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Absolute quantification of viable bacteria abundances in food by next-generation sequencing
Quantitative NGS of viable microbes

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ABSTRACT

Next-generation sequencing (NGS) is an important tool for taxonomical bacteria identification. Recent technological developments have led to its improvement and availability. Despite the undeniable advantages of this approach, it has several limitations and shortcomings. The usual outcome of microbiota sequencing is a relative abundance of bacterial taxa. The information about bacteria viability or enumeration is missing. However, this knowledge is crucial for many applications. In the current study, we elaborated the complete workflow for the absolute quantification of living bacteria based on 16S rRNA gene amplicon sequencing. A fluorescent PMAxx reagent penetrating a damaged cell membrane was used to discriminate between the total and viable bacterial population. Bacteria enumeration was estimated by the spike-in technique or qPCR quantification. For method optimization, twenty bacterial species were taken, and the results of the workflow were validated by widely accepted methodologies: flow cytometry, microbiological plating, and viability-qPCR. Despite the minor discrepancy between all methods used, they all showed compatible results. Finally, we tested the workflow with actual food samples and received a good correlation between the methods regarding the estimation of the number of viable bacteria. Overall, the elaborated and integrated NGS approach could be the next step in perceiving a holistic picture of a sample microbiota.

1. Introduction

The knowledge and reliability of quantitative data on viable microbes are crucial for decision-making in many fields. Whether the microbes are pathogenic, neutral or probiotic, the quantitative information and their viable state matter the most. Currently available methods have different limitations and biases and therefore do not provide enough essential knowledge. Despite the fast development of molecular techniques, the most widely accepted methodology in food microbiology is plating. Cell plating is an affordable and simple method that describes the number of cultivable organisms in the product. However, not all bacteria are cultivable, taxonomic characterization is often missing, and it might take up to a week to obtain the results (Stewart 2012). Modern molecular methods to detect bacteria or metabolites such as next-generation sequencing (NGS), PCR or immunoassays provide more extended information about the number of specific bacteria, but they also have drawbacks (Fanning et al., 2017), (Muyzer and Ramsing 1995). ELISA and PCR methods are good choices for the detection of specific and preselected microorganisms. These techniques allow a quantitative approach and are quick, but usually sensitive only after the cultural enrichment stage and evaluate only predefined bacteria in a sample. The only method that has the potential to characterize the food microbiota in full is NGS (Mayo et al., 2014), (Jagadeesan et al., 2019). 16S rRNA gene (16S) amplicon NGS provides a relative abundance of all bacteria, but their quantification is often stalled by the large variety of 16S copy numbers in different taxonomic groups. The usage of the amplification step by PCR in library preparation creates a bias and raises the question about the specificity and threshold choice for data analysis. Thus, the development of a methodology for the quantification of NGS data will give us valuable information about the absolute abundances of all alive bacteria in a food sample.

Recent developments related to NGS and its increased availability

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make it an excellent tool for the identification of microbiological taxa. The standard amplicon sequencing pipeline gives extensive data about the relative abundances of bacteria. However, an absolute quantification is often necessary. The first mention of Quantitative Microbiome Profiling (QMP) based on absolute quantification of microbial abundances from NGS data was introduced by Vandeputte (Vandeputte et al., 2017). They applied flow cytometry (FC) for the normalization of 16S rRNA gene sequencing data to determine the bacterial load of the gut microbiome samples. Different methodologies have been employed to quantify the sequencing data so far (Galazzo et al., 2020), (Props et al., 2017), and (Jin et al., 2022). The most widely applied methods are based on quantitative PCR and the usage of internal controls (Smets et al., 2016), (Stämmler et al., 2016), (D. M. Tourlousse et al., 2017), (Azarbad et al., 2022).

Quantitative real-time PCR (qPCR) is an affordable and technologically simple method to move from relative to absolute data. However, it possesses the same biases during a sequencing library preparation – amplification of the specific region of the 16S rRNA bacterial gene by conservative primers. The most used primers for bacterial quantification often are the same ones that work at the library preparation stage, namely universal primers specific for the hypervariable region of the 16S rRNA gene (Liu et al., 2012), (Kim et al. 2013). Imbalance in the amplification of different taxa and variation of 16S gene copy number are the main sources of errors in this type of quantification. Droplet digital PCR (ddPCR) is the most effective existing technology to get absolute DNA values. It does not need controls and provides precise concentrations. Although ddPCR is an ideal choice in the case of single bacterial species quantification, it is not suitable for consortia enumeration. Moreover, the method is quite expensive and not every laboratory can afford it.

The addition of internal standards or spike-in controls already at the stage of DNA extraction might be an excellent approach to check the whole sequencing workflow from the beginning and eliminate all biases connected with the loss of DNA during the isolation from complex matrices. Besides, the spike-in might be used for data quantification. Two major types of spike-in controls have been implicated so far – synthetic DNA molecules (Zemb et al., 2020), (Di. M. Tourlousse et al. 2018), (Tkacz et al. 2018) or cells (Smets et al., 2016), (Piwosz et al., 2018). Synthetic DNAs are more accurate and versatile but do not tackle all of the issues, such as cell lysis efficiency during microbial DNA extraction or PCR-based bias. Spike-in cells usually represent a mixture of different proportions and amounts of Gram-positive and Gram-negative bacteria that are not specific to the studied environment. Knowing the input spike-in quantity and relative abundance of bacteria in the studied sample enables the calculation of the absolute numbers.

However, even obtaining the absolute NGS data is often misleading and does not show the real microbiological situation. Standard NGS pipeline provides information about the total microbiota yet does not provide the number of live bacteria in a food sample. At the same time, knowledge about the variety of viable microbial consortia is what most microbiological tasks are aimed at. For example, sterilization and pasteurization kill the majority of viable microbes but do not often affect spores, and the detection of total consortia says little about the food safety. Even a low number of viable pathogens could reach critical numbers and pose a health risk in an environment that supports their growth. With these data, a more accurate model for the spread of diseases and health prognoses could be done. Moreover, the exact enumeration of viable bacteria is valuable to estimate the health benefits of fermented foods.

