore, it is important to develop more innovative methods such as amplification-free and contamination-free methods to improve the accuracy. Additionally, it would be a good option to choose one optimal internal standard or a series of multiple indigenous internal standards with different order of magnitudes of abundance to calibrate the quantification result.

In conclusion, quantitative microbiome profiling can quantify all microbial taxa easily by estimating the abundance of spike-in standards, total microbiota or indigenous internal standards. Quantitative microbiome profiling is until now the best and ultimate method to understand microbiota structure, and to further realize rational and optimal control of food fermentations. However, disadvantages still exist in quantitative microbiome profiling. Consequently, it still

deserves efforts to overcome drawbacks such as amplification biases by qPCR or other interference factors. Quantitative microbiome profiling provides numerous important new insights into the microbiota structure, and its applications in food fermentations are gradually increasing.

Application of microbial quantitative methods in food fermentations

At present, microbial quantitative methods enable us to understand the abundance of total microbial load or specific microbial taxa in various food fermentations. Table 3 gives an overview of application of microbial quantitative methods used in food fermentations. Here we address these applications in more detail.

Fermented vegetable

Fermented vegetables (known as pickles), produced by semi-solid state fermentation, include fermented bamboo shoots, fermented radish, fermented cabbage, and fermented cucumber (Liu and Tong 2017). The microbiota in vegetable fermentation mainly include bacteria (lactic acid bacteria, Micrococcaceae, Bacilli), yeasts, and filamentous fungi (Behera et al. 2020).

In the past, plate counting is a common method to quantify bacterial abundance in fermented vegetables (Pérez-Díaz et al. 2019). For example, aerobic bacterial counts and lactic acid bacterial counts are 7.08 lg CFU/g and 6.40 lg CFU/g in finished fermented vegetables observed by plate counting, respectively (Kang et al. 2019). Phospholipid fatty acid analysis reveals contents of both Gram-positive and Gram-negative microorganisms during the production of Chinese sauerkraut (Wu et al. 2014b).

The absolute abundances of specific microorganisms are also determined in vegetable fermentations. For example, qPCR analysis reveals that the abundances of *Lactobacillus* and *Debaryomyces* range from 9.00 lg to 12.00 lg copies/mL and 6.00 lg to 10.00 lg copies/mL, respectively, during the fermentation of industrialized *Qingcai paocai* (a sort of pickle made of cabbage) (Liang et al. 2018), and the abundance of lactic acid bacteria ranges from 7.00 lg to 9.00 lg copies/mL in industrially matured Chinese *paocai* (a sort of pickle) of different factories (Liang et al. 2016a).

Recently, high-throughput sequencing is used to quantify microbiota by relative abundance in the vegetable fermentation (Liu et al. 2019b). For example, high-throughput sequencing analysis shows that Lactobacilli accounts up to 77.60% in 3 major types of traditional Chinese fermented vegetables (Xiao et al. 2020), *L. acetotolerans*, *Pichia kudriavzevii*, *Pichia norvegensis*, *Debaryomyces hansenii*, *Kazachstania exigua,* and *Kazachstania humilis* are dominant (average relative abundance > 1%) in Suansun (a sort of fermented bamboo shoot), and *Lactobacillus delbrueckii*, *Lactobacillus fermentum*, *Lactobacillus aviarius*, *P. kudriavzevii*, and *D. hansenii* are dominant in Suancai (a sort of pickle) (Guan et al. 2020). It determines the relative abundance of the microbiota involved in fermented pickles with different containers, and shows that container materials affect the abundance of specific genera, *Lactococcus* and *Pediococcus* (Liu et al. 2020b).

Fermented meat

Fermented sausage, as a representative fermented meat product, is produced by high temperature drying and natural fermentation (Wang, Jiang, and LiN 1995). Many studies quantify the microbiota in fermented sausages. Plate counting is originally used to quantify aerobic and specific microorganisms such as *Lactobacillus* and *Staphylococcus* during sausage fermentation (Settanni et al. 2020). qPCR is used to quantify the absolute abundance of species *Weissella viridescens* in blood sausages (Gómez-Rojo et al. 2015), *Cladosporium oxysporum* related to the black spot formation in sausages (Lozano-Ojalvo et al. 2015), *Listeria monocytogenes* in fermented sausages (Rantsiou et al. 2008), *Mycobacterium avium* subspecies in meat product salami (Klanicova et al. 2011), *Staphylococcus* spp. and *Staphylococcus equorum* during the ripening of Spanish sausage (Fonseca et al. 2013).

PCR-denaturing gradient gel electrophoresis technique is used to acquire microbial relative abundance in fermented sausages (Cocolin et al. 2001). For example, it is adopted to study the bacterial diversity in Sichuan-style sausage fermentation, and reveals that *Weissella* and *Lactobacillus* are dominant in the later stage (Wang et al. 2021b). High-throughput sequencing is used later to obtain better relative microbiome profiling results. For example, Firmicutes, Cyanophyta, Proteobacteria are determined to be dominant bacterial phylum, and they account for 20.20 to 78.39%, 13.13 to 58.16%, and 7.14 to 28.04% in various traditional fermented sausages, respectively (Huang et al. 2021). Bacterial communities are compared in different sausages by high-throughput sequencing. In salami the relative abundance of the genus *Staphylococcus* reaches 97.45%, and in Chinese smoked-cured sausage the relative abundance of *Weissella* spp. reaches 25.32% and *Pediococcus* spp. reaches 16.67% (Wang et al. 2018b).

Fermented dairy

Yogurt

Yogurt is a coagulated milk product resulting from the solid-state fermentation of lactose in milk by lactic acid bacteria (Moh, Etienne, and Jules-Roger 2021). In early studies, microscopic observation and plate counting are main quantitative methods for yogurt, and they are used to enumerate characteristic microorganisms such as *Streptococcus thermophilus* and *Lactobacillus bulgaricus* (Bracquart 1981). Later, the ATP assay method is developed for rapid determination of microbial counts in yogurt. After 8 hours of Lauryl Sulfate Tryptose broth with sodium deoxycholate cultivation, the content of coliforms is counted from 10 to 100 CFU/g in yogurt (Takahashi et al. 2018).

