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Temporal dynamics of probiotic *Lacticaseibacillus casei* and *rhamnosus* abundance in a fermented dairy product evaluated using a combination of cultivation-dependent and -independent methods

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ABSTRACT

Benchmark of cost-efficient and accurate methods for quantifying probiotics in dairy products represents great interest to the food industry. The advantages of cultivation-independent techniques over the traditionally used cultivation-based ones are to be investigated in this context.

We evaluated the levels of *Lacticaseibacillus casei* and *rhamnosus* in multiple formulations of a fermented dairy product fortified with these probiotics during the shelf-life using cultivation, taxon-specific qPCR augmented with propidium monoazide (PMA) viability test and 16S rRNA gene sequencing. The analyzed products were the yogurts produced with traditional yogurts starter cultures including *Bifidobacteria* or without them. The effect of the starter culture on probiotics viability and abundance was assessed. The methods for probiotic profiling were compared.

All methods confirmed high levels for the probiotics throughout the shelf-life. The PMA-qPCR showed that their non-viable proportion was low. The formulations with the starter cultures including *Streptococcus* and *Lactobacillus* were associated with a lower abundance of each probiotic compared to those that additionally had *Bifidobacterium* in the starter culture. The total microbial composition according to the sequencing was generally as expected, but the method was of limited use for profiling the probiotic levels due to the data compositionality and dominance of the starter culture taxa.

1. Introduction

Since the 20th century, the microorganisms used in the food industry are in the focus of scientific interest (Fuller, 1992), which led to the emergence of the concept of "probiotics" - live microorganisms that, when administered in adequate amounts, benefit the host health (Hill et al., 2014). Although there are many positive effects including normalization of transit time and gut permeability, anti-inflammatory activity and protection against pathogens, to date no health claims for probiotics were accepted according to the European Food Safety Authority (EFSA) (de Simone, 2019) and the evidence of their effectiveness for disease treatment is insufficient (Bernaola Aponte et al., 2013; Butterworth et al., 2008; Kaur et al., 2020; Ong et al., 2019).

It is obligatory for a manufacturer of fortified food products to control the probiotic abundance and viability during the shelf life. It will also ensure that a consistent material is supplied for clinical studies evaluating the physiological effects of such products (Shane et al., 2010). In fermented dairy products, factors like acidity, oxygen and nutrient levels can significantly affect the probiotic viability (Tamime et al., 2017). Although most commercial probiotic species are considered safe, the current regulation of probiotic-fortified products manufacture requires serious improvements based on a solid microbial profiling methodology (de Simone, 2019). Development and benchmarking of methods for qualitative and quantitative identification of

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probiotic bacteria in food products and analysis of their viability are important goals for manufacturers and consumers of fortified products (FAO/WHO, 2001).

There are many approaches for analyzing microbial composition of food products that can be applied routinely for this purpose. Although the cultivation-based methods (like cultivation on agar plates) are mostly used, the cultivation-independent molecular tools are gaining more popularity as they are more scalable and provide a more complete and/or precise community profiling - up to the strain-level (Callon et al., 2004). However, there is a lack of studies comparing different approaches during the shelf-life of the same product (Huys et al., 2006). This may be especially interesting in the case of multistrain probiotics because there are reports of suppression of one strain by the others in the mixture, as well as, contrarily, of synergistic effects leading to increased probiotic potential (Mikelsaar et al., 2011); besides, multiple probiotic species within the same product might confer distinct health effects.

The *Lacticaseibacillus* (recently reclassified from *Lactobacillus* (Zheng et al., 2020)) is a major microbial genus rich in species providing health benefits - either being a part of the inherent host microbiome or when introduced, i.e. with food. Safety and specific health benefits have been demonstrated for many *Lacticaseibacillus* strains (Bubnov et al., 2018; Hill et al., 2014; Reid et al., 2017; Salminen & Deighton, 1992). Particularly, certain strains of *L. casei* and *rhamnosus* showed anti-inflammatory (Schultz et al., 2004; Watterlot et al., 2010) and cancer preventing effects (Aso et al., 1995; Gamallat et al., 2016; Ishikawa et al., 2005; Jacouton et al., 2017), protection against pathogens (Aggarwal et al., 2014) and alleviation of allergic diseases symptoms (Kalliomäki et al., 2003). Putative mechanisms of their probiotic action include production of lactate - an inhibitor of pathogens growth and a precursor of butyrate for commensal microorganisms (Louis & Flint, 2009), regulation of intestinal permeability and host immunity presumably via bacteriocins or pili (Dobson et al., 2012) and alteration of host gene expression (Sanders et al., 2019; van Baarlen et al., 2013). Due to the reported health benefits and prominent abilities to survive in diverse niches, *Lacticaseibacilli* are added to a wide range of functional food products.

We performed comparative analysis of 7 methods for enumerating probiotics in food by analyzing the dynamics of probiotic *Lacticaseibacillus casei* and *rhamnosus* levels in a fermented dairy product during the shelf-life. The links of probiotic abundance with technological factors like variability across formulations and production batches were investigated.

2. Materials and methods

2.1. Study design

The investigated product was the Imunele ® (PepsiCo, Russia) drinkable yogurt with fruit premix fortified with bacterial strains declared by the manufacturer as probiotics (*Lacticaseibacillus casei* and *L. rhamnosus*) and a vitamin complex. The concentration of lactic acid microorganisms (including the starter culture and probiotic bacteria) was at least $1{\cdot}10^7$ CFU/g. The concentration of the probiotic cultures was at least $1{\cdot}10^6$ CFU/g.