Despite the obvious benefits and common use of culture-based methodology, it is not so sensitive and does not provide the exact information about the presence of definite taxonomic groups. The second type of technique for living microbes' detection is based on membrane integrity of bacteria. The membrane of dead cells is permeable, and the addition of fluorescent dyes such as propidium iodide (PI), ethidium monoazide (EMA), or propidium monoazide (PMA) can discriminate between live and dead microorganisms. So-called viability staining can be evaluated further by epifluorescence microscopy, flow cytometry, or is applied in viability qPCR. The last possibility to describe viable microbes only is RNA-based transcriptomics. As RNAs are unstable molecules and present only in alive bacteria, thereby the whole sequencing pipeline can start from RNA isolation. However, due to the short lifespan of RNA and limitations connected with it, this method is not commonly used. Despite high resolution, sensitivity, and compatibility with low biomass samples, RNASeq is an expensive and time-consuming analysis and is not widely used as a routine approach.

In our study, we modified the NGS pipeline by applying the fluorescent permeable dye PMAxx (improved version of PMA) for the detection of viable microbes only and the NGS data quantification by spike-in controls or qPCR using 16S rRNA gene V4 region-specific primers. The simple and reliable approach that is based on modification of broadly accepted amplicon NGS technology would allow the accurate estimation of the absolute number of alive bacterial cells that will significantly improve the quality of future microbiome studies.

Table 1
Bacterial strains used in the study and their viability measured by flow cytometry and qPCR.

Acronym	Bacterial species	Gram stain	Viability by FC, %	Viability by qPCR, %
AM	*Akkermansia muciniphila*	negative	66.11 ± 0.03	81.22 ± 13.47
AS	*Alistipes shahii*	negative	62.40 ± 7.63	78.51 ± 12.18
AC	*Anaerostipes caccae*	variable	60.69 ± 9.86	90.01 ± 5.02
ACo	*Anaerotruncus colihominis*	positive	23.35 ± 0.90	40.15 ± 13.87
BC	*Bacteroides caccae*	negative	3.76 ± 2.16	9.28 ± 0.26
BT	*Bacteroides thetaiotaomicron*	negative	63.88 ± 8.26	56.87 ± 2.51
BU	*Bacteroides uniformis*	negative	88.59 ± 1.82	100.00 ± 8.84
BA	*Bifidobacterium adolecentis*	positive	83.72 ± 1.55	90.26 ± 7.07
BH	*Blautia hydrogenotrophica*	positive	35.06 ± 14.86	24.63 ± 1.62
BF	*Butyricimonas faecihominis*	negative	95.88 ± 0.85	92.47 ± 27.24
CM	*Catenibacterium mitsuokai*	positive	6.14 ± 1.86	0.46 ± 0.01
ChM	*Christensenella minuta*	negative	40.40 ± 0.46	34.66 ± 7.18
CA	*Collinsella aerofaciens*	positive	4.31 ± 0.13	11.25 ± 1.51
DF	*Dorea formicigenerans*	positive	81.80 ± 1.19	90.18 ± 8.42
DL	*Dorea longicatena*	positive	14.91 ± 1.49	22.70 ± 0.89
ET	*Eisenbergiella tayi*	positive but Gram-stain negative	8.95 ± 1.57	43.96 ± 2.16
FP	*Faecalibacterium prausnitzii*	positive	72.23 ± 5.40	74.70 ± 14.27
OS	*Odoribacter splanchnicus*	negative	87.06 ± 0.54	93.38 ± 4.83
PC	*Prevotella copri*	negative	32.88 ± 4.00	73.56 ± 21.67
RF	*Roseburia faecis*	negative or variable	67.84 ± 4.32	87.07 ± 2.16
	Mix of 20 strains		**68.41 ± 0.18**	**60.92 ± 3.98**[a]

[a] Universal 16S rRNA gene V4 primers were used instead of specific ones.

2. Materials and methods

2.1. Bacterial strains

All 20 strains (Table 1) used in this study were purchased from DSMZ (German Collection of Microorganisms and Cell Cultures GmbH). The strains were grown on modified YCM (yeast extract, casitone, mucin) or MRS (de Man, Rogosa and Sharpe) medium until the stationary growth phase. Cells were harvested by centrifugation at 14 000×*g* for 5 min at 4 °C and washed once with DPBS (Dulbecco's phosphate-buffered saline, PAN-Biotech GmbH, Germany). Cell pellets were suspended in a solution of DPBS with 20% glycerol and 1% L-cysteine hydrochloride, frozen in liquid nitrogen and stored at −80 °C in aliquots. The number of cells in the stock culture was estimated by optical density at 600 nm (OD600) with the assumption that OD600 of 1 equates approximately cfu/mL. 5 × 10^8 cells were taken from each strain stock and added to the mixture. The final OD600 of the mixed stock culture was 0.98.

For the viability analysis by qPCR and NGS, five multi-strain glycerol stocks were prepared in aseptic conditions. For that, the stocks were centrifuged at 5000×*g* for 10 min at 4 °C and the supernatant was discarded. Each pellet was resuspended in 1 mL of 0.85% NaCl solution and centrifuged at 5000×*g* for 10 min at 4 °C. Then two pellets were suspended in 200 μl of 1 × PBS (Phosphate-buffered saline, BIO-RAD, CA, USA) and continued with genomic DNA (gDNA) extraction for the total cell analysis. The other three pellets were suspended in 400 μl of 0.85% NaCl solution for the following PMAxx treatment, spike-in control inserting and gDNA isolation to predict viable cells' presence.