Table 3. Application of microbial quantitative methods in food fermentations.

Fermented food samples	Microbial quantitative methods	Targets of quantification	Results of microbial quantification	References
Fermented vegetables				
Finished fermented vegetable	Plate counting	Aerobic bacteria	7.08 lg CFU/g[a]	(Kang et al. 2019)
		Lactic acid bacteria	6.40 lg CFU/g[a]	
Brine of radish during Chinese sauerkraut manufacture	Phospholipid fatty acid analysis	Bacteria	–[b]	(Wu et al. 2014b)
		Fungi	–[b]	
		Total microbiota	–[b]	
Qingcai paocai (pickle) fermentation	qPCR	*Lactobacillus*	9.00 lg to 12.00 lg copies/mL[a]	(Liang et al. 2018)
		Debaryomyces	6.00 lg to 10.00 lg copies/mL[a]	
Matured Chinese *paocai* (pickle)	qPCR	Lactic acid bacteria	7.00 lg to 9.00 lg copies/mL[a]	(Liang et al. 2016a)
Chinese fermented vegetable	High-throughput sequencing	Lactobacilli	77.60%	(Xiao et al. 2020)
Suansun (fermented bamboo shoot)	High-throughput sequencing	*Lactobacillus acetotolerans*	56.08%	(Guan et al. 2020)
		Serratia marcescens	8.04%	
		Lactobacillus sakei	2.21%	
Suancai (pickle)	High-throughput sequencing	*Lactobacillus delbrueckii*	18.92%	
		Lactobacillus delbrueckii	17.93%	
		Lactobacillus aviarius	16.79%	
Fermented meat				
Fermented sausage (fermented beef, horse, wild boar, and pork salamis) after ripening	Plate counting	Mesophilic rod lactic acid bacteria	7.08 lg to 7.77 lg CFU/g	(Settanni et al. 2020)
		Yeasts	5.39 lg to 6.24 lg CFU/g	
		Staphylococci	5.14 lg to 5.93 lg CFU/g	
Dry-cured fermented sausage 'salchichón' after ripening	qPCR	*Cladosporium oxysporum*	7.09 ± 0.07 lg CFU/cm^2	(Lozano-Ojalvo et al. 2015)
Salami	qPCR	*Mycobacterium avium* subsp. *hominissuis*	2.24 lg copies/g[a]	(Klanicova et al. 2011)
Dry fermented Spanish sausage	qPCR	*Staphylococcus* spp.	5.28 lg CFU/g	(Fonseca et al. 2013)
		Staphylococcus equorum	2.87 lg CFU/g	
Fermented sausage	High-throughput sequencing	Firmicutes	20.20 to 78.39%	(Huang et al. 2021)
		Cyanophyta	13.13 to 58.16%	
		Proteobacteria	7.14 to 28.04%	
Salami	High-throughput sequencing	*Staphylococcus*	97.45%	(Wang et al. 2018b)
Chinese smoked-cured sausage		*Weissella* spp.	25.32%	
		Pediococcus spp.	16.67%	
		Lactobacillus spp.	7.93%	
Fermented dairy products				
Natural yogurt	Plate counting	*Streptococcus thermophilus*	8.28 lg to 8.63 lg CFU/mL[a]	(Bracquart 1981)
		Lactobacillus bulgaricus	8.07 lg to 8.60 lg CFU/mL[a]	
Yogurt	ATP	Coliforms	10 to 100 CFU/g	(Takahashi et al. 2018)
Probiotic foods and yoghurt	qPCR	*Lactobacillus* spp.	6.00 lg to 7.00 lg copies/g[a]	(Angelakis et al. 2011)
Yogurt starter culture	qPCR	*Streptococcus thermophilus* DGCC7796	–[b]	(Miller, Dudley, and Roberts 2012)
		Streptococcus thermophilus DGCC7710	–[b]	
		Lactobacillus delbrueckii ssp. *bulgaricus* DGCC4078	–[b]	
		Lactobacillus delbrueckii ssp. *lactis* DGCC4550	–[b]	
Yogurt	flow cytometry	Lactic acid bacteria	8.90 lg to 9.43 lg cells/mL[a]	(He et al. 2017)
Yogurt	High-throughput sequencing	Firmicutes	99.60%	(Zhi et al. 2016)
		Streptococcus	87.10%	
		Lactobacillus	10.30%	
		Lactococcus	0.30%	
Yogurt from Korea	High-throughput sequencing	*Streptococcus thermophilus*	67.98%	(Suh and Kim 2021)
		Lactobacillus delbrueckii	0.12%	
Kefir	Plate counting	*Lactobacillus sp.*	5.00 lg to 6.00 lg CFU/g[a]	(Lee et al. 2018)
		Lactococcus sp.	5.00 lg to 6.00 lg CFU/g[a]	
Kefir	qPCR	*Lactobacillus kefiri*	5.63 lg CFU/mL	(Kim et al. 2016)
Kefir grains from Germany, Turkey, Korea, and UK	High-throughput sequencing	*Acetobacter*, *Lactobacillus*, *Lactococcus*, and *Leuconostoc* (together)	>95.00%	(Blasche et al. 2021)
Kefir fermentation	Quantitative microbiome profiling	All bacteria and yeasts	–[b]	
White cheese vats after cleaning procedures of processing lines	Plate counting	*Bacillus spp.*	3.78 lg CFU/100 cm^2	(Ipek and Zorba 2018)
Parmigiano Reggiano cheese fermentation	qPCR	*Lactobacillus helveticus*	6.41 ± 0.20 lg copies/mL	(Bertani et al. 2020)
		Lactobacillus delbrueckii ssp. *lactis*	6.98 ± 0.42 lg copies/mL	

(Continued)

Table 3. (continued).

Fermented food samples	Microbial quantitative methods	Targets of quantification	Results of microbial quantification	References
Fresh cheese	qPCR	*Listeria monocytogenes*	3.60 lg CFU/g[a]	(Rantsiou et al. 2008)
Edam cheese	High-throughput sequencing	*Lactococcus*	43.78% (autumn); 53.35% (spring)	(Nalepa, Ciesielski, and Aljewicz 2020)
Fermented alcohol beverages				
Daqu (starter) for Chinese liquor fermentation	Plate counting	Total bacteria	5.00 lg to 6.00 lg CFU/g[a]	(Hu et al. 2017)
Pit mud for Chinese liquor fermentation	Phospholipid fatty acid analysis	Total microbiota	25.52 to 103.38 nmol/g	(Ding et al. 2015)
	PCR-denaturing gradient gel electrophoresis and high-throughput sequencing	Clostridiaceae	44.67%	
Stacking and liquor fermentations for Sesame-flavor Chinese liquor	qPCR	*Bacillus*	6.24 lg to 6.61 lg copies/g	(Shen et al. 2020)
Sauce-flavor Chinese liquor fermentation	High-throughput sequencing	*Virgibacillus* *Kroppenstedtia* *Bacillus*	25.43% 13.97% 22.12%	(Zhang et al. 2021)
Chinese liquor fermentation	Quantitative microbiome profiling	All bacteria and fungi	–[b]	(Wang et al. 2021a)
Chinese liquor fermentation	Quantitative microbiome profiling	All bacteria and fungi	–[b]	(Du, Wu, and Xu 2020a)
Wine fermentation	Microscopic observation	Yeasts	~ 8.00 lg CFU/mL[a,b]	(Andorrà et al. 2012)
Touriga Nacional and Cabernet Sauvignon wines	Plate counting	Yeasts	4.00 lg to 6.00lg CFU/mL[a]	(Nunes de Lima et al. 2021)
Red Wine	qPCR	*Brettanomyces bruxellensis*	1.00 lg to 4.00 lg CFU/mL[a]	(Tofalo et al. 2012)
Fermented Wine	Flow cytometry	*Oenococcus oeni*	9.08 lg to 9.50 lg cells/mL[a]	(Bartle, Mitchell, and Paterson 2021)
Red and white wine fermentation	High-throughput sequencing	*Tatumella*	26.00 ± 3.00%	(Bubeck et al. 2020)
Other fermented foods				
Vinegar	Microscopic observation	Total bacteria	–[b]	(Mesa et al. 2003)
Vinegar fermentation	qPCR	Acetic acid bacteria	8.82 lg copies/g	(Li et al. 2016)
Fumigated vinegar fermentation	High-throughput sequencing	*Lactobacillus* and *Acetobacter* (together)	>90.00%	(Yun et al. 2019)
Sourdough fermentation	Plate counting	Lactic acid bacteria	6.00 lg CFU/g[a]	(Settanni et al. 2013)
Sourdough fermentation	qPCR	*Lactobacillus sanfranciscensis*	–[b]	(Baek et al. 2021)
Chinese sourdough	High-throughput sequencing	*Pediococcus pentosaceus*	58.00%	(Xing et al. 2020)

[a]Data is converted by this review.
[b]No definite numerical values in the reference.

Moreover, a qPCR method is developed to quantify *Lactobacillus* spp. by designing species-specific primers to count 6.00 lg to 7.00 lg copies/g of *Lactobacillus* spp. in 13 probiotic foods and yogurt (Angelakis et al. 2011). By designing strain-specific primers, dynamics of *S. thermophilus* spp. (DGCC7796, DGCC7710), *L. bulgaricus* spp. (DGCC4078), and *L. delbrueckii* ssp. lactis strain (DGCC4550) can be quantified in a commercial yogurt starter culture (Miller, Dudley, and Roberts 2012). High-sensitivity flow cytometry is used to rapidly quantify live lactic acid bacteria in yogurt. Viable lactic acid bacteria counts measured by flow cytometry agree well with those by plate counting method (He et al. 2017).

Further, the relative microbiome profiling method is developed to quantify different microbial taxonomy in yogurt (Gong et al. 2020). High-throughput amplicon sequencing analysis shows that the percentage of *Streptococcus* reaches 87.10%, *Lactobacillus* 10.30%, and *Lactococcus* 0.30% in yogurt (Zhi et al. 2016). Metagenomic sequencing analysis reveals that *S. thermophilus* is the dominant bacterium (67.98%) in commercial yogurts from Korea (Suh and Kim 2021).

Kefir

Kefir is produced by milk fermentation with kefir grains (a starter) containing lactic acid bacteria, acetic acid bacteria, and yeasts (Kim et al. 2015). Recently, the plate counting method is applied to quantify microorganisms in a mixed-starter culture for kefir fermentation. The result shows that the initial viable cell counts of *Lactobacillus* sp. and *Lactococcus* sp. both range from 5.00 lg to 6.00 lg CFU/g (Lee et al. 2018). Further, many other methods are developed to quantify the abundance of microorganisms in kefir grains and kefir more effectively and rapidly. For example, the qPCR method is used to quantify functional microorganisms by absolute abundance in kefir (Nejati et al. 2020; Wang et al. 2018a). A qPCR primer-set is designed to quantify *Lactobacillus kefiri*, and the abundance of 5.63 lg CFU/mL is observed in kefir fermented for 48 hours (Kim et al. 2016). The PCR-denaturing gradient gel electrophoresis technique and high-throughput sequencing are used to quantify the microbiota by relative abundance in kefir grains and kefir fermentation (Chen, Wang, and Chen 2008). For example, *Lactobacillus kefiranofaciens*

and *L. kefiri* are the dominant bacteria, and *Saccharomyces cerevisiae* is the dominant yeast in three kefir grains from Brazil (Leite et al. 2012). Additionally, quantitative microbiome profiling analysis is used in kefir fermentation. The relative microbiome profiling is converted to quantitative microbiome profiling results by integrating total DNA abundance. This method reveals the dynamics of different Lactobacilli species during kefir fermentation (Blasche et al. 2021).