2.2. Bacterial cells separation from kimchi and sauerkraut samples

Microbial cells from kimchi and sauerkraut were isolated in sterile conditions. 50 g of each sample were agitated in 50 ml of 0.85% NaCl, 0.05% Tween 20 solution (Sigma) at 200 rpm for 15 min at room temperature on Yellow Line OS 5 Basic Orbital Shaker (IKA Works Inc, Wilmington, NC, USA). The samples were filtered through the Whatman filter paper, and the filtrate was centrifuged at 5000×*g* for 15 min at 4 °C. The cell pellets were washed with 1 ml of 0.85% NaCl solution and centrifuged at 5000×*g* for 15 min at 4 °C. The final pellet was resuspended in 2 ml of 0.85% NaCl solution and divided into five 400 μl aliquots. The first aliquot was subjected to flow cytometry analysis. Another four aliquots were used for viable and total cell analysis with qPCR and NGS. Two of the four aliquots were centrifuged at 5000×*g* for 15 min at 4 °C, the supernatant was discarded, and the pellets were frozen at −20 °C until gDNA extraction for total cell number estimation. For the remaining two aliquots, the PMAxx treatment was applied for alive cell consortia analysis, then cells were frozen until gDNA extraction.

2.3. Flow cytometry analysis

Samples were diluted to a final cell concentration of approximately 1 × 10^6 cells/mL with filter-sterilized (0.22 μm) PBS (PAN-Biotech GmbH, Germany). Membrane integrity was evaluated using double staining with green fluorescent-dye SYTO24 (SYTO™ 24 Green Fluorescent Nucleic Acid Stain - 5 mM Solution in DMSO, Invitrogen, USA) labelling all bacteria, and red fluorescent propidium iodide (PI) (Propidium Iodide - 1.0 mg/mL Solution in Water, Invitrogen, USA), which permeates only cells with damaged membranes. The final concentrations of SYTO 24 and PI were 1 μM and 2 μM, respectively. For staining, the cells were incubated in the dark for 20 min at 37 °C and 10 min on ice. Analyses were performed using A50-Micro Flow Cytometer (Apogee Flow Systems, UK) with a 20 mW laser at 488 nm. Over 10 000 events were collected per sample at a flow rate set to 3 μL/min. Green fluorescence was acquired using a bandpass filter FL-2 (517–553 nm), and red fluorescence was acquired using a long-wavelength pass filter FL-3 (>575 nm). All parameters were collected as logarithmic signals. Thresholds were adjusted for forward (FSC) and side scatter (SSC) to exclude noise and debris. Gating of red fluorescence versus green fluorescence dot plot was used to obtain cell count data. Event count was obtained for both dyes and was used to calculate the absolute number as well as the ratio of viable and dead cells.

2.4. Microscopy

Single-strain bacterial stock cultures were adjusted to OD 1 at 600 nm by DPBS solution with 1% L-Cysteine hydrochloride. Samples were vortexed and pipetted onto microscope slides followed by Gram staining by BioGram 4 kit (Biognost Ltd, Croatia) according to the manufacturer's instructions. The strains were examined under the Eclipse E200-LED microscope (Nikon, Minato City, Tokyo, Japan) equipped with a 100-fold magnifying oil immersion objective lens. Nikon D5200 camera was used to take brightfield images.

2.5. Plating

Five grams of kimchi and sauerkraut samples were diluted in 45 ml sterile 0.85% NaCl solution, subsequently, serial 10-fold dilutions of the samples were made. The number of bacteria was determined by plating 100 μl of kimchi and sauerkraut samples dilutions on PCA (Plate Count Agar, Neogen, Lansing, MI, USA) and incubating for 72 h at 30 °C. Plating was carried out in two technical replicates. Results were presented as colony-forming units per gram of the sample (cfu/g).

2.6. PMAxx treatment and addition of spike-in control

Twenty-strain consortia, isolated kimchi and sauerkraut cell cultures were prepared as aforementioned. For viability testing, PMAxx™ solution in H_2O (Biotium, Fremont, CA, USA) was added to 400 μL of the cell suspension to obtain a 25 μM final concentration. The PMAxx-cells suspension was incubated in dark for 10 min on a shaker. After that, the samples were exposed to blue light by PMA-Lite™ LED Photolysis device (Biotium) for 20 min with intermittent inversion. The samples were centrifuged at 5000×*g* for 15 min at 4 °C, the pellets were resuspended in 200 μL of 1 × PBS. If applicable, a defined number of ZymoBIOMICS™ Spike-in Control I (High Microbial Load, Zymo Research, Irvine, CA, USA) was added. For the titration experiment, the number of the added spike-in control is shown in Table 2; the for method validation study, 4 × 10^7 spike-in cells were added to each aliquoted kimchi/sauerkraut samples. The cells were then immediately subjected to DNA extraction.

2.7. DNA extraction

The gDNAs of the 20-strain consortia samples were isolated by ZymoBIOMICS™ DNA Miniprep Kit (Zymo Research) and of the kimchi/sauerkraut samples by Quick-DNA™ Fungal/Bacterial Miniprep Kit (Zymo Research) according to the manufacturer's instructions. The

Table 2
Application of the spike-in standard for cell number absolute quantification.

		20St	20St 0.5%	20St 1%	20St 2.5%
Number of added spike-in cells		0.00	7.50 × 10^6	1.50 × 10^7	3.75 × 10^7
Calculated abundance of added spike-in, %	*Allobacillus halotolerans*	0.00	0.25	0.72	1.13
	Imtechella halotolerans	0.00	0.18	0.35	1.28
SUM of spike-in, %		**0.00**	**0.43**	**1.07**	**2.42**
Calculated abundance of 20 species consortia, %		100	99.57	98.93	97.58
Calculated number of total cells in consortia		**N/A**	**1.74 × 10^9**	**1.39 × 10^9**	**1.51 × 10^9**

concentrations of the extracted DNAs were quantified by a Qubit™ 4 Fluorometer (Thermo Fisher Scientific, Waltham, MA, USA) using Qubit dsDNA BR Assay Kit (Thermo Fisher Scientific).