Cheese

Cheese is a milk product by solid-state fermentation with bacteria and fungi (Walsh et al. 2020). The abundance of the microbiota in cheese fermentation is an important parameter to influence the quality of cheese. Conventional plate counting and direct microscopic enumeration are initial and common quantitative methods for *Lactobacillus paracasei* and *Bifidobacterium* sp. in cheese (Auty et al. 2001; Haque, Kucukoner, and Aryana 1997). Plate counting method is used to determine microbial abundance in white cheese processing before and after cleaning procedure (Ipek and Zorba 2018).

Recently, quantitative methods targeting specific microorganisms are applied in cheese making. An example is to determine the absolute abundance of bacterial species by qPCR in Parmigiano Reggiano cheese making process. The mean abundance of *Lactobacillus helveticus* reaches 6.41 ± 0.20, *L. delbrueckii* ssp. *lactis* 6.98 ± 0.42, *S. thermophilus* 5.55 ± 0.98 and *L. fermentum* 3.33 ± 0.98 lg copies/mL in conventional and organic production lines (Bertani et al. 2020). Viable *Salmonella typhimurium*, inoculated in coalho cheese, can be quantified by qPCR with ethidium bromide monoazide, and as low as 10 CFU/10 g viable *S. typhimurium* cells can be detected (Monteiro de Mendonca et al. 2019). Currently, high-throughput qPCR can provide a rapid result in a way of microfluidics. For example, a rapid microbial quantification for 24 species/subspecies by qPCR is established in cheese samples with good specificity and efficiency (Dreier et al. 2021).

With the development of high-throughput sequencing, relative abundances of microbiota taxa are revealed in various cheeses (Dugat-Bony et al. 2016; Murugesan et al. 2018) and their fermentations (Nam et al. 2021). For example, high-throughput sequencing shows that *Lactobacillus rhamnosus*, *L. kefiri*, *L. kefiranofaciens*, *Lactobacillus casei*, *S. thermophilus*, and *Bifidobacterium* have the highest relative abundances in Edam cheeses (Nalepa, Ciesielski, and Aljewicz 2020).

Fermented alcohol beverages

Chinese liquor

Chinese liquor (called *Baijiu* in Chinese), is a popular fermented alcoholic beverage in China (Jin, Zhu, and Xu 2017; Yang, Fan, and Xu 2020). It is produced by a spontaneous solid-state fermentation by microbiota including filamentous fungi, yeasts, and bacteria (Wu et al. 2021). Plate counting is a common method to quantify microorganisms in Chinese liquor fermentation. It is applied to enumerate viable cell counts of total bacteria, *Bacillus* spores, yeasts, and molds in the starter (Hu et al. 2017) and *Bacillus licheniformis* and *S. cerevisiae* in Chinese liquor fermentation (Meng et al. 2015). Phospholipid fatty acid analysis is used to explore the fungal biomass in three finished *Daqu* (a starter or Koji for Chinese liquor fermentation) (Wu et al. 2014a). It is also combined with PCR-denaturing gradient gel electrophoresis to characterize microbiota profiling in Chinese liquor fermentation. By phospholipid fatty acid analysis, the total microbial biomass is determined ranging from 25.52 to 103.38 nmol/g in the pit (a sort of fermentor) mud and from 29.96 to 64.50 nmol/g in fermented grains. By PCR-denaturing gradient gel electrophoresis analysis, *Clostridiaceae*, *Lactobacillaceae*, *Methanoculleus*, and *Pichia* are identified as predominant (average relative abundance > 10%) microorganisms in pit mud and fermented grains (Ding et al. 2015). The qPCR method is also used in Chinese liquor fermentation. For example, the absolute abundance of *Bacillus* is determined ranging from 6.24 lg to 6.61 lg copies/g after inoculation during stacking and alcoholic fermentation (Shen et al. 2020). With the development of high-throughput sequencing technology, the relative abundance of microbiota is revealed in Chinese liquor fermentation (Wang et al. 2019). In a recent study, *Virgibacillus* (25.43%), *Bacillus* (22.12%), *Oceanobacillus* (16.57%), and *Kroppenstedtia* (13.97%) are characterized to be dominant in the initial stacking fermentation of sauce-flavor Chinese liquor (Zhang et al. 2021).

Quantitative microbiome profiling based on exogenous internal standards is used to reveal the absolute abundance of all microbiota taxa in Chinese liquor fermentation (Wang et al. 2021a). It reveals that the absolute abundance of *Lactobacillus* reaches its maximum after 7 days of fermentation, different from that after 20 days by relative microbiome profiling. Furthermore, quantitative microbiome profiling with indigenous internal standards is also used, and it identifies *Lactobacillus* as a key microorganism to produce flavor compounds (Du, Wu, and Xu 2020a). Likewise, in *Daqu* fermentation, *S. cerevisiae* is selected as an indigenous internal standard of quantitative microbiome profiling. Consequently, *Wickerhamomyces*, *Rhizopus, Aspergillus*, *Saccharomyces*, and *Pichia* are confirmed as dominant yeast genera and *Saccharomycopsis* is confirmed as a predominant genus during *Daqu* fermentation (Ban et al. 2022).

Wine

Wine, as a popular alcoholic beverage, is mainly attributed to the substantial yeasts and bacteria involved with alcohol and malate-lactic acid fermentation (Liu et al. 2017). Microscopic observation is a usual microbial quantitative method to determine counts of yeasts in wine making (Andorrà et al. 2012). Plate counting is another frequent quantitative method used to determine yeasts (Nunes de Lima et al. 2021), bacterial and lactic acid bacterial populations in wine fermentation (Fernández-Pérez, Rodríguez, and Ruiz-Larrea 2019). ATP analysis is developed to quantify at least 1000 CFU/L of bacteria and 50 CFU/L of yeasts in

artificially contaminated wine (Monica et al. 2021). Furthermore, qPCR technique is used to quantify *Brettanomyces* (Tessonnière et al. 2009; Tofalo et al. 2012), *Lactobacillus brevis*, *Lactobacillus hilgardii*, and *Lactobacillus plantarum* in Slovak red wines after fermentation (Kántor et al. 2016). Flow cytometry is used to enumerate *Oenococcus oeni* in wine fermentation (Bartle, Mitchell, and Paterson 2021). The method combining flow cytometry with fluorescence in situ hybridization is developed to quantify *Brettanomyces* in red wine (Serpaggi et al. 2010). Later, the application of high-throughput amplification sequencing is gradually increasing in wine fermentation. It reveals that *Tatumella* is the most abundant bacteria with the relative abundance of 26.00% during red and white wine fermentations (Bubeck et al. 2020). The relative abundance of *S. cerevisiae* grows from 5.45 to 80.90% during fermentation (Liu et al. 2021).

Other fermented foods

Vinegar

Vinegar, as a representative flavoring agent around the world, is usually produced by transferring sugar to ethanol, and subsequently to acetic acid (Tamang et al. 2020). Microscopic observation with fluorescence staining is an initial technique for measuring microbial biomass in vinegar fermentation. With the help of the LIVE/DEAD® *Bac*Light™ Bacterial Viability kit, both viable and total populations of acetic acid bacteria can be obtained in vinegar fermentation (Mesa et al. 2003). The qPCR assay is widely used to quantify specific microorganisms during vinegar fermentation. For example, it is used to study the absolute abundance of total bacteria, acetic acid bacteria, lactic acid bacteria, and *Bacillus* during the solid-state acetic acid fermentation of vinegar. Among them, the abundance of acetic acid bacteria increases rapidly and reaches the maximum with 8.82 lg copies/g at day 7 (Li et al. 2016).

The PCR-denaturing gradient gel electrophoresis technique is developed to detect many uncultured microorganisms by relative abundance. For example, it is used to effectively differentiate 19 acetic acid bacteria strains from traditional balsamic vinegar fermentation (Vero et al. 2006). High-throughput sequencing technology is used to quantify the microbial taxa by relative abundance in vinegar fermentation. The total abundance of *Lactobacillus* and *Acetobacter* reaches above 90.00% in Liangzhou fumigated vinegar fermentation (Yun et al. 2019).

Sourdough

Sourdough is used as a leavening agent in artisanal bread making. Sourdough comes from a solid-state fermentation including lactic acid bacteria and yeasts (Martín-Garcia, Riu-Aumatell, and López-Tamames 2021). Unlike general bread making by the dough process with baker's yeast *S. cerevisiae*, sourdough can further enhance the taste and nutrition of sourdough bread (Gänzle and Ripari 2016). During sourdough fermentation, the classical plate counting method is applied to quantify lactic acid bacteria (Settanni et al. 2013). In addition, the qPCR technique is applied to acquire absolute abundance of total microbiota or specific species. By designing universal primers of lactic acid bacteria in sourdough fermentation, the total lactic acid bacteria can be quantified (Pontonio et al. 2017). Species-specific qPCR is used to quantify *Lactobacillus sanfranciscensis*, *L. brevis*, and *Lactobacillus curvatus* in sourdough fermentation (Baek et al. 2021).

The PCR-denaturing gradient gel electrophoresis analysis is used to study the relative microbiome profiling. *L. sanfranciscensis*, *L. brevis* and *Candida humilis* are the dominant species during the fermentations of three kinds of Italian Panettone goods from sourdough (Garofalo et al. 2008). In addition, high-throughput sequencing technology is used to clarify the relative abundance of microbiota. For example, *Pediococcus pentosaceus* (58.00%) is determined to be the predominant species in Chinese traditional sourdough samples collected from three terrain conditions (mountain, plain, and basin) (Xing et al. 2020).

In conclusion, conventional microbial quantitative methods are widely adopted in various food fermentations, and provide primary insights into microbiota structure and function. However, these conventional methods are unable to reveal the real microbiota structure. Although quantitative microbiome profiling methods can reveal the real structure of the microbiota, it is so far rarely used in food fermentations, only pioneer work is reported in kefir and Chinese liquor fermentations (Blasche et al. 2021; Du, Wu, and Xu 2020a; Wang et al. 2021a). In kefir, quantitative microbiome profiling successfully uncovers the changes of microbiota composition in kefir grain and kefir fermentation (Blasche et al. 2021). In Chinese liquor fermentations, quantitative microbiome profiling confirms the misinterpretation from relative microbiome profiling (Wang et al. 2021a), and identifies *Lactobacillus* as a dominant genus in *Daqu* fermentation (Ban et al. 2022) and a key producer of flavor compounds (Du, Wu, and Xu 2020a). Consequently, these applications of quantitative microbiome profiling provide more accurate microbial targets and achieve better results in regulating fermentations to improve quality of fermented foods. It is urgent and necessary to expand the application of quantitative microbiome profiling into more food fermentations.