2.8. Quantitative real-time PCR

For the viability study, quantitative real-time PCR (qPCR) was performed using 5 × HOT FIREPol® EvaGreen qPCR Mix Plus (no ROX, Solis BioDyne, Tartu, Estonia). The reaction was done in triplicates on qTOWER3 thermal cycler (Analytik Jena, Jena, Germany) using a qPCRsoft 4.0 software. Each qPCR run contained no-template control for checking external contaminations. The cycling conditions included a preliminary denaturation at 95 °C for 12 min, then 40 cycles of denaturation at 95 °C for 30 s, annealing at temperature corresponding used primer pair (Supplementary Table S1) for 30 s, and extension at 72 °C for 1 min. During qPCR cycles, the fluorescence was measured at the end of each annealing step, and a melting curve analysis step (at a ramp from 55 °C to 95 °C) was included to assess the specificity of the amplification.

Viability qPCR for single strains of the 20-strain consortia was performed using strain-specific primers (Supplementary Table S1). To evaluate the cell viability, the ΔCq based on the quantification cycle (Cq) of the PMAxx-treated and untreated sample was calculated as follows:

$$\Delta Cq = Cq_{\text{PMAxx-treated}} - Cq_{\text{non-treated}}.$$

The percentage of the viable cells was computed based on the equation:

$$\%\ \textbf{viable} = \frac{\mathbf{100}}{\mathbf{2}^{\Delta \mathbf{Cq}}}.$$

Total and viable cell gDNA concentrations were measured based on the standard calibration curve using 515F/806R primers, specific for the bacterial V4 region of the 16S rRNA gene (Caporaso et al., 2011) as used during the preparation of the sequencing libraries but without adapter nucleotides (Supplementary Table S1). ZymoBIOMICS™ Microbial Community DNA Standard (Zymo Research) was used for calibration curve creation. The microbial cell number was calculated considering the amount of sample taken for the DNA extraction, a dilution factor and an approximation expressed as 1 ng of bacterial DNA ≈2–4 × 10^5 cells, if the average size of the bacterial genome is taken as 2.2 Mbp for LAB (lactic acid bacteria) (Klaenhammer et al., 2002) and 4.5 Mbp for bacteria of 20-strain consortia. The equation for single-cell bacterial DNA will be:

$$\mathbf{m}_{=}\frac{\textbf{Genome size} \times \mathbf{M}}{\mathbf{N}_A},$$

where M is the average molar mass of a base pair equal to 660 g/mol, and N_A is the Avogadro constant 6.02 × 10^{23} mol^{-1}.

2.9. 16S rRNA gene amplicon sequencing

Amplicon libraries targeting the V4 region of the 16S rRNA gene by the primer pair 515F/806R were prepared according to Illumina's dual indexing protocol as published in Kazantseva et al., (2021) (Kazantseva et al., 2021). Multiplexed and normalized libraries were sequenced on iSeq 100 System using i2 kit (Illumina, San Diego, CA, USA). DNA sequence data were analyzed as published before (Kazantseva et al., 2021), (Espinosa-Gongora et al., 2016), (McDonald et al., 2016) by BION-meta program (https://github.com/nielsl/mcdonald-et-al, Danish Genome Institute, Denmark) according to the current instructions. All sequencing data are available on the SRA database with the PRJNA861123 reference.

2.10. Data processing

Statistical analysis and visualization were carried out using Excel software (Microsoft 365 Apps for business, Microsoft Corp., Redmond, WA). qPCR and FC experiments as well as spike-in sequencing protocol were performed in triplicate. All data were independently analyzed using paired Student's *t*-test and represented where appropriate as means ± standard deviation.

3. Results and discussion

The aim of the study was to develop and validate the methodology for quantitative taxonomical determination of bacterial species that distinguishes between viable and the whole microbiota using next-generation sequencing approach. For that, PMAxx reagent that discriminates between alive and total bacteria consortia was validated for 16S library preparation and two ways of bacteria enumeration including qPCR and spike-in cell addition were applied.

3.1. Assessment of bacteria viability by PMAxx-qPCR

For the first viability step validation, twenty bacterial species that differ by their Gram-stain (Table 1) were chosen. To discriminate between viable and dead bacteria, a new generation viability fluorescent reagent PMAxx was used. This reagent is a photoreactive dye that selectively penetrates dead cells through compromised membrane and covalently binds DNA upon photolysis with visible blue light. Dye-modified DNA cannot be amplified by PCR and thereby is eliminated from the following procedure. Cells for the analysis were taken from glycerol stocks and due to specific features, their viability after the storage was variable. For viability control, convenient flow cytometry (FC) with SYTO24 and PI dyes was carried out. According to the FC analysis (Table 1), viability for individual species varied from 4 to 96%, but the mix of 20 strains consortia had the viability of 68%.

The next step was to estimate the bacteria viability by PMAxx reagent and qPCR. For that, cells from glycerol stocks were mixed in some proportion according to their OD and half of them were treated by PMAxx (viable cells), while another half stayed untreated (total cells). Extracted gDNA was analyzed by viability qPCR using strain-specific primers. Despite the relatively good correlation between FC and qPCR data (Fig. 1, R^2 = 0.884 with two outliers excluded and 0.789 when outliers are used), some differences were observed. Since microbial populations are usually heterogenic (Hewitt and Nebe-Von-Caron 2001), the FC data are not completely reliable. The formation of cellular chains and aggregates may lead to underestimation of cell number, but PCR enumeration depends on DNA amount only. Besides, both viability tests are based on the similar fluorescence dye penetration that could be a limitation for some bacterial species. This may be the reason why qPCR data using PMAxx reagent and strain-specific primers tended to show higher level of viable cells than FC.

To understand the nature of dissimilarities, microscopic evaluation of several bacterial strains was performed (Fig. 2). As seen from Fig. 2B, cells that showed a discrepancy between FC and qPCR data are more prone to generate clusters and fiber-like structures that can affect definite cloud formation and the subsequent FC data interpretation.