Challenges and perspectives of quantitative microbiome profiling

Although quantitative microbiome profiling methods have a great superiority in uncovering microbiota structure and function, its application in microbial ecosystems of food fermentations is still rather limited. To better apply this advanced method to quantify microbiota in food fermentations, some technology challenges should be overcome. Figure 3 concludes current challenges and potential solutions of quantitative microbiome profiling. Meanwhile, Figure 4 shows a roadmap of application perspectives of quantitative microbiome profiling in food fermentations. With these innovative directions, it will come true to solve problems in quantitative microbiome profiling and realize the ultimate

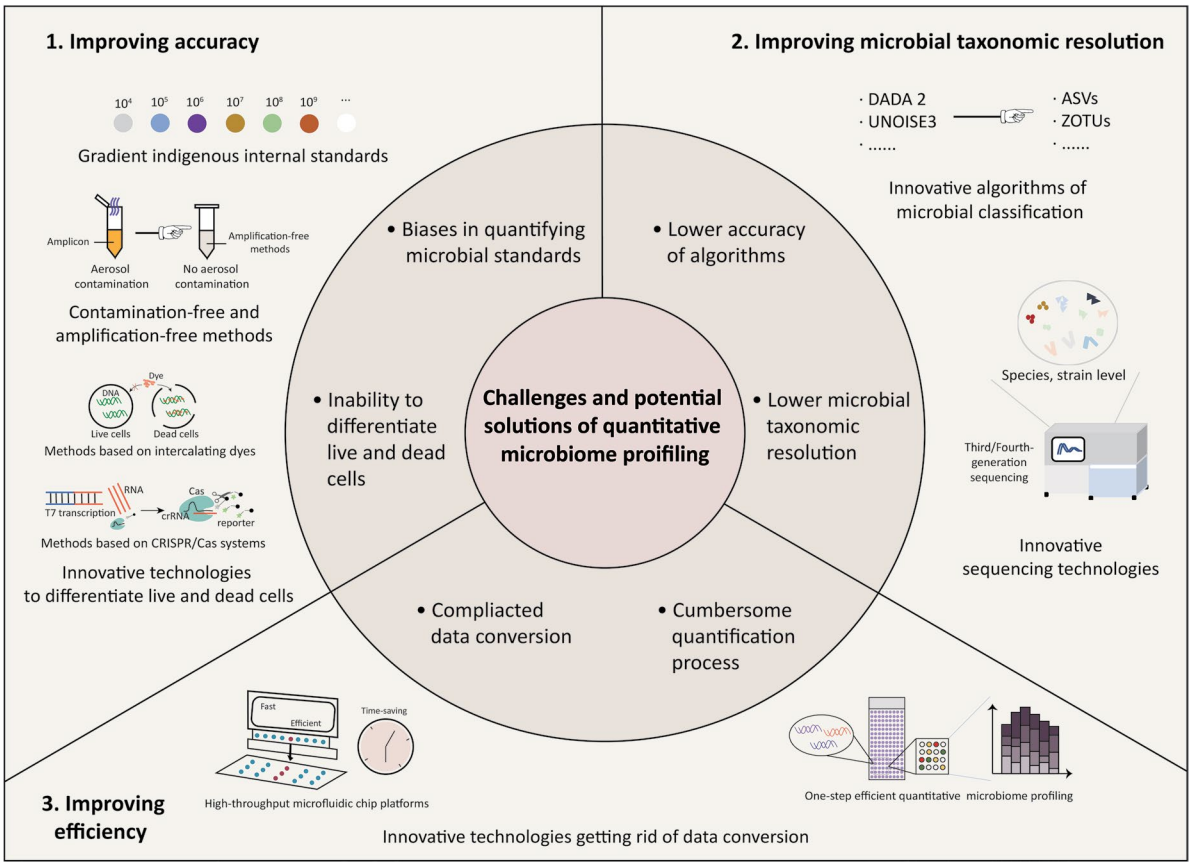


Figure 3. Challenges and potential solutions of quantitative microbiome profiling in food fermentations.

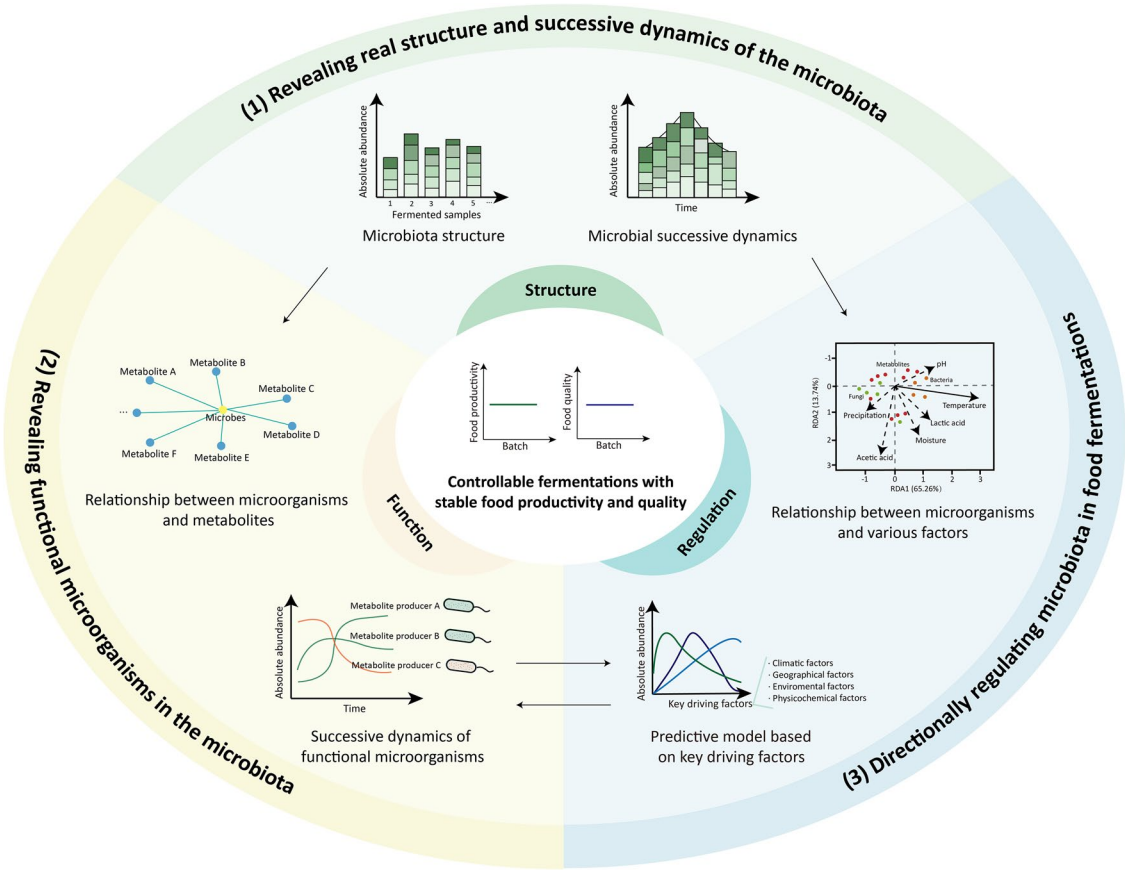


Figure 4. Application potential of quantitative microbiome profiling in food fermentations.