The study by Vandeputte et al. (2017) (Vandeputte et al., 2017) also showed that qPCR quantification is significantly comparable and negatively correlated with FC for absolute quantification of fecal microbiota by the same amplicon sequencing protocol. However, in the case of FC, when more than one cell population is observed, single microbial cells interact with each other forming aggregates, or cell and matrix debris are not completely excluded from the analyzed pellet. The obtained data are not entirely accurate and abnormal populations could be missed or misinterpreted. The complete dissociation of cells is not trivial for samples where bacteria are attached to each other or to the substrate, which makes an accurate counting by FC difficult. On the other hand,

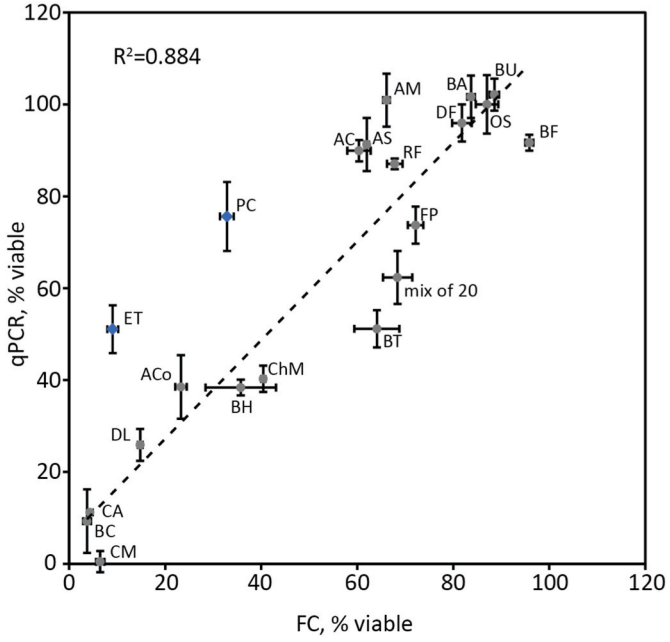

Fig. 1. Scatterplot for cell viability measured by FC and qPCR using strain-specific primers. The dotted line represents a linear regression trendline and R^2 shows the correlation. Bacterial strain acronyms can be found in Table 1. The grey circles represent points that were used for the trendline calculations, while the blue squares represent outliers. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

the efficiency of PMAxx or any other reagent's penetration into a damaged cell could be limited. Also, the strength of dye-DNA covalent binding depends on the transparency of the cell's suspension. For colored matrices this efficiency could be decreased. At the same time, for not intensively colored matrixes, the covalent dye-DNA binding should be effective.

Overall, PMAxx dye is a reliable reagent that can be used to discriminate between viable and dead microbes in the NGS library preparation step, excluding damaged cells from the following analysis.

3.2. Bacterial enumeration of NGS

Next-generation sequencing is a powerful and reliable technology for taxonomic identification of bacteria. However, the common protocol reveals only relative microbial distribution of the sample but does not provide information regarding absolute cell numbers. To evaluate the number of bacteria taken for sequencing, we applied spike-in cell control adding to the sample before DNA extraction procedure. We used commercial High-load Spike-in control from ZymoResearch with a defined amount of rarely occurred bacterial strains: *Imtechella halotolerans* and *Allobacillus halotolerans*. These spiked strains went through the whole NGS workflow and were used as *in situ* positive controls for the whole analysis. Knowing the exact amount of definite added cells, it is possible to recalculate the number of all bacteria in the sample.

First, we performed the titration of the spike-in control to confirm the linear dependence of the added cells in the number of the whole bacteria population. As seen from Fig. 3, a different percentage of added spike-in control linearly correlates ($R^2 = 0.9962$) with the total bacteria number. Thus, it is not necessary to add spike-in at an exact amount to obtain a reliable estimation of a microbial cell number in the sample.

According to the OD600 measurement of the cultures, the theoretical total number of consortia cells taken for analysis was about 1.50×10^9. To estimate the total number of cells by the NGS, we used the number of reads of spike-in control related to the reads of consortia species from NGS data. Knowing the exact number of spike-in cells taken for analysis, we calculated the total bacterial cells in the sample (Table 2). Using a different amount of added spike-in cells (0.5, 1.0, and 2.5%), the average calculated total bacterial load was $1.55 \pm 0.18 \times 10^9$, which is quite accurate.

Adding the spike-in cells as an internal standard prior to the DNA extraction step is crucial as the standard is subjected to cell lysis and DNA extraction efficiency together with target bacteria by the same methodological workflow. Both are important for absolute quantification, especially the method of cell destroying for complex microbiota. The bead-beating step is recommended for full DNA recovery and is included in most reliable DNA extraction kits. Adding cells as a spike-in control for bacteria enumeration has one more universal merit – it is possible to use different regions or even the whole of 16S rRNA gene for sequencing and not be limited by 515F/806R primer pairs. This is important in the case of some species, where V4 region is not suitable for their identification and different fragments for higher resolution of lower-rank taxa should be used (Bukin et al., 2019).

It is important to mention that spike-in addition should be done before the DNA extraction step, which is not always possible, or the knowledge about the number of bacteria in a sample is necessary in retrospective. For that, the only chance to obtain an estimated bacterial concentration in the sample is from microbial gDNA concentration. Real-time PCR is an accepted methodology to obtain quantitative data