goal of rational and optimal control of spontaneous food fermentations in the near future.

Challenges of quantitative microbiome profiling

(1) Improving the accuracy of quantitative microbiome profiling

The quantitative microbiome profiling is calculated from relative microbiome profiling and the abundance of spike-in standards, total microbiota or indigenous internal standards. The quantification of microbial standards could be error-prone. For the method with spike-in standards, the quantitative microbiome profiling result would be affected by both the DNA loss in the extraction of spike-in standards and the concentration level of spike-in standards. Gradient spike-in standards with optimal concentrations can calibrate errors and further improve the accuracy of quantitative microbiome profiling. For the method based on total microbiota or indigenous internal standards, the quantitative microbiome profiling result would also be influenced by the disadvantages of different quantification methods, such as amplification biases of qPCR, background noises of flow cytometry assays. Therefore, it is necessary to further develop more amplification-free and contamination-free methods to avoid these problems, and to combine multiple microbial quantification methods to quantify the total microbiota or internal standard abundance. Furthermore, for the method based on indigenous internal standard, selecting optimal gradient indigenous internal standards would help calibrate the quantification result, and further improve the accuracy of quantitative microbiome profiling in food fermentations.

In addition, current quantitative microbiome profiling methods mainly rely on extracted DNA sequences. However, these methods are unable to differentiate live and dead cells. Therefore, it is of great importance to exclude the influence of dead cells on the quantification of viable microbiota. Propidium monoazide can be introduced into conventional qPCR amplification to reduce the interference of dead cells. Furthermore, clustered regularly interspaced palindromic repeats (CRISPR)/CRISPR-associated protein 13a (Cas13a) system has been widely used to recognize RNA due to its specific target-activated *trans*-cleavage ability (Xue et al. 2022). Live cells can be identified and detected with CRISPR/Cas13a systems, because RNA sequences degrade quickly in dead cells. Recently, a method based on CRISPR/Cas13a and RNA sequence-based amplification is developed to quantify viable *Salmonella enterica* (Xue et al. 2022). As a result, more innovative approaches, such as amplification-free methods and methods based on novel CRISPR/Cas systems, should be developed to reduce the bias and interference of dead cells to improve the accuracy of quantitative microbiome profiling analysis in food fermentations.

(2) Improving the microbial taxonomic resolution of quantitative microbiome profiling

Absolute quantification of the microbiota with a higher taxonomic resolution could improve our understanding of the key microbial taxa and the variation. This higher microbial taxonomic resolution analysis will stimulate to characterize microbial diversity and dynamics. However, quantitative microbiome profiling based on high-throughput amplicon sequencing by operational taxonomic units (OTUs) classification, can only quantify microbial taxa at phylum or genus level. Therefore, it is essential to improve the microbial taxonomic resolution of quantitative microbiome profiling. Recently, some strategies are proposed to improve taxonomic resolution. For example, an algorithm named divisive amplicon denoising algorithm 2 (DADA 2) is developed to classify reads into amplicon sequence variants (ASVs) at 100% similarity level (Callahan et al. 2016). Another algorithm named UNOISE3 is also developed to cluster reads into zero-radius operational taxonomic units (ZOTUs) at 100% sequence similarity (Edgar 2016). Additionally, third-generation sequencing technology, sequencing the full-length 16S rRNA gene, can quantify the microbial taxa at species level (Johnson et al. 2019). Moreover, a metagenomic binning method is developed to bin DNA at strain level (Ma, Xiao, and Xing 2020). Furthermore, a novel sequencing technology classifies microbiota at the single-cell level by designing cellular barcodes (Jin et al. 2022). These methods can improve the microbial taxonomic resolution from genus to species, or strain level of quantitative microbiome profiling in food fermentations. In the future, it is expected to adopt more advanced algorithms and sequencing technologies to improve microbial taxonomic resolution in quantitative microbiome profiling, and to further illustrate the roles of key microbial species or even strains in food fermentations.

(3) Improving the efficiency of quantitative microbiome profiling

Current quantitative microbiome profiling methods are mainly based on the data conversion from high-throughput amplicon sequencing results with the abundance of standards. The quantification process is cumbersome, and more innovate methods are urgently needed to get rid of the amplicon sequencing and conversion process. Recently, high-throughput microfluidic chip platforms are developed to improve the efficiency of microbial quantification and scale up multi-targets analysis (Ackerman et al. 2020). For example, a one-step detection method based on a high-throughput microfluidic chip can quantify 32 microbial targets rapidly, that is highly automatic and convenient (Xiang et al. 2022). Another microfluidic platform called microfluidic Combinatorial Arrayed Reactions for Multiplexed Evaluation of Nucleic acids (mCARMEN) can differentiate and quantify multiple viruses simultaneously (Welch et al. 2022), and can be used in microbial quantification in food fermentations. Besides, a microfluidic chip combing with real-time cellular recognition can isolate single cells in a high throughput and efficiency way (Wang et al. 2021c). Although microfluidic chips cannot quantify all microbial taxa at once, it shows the benefits of high-throughput. In the future, it is anticipated to develop more innovative technologies based on existing multiplex analysis strategies. These innovative technologies would get rid of the present amplicon sequencing to obtain one-step efficient quantitative microbiome profiling.