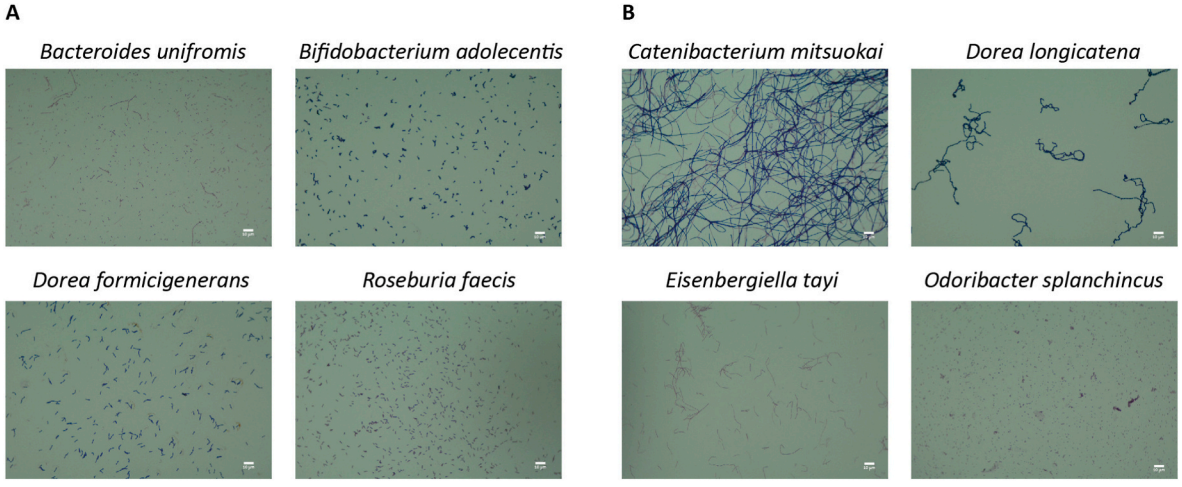

Fig. 2. Microscopy images of bacterial strains specific for (A) similar FC and qPCR data, and (B) different between FC and qPCR data. The white bar on the bottom right corner represents the length of 10 μm.

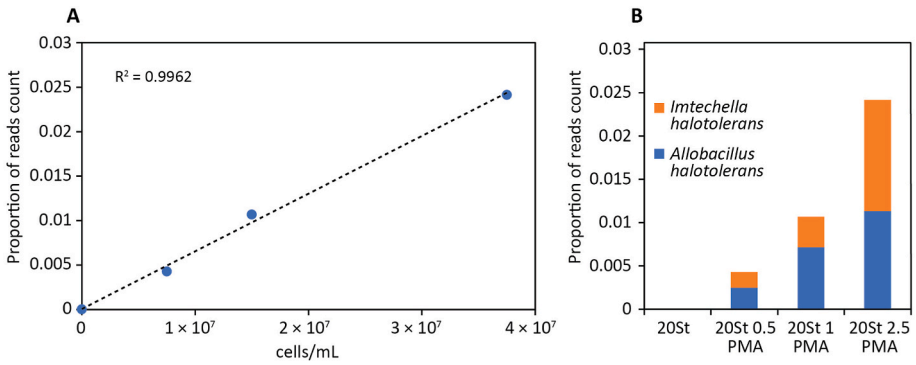

Fig. 3. Assessment of the quantitative performance of the spike-in standard for varying amounts of spike-in control added using 16S V4 amplicon NGS. (A) Dose-response correlation graph. (B) Relative abundance of spike-in species.

from DNA, as microbial gDNA concentration is directly proportional to bacteria cell number. To test the latter, we carried out qPCR using conservative 16S V4 region and degenerative primers for enumeration of bacteria in the samples (Table 3). Received by calibration curve methodology gDNA microbial concentration was converted to the total bacterial cell number which was $1.16 \pm 0.12 \times 10^9$ cells (20St qPCR). To compare with the theoretical value, the number of total cells calculated by the qPCR equation is of the same order but a lower number.

In our study, spike-in application led to very precise microbes' enumeration similar to FC, while data received by qPCR were slightly lower. Similar results were obtained by O. Zemb et al., (2020), where qPCR gave 1.9 times lower bacterial number estimates than artificial internal spike-in standard. In their work, qPCR allowed the increase of the sensitivity of the technology and minimize the amount of internal standard added when compared to the work published by Tkacz et al. (2018) (Tkacz et al. 2018), where they had to add 20–80% of the synthetic spike-in and thereby sacrifice the total sequencing input. The difference between qPCR and spike-in methodologies may be due to the degenerative primer usage, variances between 16S rRNA gene copy numbers for different species, or approximation between DNA mass and average cell number taken for analysis. Indeed, 16S rRNA gene copy number is determined by the dynamics of DNA replication and cell division (Cooper and Helmstetter 1968) and can change from 7 to 38 copies per cell even for *E. coli* (Bremer and Dennis 2008). It means that any estimation of cell number based on 16S rRNA gene copy number is biased if the population is growing, which should be considered for fast growing environments. Furthermore, PCR inhibitors would impact the bacterial estimation by qPCR, but it is fair to say that the same bias is appropriate for the whole amplicon-based sequencing pipeline. Despite all these bottlenecks, even qPCR combined with a standard curve quantification gave reliable data that can be considered as complementary quantification methods for bacterial count.

In general, both technologies showed comparable and reliable data, and thereby complemented each other.

Table 3
Assessment of various methods for cell number estimation for total and viable bacterial consortia.

Sample name	Cell number	Viability, %
20St theoretical	1.50×10^9	
20St FC	$1.56 \pm 0.17 \times 10^9$	68.41 ± 0.18
20St qPCR	$1.16 \pm 0.12 \times 10^9$	60.92 ± 3.98
20St spike-in	$1.55 \pm 0.18 \times 10^9$	67.79 ± 8.19
20St alive FC	$1.06 \pm 0.17 \times 10^9$	
20St alive qPCR	$7.03 \pm 0.25 \times 10^8$	
20St alive spike-in	$1.5 \pm 0.12 \times 10^9$	

*based on three parallel measurements.

3.3. Combined approach

The next step in the protocol development was to combine PMAxx treatment and bacteria enumeration procedures. For that, PMAxx-treated (for viable cell detection), and untreated (for total) microbial cells were subjected to spike-in addition, and the whole workflow of 16S amplicon sequencing was carried out. For alive bacteria enumeration, the analysis was performed in three parallels (20St 0.5PMA, 20St 1PMA, 20St 2.5PMA) that differed by the amount of *in situ* spike-in control added. The DNA sequencing data analysis showed that all 20 bacterial species were detected (Fig. 4). Moreover, the taxonomical distribution of viable bacteria performed in three parallels was consistent but differed from total bacteria profile. Thus, *Catenibacterium mitsuokai* and *Collinsella aerofaciens* were detected in total consortia but were absent among the viable cells, which correlates with FC and qPCR data (Fig. 4A, Table 1). Also, the number of *Bacteroides caccae* decreased in viable cells consortia in accordance with FC and qPCR analyses. In general, the viability of all members of 20-strain consortia assessed by NGS showed the pattern completely correlated with FC and qPCR data.

To represent results in absolute cell numbers, the data were normalized according to spike-in or qPCR quantification (Fig. 4B). The total amount of detected bacteria measured by spike-in control was $1.55 \pm 0.18 \times 10^9$ (Table 3), which is similar to that evaluated by FC quantity of 1.56×10^9 cells and close to the theoretical value. The average number of alive bacteria in the samples according to the spike-in control was $1.05 \pm 0.12 \times 10^9$ (20St alive Spike-in) while using qPCR approach – $7.03 \pm 0.25 \times 10^8$ cells (20St alive qPCR). FC data regarding bacterial viability estimation significantly correlated with spike-in enumeration and showed 1.06×10^9 alive cells in the sample. The calculated percentage of viability (Table 3) stayed in the range of 61–68% for all methods used.

Overall, for total and viable consortia, the cell number estimation was very similar between FC and spike-in sequencing methodology but the usage of qPCR as a method for cell number measurement resulted with slightly lower values.

3.4. Quantitative alive bacteria NGS for food samples

To demonstrate the potential of the methodology as applicable for real food samples, we chose three fermented foods for analysis – two kimchi prepared from Chinese and White cabbage (K4 and K5), and classical sauerkraut (K6). These food matrices can be considered complicated as they consist of plant material and are enriched with colorful tiny particles of spices that in case of kimchi gives an intensive color to cell pellets and interferes with DNA-PMAxx covalent binding. However, these are naturally fermented models and have enough bacteria for a proper analysis. Thus, the main task was to understand how our pipeline works for complex food matrices, describe their microbiological profiles, and find out the limitations and bottlenecks of the

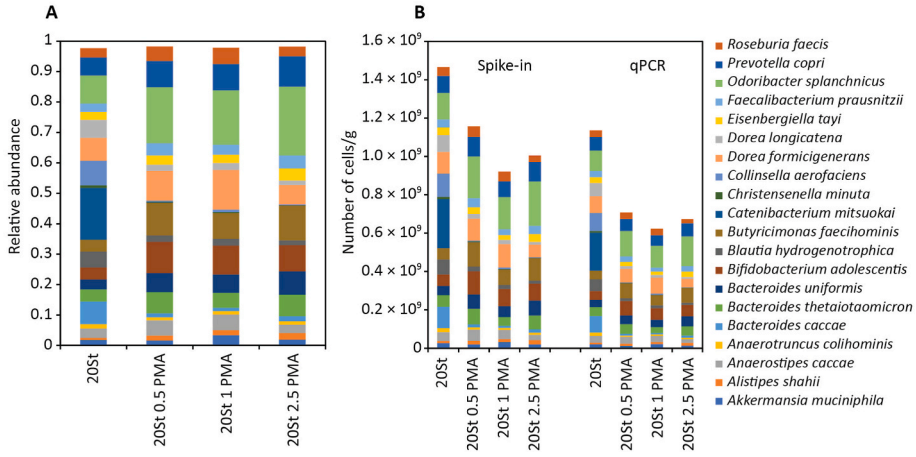

Fig. 4. Bacterial identification of total (20St) and alive (20St 0.5PMA, 20St 1PMA, 20St 2.5PMA) consortia samples based on 16S V4 amplicon NGS. (A) Relative abundance of bacterial distribution. (B) NGS data normalized to spike-in control and qPCR-based quantification.

technology.

For the method validation, classical microbiological methods such as PCA plating and FC were employed to estimate the number of viable bacteria and compared with the developed technology. Cells analyzed by NGS were divided by half and PMAxx treatment was applied for one part of the cell pellet (viable bacteria), while untreated cells were considered as total cell population. The results of the study represented in Table 4 show that the estimated number of cells measured by different methodologies does not differ significantly. In general, plating gave the lowest cell values, while spike-in technology operates with the highest numbers. As the majority of bacteria in the analyzed food samples belonged to LAB, for cell numbers calculation with qPCR we took the average genome size in the equation as 2.2 Mb (Klaenhammer et al., 2002). This led, in general, to similar values as for the spike-in approach. Thus, for kimchi samples, the numbers differed non-significantly but were lower in case of total bacteria number for sauerkraut (3.06×10^8 against 1.86×10^9 for qPCR and spike-in correspondingly), and higher for viable bacteria enumeration (Table 4). The common tendency is that the viable bacterial number was always lower compared to the total bacteria. The largest difference between all methodologies was observed for sauerkraut K6 samples, where the proportion of viable cell consortia was minimal for studied fermented food. In general, standard microbiological plating showed the lowest bacteria count. It is explained by the fact that not all bacteria are culturable or capable of growth on a specific unified medium within predefined conditions.

The whole elaborated sequencing pipeline that discriminates viable (PMA) and total (TOT) consortia and establishes bacteria enumeration (B) is introduced in Fig. 5. Analysis of viable and total bacterial distribution indicated that some bacteria disappeared from the total cell consortia. Thus, *Leuconostoc mesenteroides* was mainly detected among the total cells but not in viable cell profile. At the same time, the proportion of *Levilactobacillus* spp and *Lactiplantibacillus plantarum* were higher for viable bacteria. The differences between the total and viable taxonomical profiles are better evaluated by relative distribution analysis (Fig. 5A), while the real picture is clear only after the application of spike-in normalization methodology (Fig. 5B). This picture shows the actual pattern of total and viable bacteria in the samples. Overall, it means that both relative and absolute values must be evaluated and represented to make a conclusion regarding an analysis.

The next step for the method development could be the estimation of spike-in standard by qPCR with species-specific primers and assessment of more accurate DNA recovery yield. Another approach for more accurate qPCR quantification could be an application of individual genome sizes for identified bacteria instead of the average for the population for recalculating relative to absolute abundances. It will give more precise calculation of cell numbers in qPCR equation. It was shown in the case of kimchi and sauerkraut bacteria quantification, where the food samples contained mainly lactic acid bacteria. Correction of genome size for the LAB specific number in the equation led to more accuracy (similar to other methods of bacteria enumeration).