Perspectives of quantitative microbiome profiling in food fermentations

At present, quantitative microbiome profiling is applied to assess the actual microbiota profile and it reveals functional microorganisms in different microbial ecosystems (Galazzo et al. 2020; Lin et al. 2019; Tkacz, Hortala, and Poole 2018). This method reveals the real microbial genera related with disease in gut microbiota (Vandeputte et al. 2017), assesses actual marine microbiota profiles and identifies the key metabolite producers (Lin et al. 2019), measures actual microbiota composition in different soils (Tkacz, Hortala, and Poole 2018), and identifies functional microorganisms to construct synthetic microbiota to improve crop productivity (Chang et al. 2017). Quantitative microbiome profiling is the ultimate and best method so far to assess the actual complex microbiota structure. The method can help understand the relationship between microbial interactions and their metabolic potential. Spontaneous food fermentations involve a rather complex microbiota. We cannot fully understand the actual microbiota structure and function by relying solely on conventional quantitative methods, and thus cannot efficiently control the fermentation process. Therefore, we should explore the further application of quantitative microbiome profiling in food fermentations with complex microbiota. Here, we propose the prospective application of quantitative microbiome profiling in food fermentations, as shown in Figure 4.

(1) Revealing real structure and successive dynamics of the microbiota in food fermentations

Quantitative microbiome profiling analysis provides the absolute abundances of microorganisms in the microbiota. It provides a more accurate insight into different species or strains among different fermented samples. It can reveal the real differential microorganisms in fermentations with different environmental factors, and identify the real microorganisms influenced by these environmental factors, such as seasonal factors, physicochemical factors or other interference factors. For example, it can identify the real differential microorganisms in fermentations with different raw materials, and reveal the effect of raw materials on the microbiota (Du et al. 2019; Liu et al. 2019a). It would also reflect the real successive dynamics of each taxon in the microbiota, reveal active taxa and their active stages in food fermentations. For example, *Saccharomyces* is identified as an active microbial taxon and its maximal absolute abundance was on day 7 during Chinese liquor fermentation (Wang et al. 2021a). As a result, quantitative microbiome profiling shows great benefits to reveal real structure and successive dynamics of the microbiota, that is critical for managing food fermentations.

(2) Revealing functional microorganisms in the microbiota in food fermentations

By combining quantitative microbiome profiling with various correlation analysis methods, we can further reveal the associations of microorganisms with metabolites, such as the flavor compounds that are considered to be crucial for food quality. The metabolites associated microorganisms can be identified as potential functional microorganisms. For example, *Lactobacillus* is identified as a functional microorganism producing flavor compounds such as ethyl phenylacetate and phenylacetic acid in Chinese liquor fermentation (Du, Wu, and Xu 2020a). The associations of microorganisms with hazardous metabolites can also be analyzed to reveal the real producer of these metabolites, such as off-flavor compounds and potential toxic compounds in food fermentations. In addition, the successive dynamics of the functional microorganisms can also be revealed to reflect its active stage in the fermentation, it will be convenient to reveal the law of the contribution of functional microorganisms to the metabolites. Once the functional microorganisms related with favorable or hazardous metabolites are identified and their successive dynamics are revealed, it will help precisely targeted control functional microorganisms in food fermentations, and consequently beneficial for improving food quality and safety.

(3) Directionally regulating microbiota in food fermentations

By correlating with quantitative microbiome profiling result with various factors, including climatic factors, geographical factors, processing factors and physicochemical factors, we can further uncover key driving factors that regulate the microbial succession during food fermentations (Liang et al. 2022; Lin et al. 2022). For example, by redundancy analysis of microbiota and climatic factors (such as wind speed, sunshine duration, daily average temperature, and precipitation), key climatic factors can be evaluated and identified, and can be used to further predict the microbial variations (Wang et al. 2020). By revealing geographical factors (such as longitude and latitude, elevation, and aspect), we can identify key geographical factors and their effects on food fermentations. It would be beneficial for further assessing and selecting optimal geographical locations for specific food fermentations (Li et al. 2021). After revealing effects of key processing factors such as room temperature and humidity on key fungal genera in starter fermentation for Chinese liquor making, the population of key fungal genera can be predicted by room temperature or humidity (Ban et al. 2022). Additionally, by identifying key physicochemical factors, such as pH, temperature, and contents of glucose, lactic acid, and acetic acid, affecting food fermentations, it will be more efficient to control the microbiota via controlling these key factors in food fermentations. In addition, based on real key driving factors, more accurate predictive models for microbiota can be constructed, and the models can be used to predict and optimize the microbiota more precisely.

Collectively, studying microbiota in food fermentations from the perspective of quantitative microbiome profiling would lead to a deeper insight into the structure and function of the microbiota in food fermentations, it would provide a theoretical basis for controlling the microbiota in food fermentations, that is of great importance for regulating the productivity and quality of fermented foods.

Conclusions

Spontaneous food fermentations face increasing challenges because of food safety and food security of the spontaneous and uncontrolled process where the crucial microbiota is complex, undefined, and unknown. To guarantee stable productivity and quality of spontaneous food fermentations, comprehensive insight into the complex microbiota is the prerequisite. Conventional microbial quantitative methods have been applied for centuries but cannot meet the requirements of accurate and actual determination of functional microbiota in food fermentations. Quantitative microbiome profiling methods are successfully applied in various microbial ecosystems although still at its pioneering stage in food fermentations. With more intensive explorations, innovations and improvements, microbial quantification methods will have their application potentials in food fermentations. Once the insight into the complex microbiota of spontaneous food fermentations can be comprehensively revealed by quantitative microbiome profiling, a rational and optimal control will be feasible for food fermentation with stable productivity and quality.

Disclosure statement

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