In summary, we can conclude that the introduced modifications are applicable to the real matrices. This complex technology is capable of differentiation between total and alive bacterial species and the acquisition of the absolute number of cells for the taxonomical description of food microbiota by NGS. This elaborated workflow provides much more information that can be used for technological process development, in shelf-life study, or for making decisions regarding safety issues.

Table 4
Bacterial enumeration of kimchi (K4 and K5) and sauerkraut (K6) microbiota by different methodologies.

Sample	Plating, cfu/g	Cell number by FC	Cell number by spike-in	Cell number by qPCR
K4 TOT		$1.49 \pm 0.26 \times 10^8$	8.56×10^8	$4.60 \pm 0.26 \times 10^8$
K4 PMA	$1.17 \pm 0.32 \times 108$	$2.61 \pm 0.05 \times 107$	3.97×108	$4.12 \pm 0.35 \times 108$
K5 TOT		$1.34 \pm 0.33 \times 10^8$	5.90×10^8	$5.66 \pm 1.65 \times 10^8$
K5 PMA	$3.64 \pm 0.12 \times 10^7$	$7.04 \pm 0.14 \times 10^7$	3.82×10^8	$3.73 \pm 0.16 \times 10^8$
K6 TOT		$1.26 \pm 0.05 \times 10^8$	1.86×10^9	$3.06 \pm 0.07 \times 10^8$
K6 PMA	$1.53 \pm 0.13 \times 10^7$	$5.96 \pm 0.02 \times 10^7$	1.28×10^8	$1.71 \pm 0.56 \times 10^8$

4. Conclusion

An integrated approach that combines new generation viability reagent usage and two alternative approaches for microbiological quantitative data assessment by spike-in control or qPCR with subsequent NGS analysis was elaborated. The full scheme of the workflow is indicated in Fig. 6. This modified sequencing workflow discriminates dead and viable microbial consortia and transforms sequencing-generated relative abundance data into a straightforward quantitative microbiota profile.

Although this method works well even in the case of real complex samples and can be routinely used for microbiological data acquisition and interpretation, it might need some further development and fine tuning. It should be noted that the methodology we proposed was tested on food samples but can be applied on any microbial sample. For example, this technology can be used for identification and evaluation of

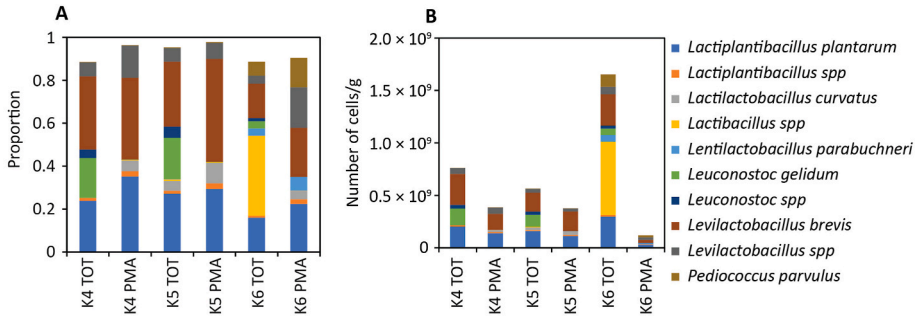

Fig. 5. The results of bacterial identification of total (TOT) and viable (PMA) kimchi and sauerkraut samples based on 16S V4 amplicon NGS. (A) Relative abundance of bacterial distribution. (B) NGS data normalized to spike-in standard.

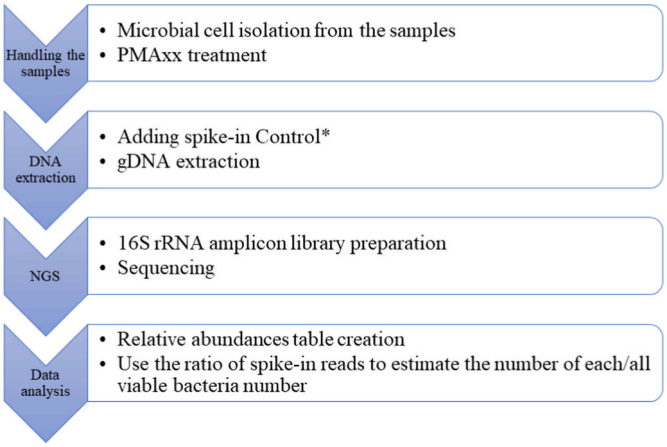

Fig. 6. The workflow used for identification and enumeration of viable microbiota by 16S rRNA NGS. *In case the spike-in control was not added, it is possible to estimate the bacterial load of the sample by qPCR approach.

viable pathogenic bacteria in any kind of environment, although it should be considered that sufficient sequencing depth per sample is designated, as NGS usually underestimates targets lower than 1% of relative abundance. Overall, an elaborated integrated workflow represents a further step in a complex approach for the application of next-generation sequencing for quantitative microbiota analysis.

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CRediT authorship contribution statement

Aili Kallastu: Methodology, Validation, Formal analysis, Investigation, Writing – review & editing, Visualization. **Esther Malv:** Methodology, Validation, Formal analysis, Investigation, Writing – review & editing, Visualization. **Valter Aro:** Formal analysis, Investigation, Writing – review & editing, Visualization. **Anne Meikas:** Methodology, Validation, Formal analysis, Investigation, Writing – review & editing. **Mariann Vendelin:** Validation, Investigation, Writing – review & editing. **Anna Kattel:** Validation, Investigation, Writing – review & editing. **Ranno Nahku:** Writing – review & editing. **Jekaterina Kazantseva:** Conceptualization, Methodology, Formal analysis, Data curation, Writing – original draft, Preparation, Writing – review & editing, Supervision, Project administration.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

We uploaded data in public database and provided the link

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.crfs.2023.100443.

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