The product was available in 9 formulations (study IDs: F1-F9) varying by the choice of a starter cultures (n = 6, IDs: S1-S6) and probiotic additives (n = 3) each containing one *L. rhamnosus* strain (Lr) and one of the three *L. casei* strains (Lc1 - Lc3) as a functional fortification (Table 1). For each recipe, two production batches were analyzed (3 replicates per batch) at 5 time points (days after manufacture: 1, 6, 11, 16 and 21 - at the end of the shelf-life). In total, there were 270 samples of the dairy product. Additionally, all 6 starter cultures and 4 probiotics were analyzed (Table 1).

The products samples were analyzed using 5 approaches (all or in subgroups): cultivation on MRS agar, qPCR for each of the 2 probiotic species, PMA-qPCR for viable cells enumeration and high-throughput 16S rRNA gene sequencing (16S rRNA-seq) with and without PMA pretreatment (Fig. 1; Table 2). Additionally, cultivation on M-RTLV media was performed for comparison with MRS agar.

Table 1
Microbial components of the investigational products.

Formulation ID	Starter culture ID	Species in the starter culture	Probiotic additive
F1	S1	*Streptococcus thermophilus* *Lactobacillus delbrueckii subscr. bulgaricus*	*Lacticaseibacillus rhamnosus* (Lr) *Lacticaseibacillus casei* strain 1 (Lc1)
F2	S2	*Streptococcus thermophilus* *Lactobacillus delbrueckii subscr. bulgaricus*	
F3	S3	*Streptococcus thermophilus* *Lactobacillus delbrueckii subscr. bulgaricus*	
F4	S1	*Streptococcus thermophilus* *Lactobacillus delbrueckii subscr. bulgaricus*	*Lacticaseibacillus rhamnosus* (Lr) *Lacticaseibacillus casei* strain 2 (Lc2)
F5	S2	*Streptococcus thermophilus* *Lactobacillus delbrueckii subscr. bulgaricus*	
F6	S3	*Streptococcus thermophilus* *Lactobacillus delbrueckii subscr. bulgaricus*	
F7	S4	*Streptococcus thermophilus* *Lactobacillus acidophilus* *Bifidobacterium animalis*	*Lacticaseibacillus rhamnosus* (Lr) *Lacticaseibacillus casei* strain 3 (Lc3)
F8	S5	*Streptococcus thermophilus* *Lactobacillus acidophilus* *Bifidobacterium animalis*	
F9	S6	*Streptococcus thermophilus* *Lactobacillus acidophilus* *Bifidobacterium animalis*	

2.2. Cultivation of bacteria and colony counting

The MRS-agar medium was prepared according to the manufacturer's instructions (Dia-M, Russia). For M-RTLV medium preparation, vancomycin hydrochloride, metronidazole, 2,3,5-triphenyltetrazolium chloride and L-rhamnose were added to the ready-mixed dry MRS-agar at recommended concentrations (Sakai et al., 2010). The Petri dishes (90 mm) were prepared under sterile conditions in a laminar box. Around 20 ml of prepared media were added to the Petri dishes. Before inoculation of a dish, sequential 10-fold dilutions in physiological saline solution were carried out. The first dilution was prepared as follows: 0.1 g of the sample was adjusted in a sterile test tube to 1 ml with sterile saline solution and homogenized by vortexing and inverting. One ml of each of the examined dilutions (from 5 to 7) was distributed uniformly over the surface of the medium and kept in a laminar box in a stream of air until getting dry. The dishes were placed in the anaerobic jar AE-01 (MagazinLab, Russia). Vacuum was created with a Millipore vacuum pump (model number 6222050 A (Merck, NJ, U.S.A.)), then replaced with anoxic atmosphere, filling the anaerobic gas with 99.999% nitrogen gas. The replacement of the atmosphere was carried out twice to reduce the amount of residual oxygen. After incubation of the dishes in a thermostat for 48 h at 37 °C, the colonies were counted on each dish (see Supplementary Notes).

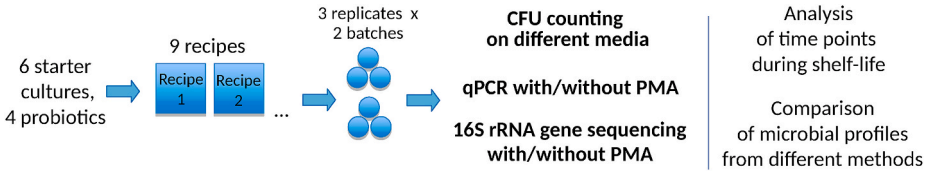

Fig. 1. Workflow of the study.

Table 2
Number of samples per analysis type.

Analysis	Number of samples
Cultivation on MRS agar	264
cultivation on M-RTLV agar	12
qPCR	270
qPCR after PMA treatment	150
16S rRNA gene sequencing	62
16S rRNA gene sequencing after PMA treatment	10

2.3. Taxon-specific primers

The sequences of primers targeting *pgi* gene fragments of *L. casei* and *L. rhamnosus* were taken from (Morovic et al., 2016). For *L. rhamnosus*, one pair of primers was selected; for *L. casei* - two, targeting two of its subspecies, to improve sensitivity (Supplementary Table 1). Specificity of the primers was checked using PCR on samples from *L. rhamnosus, L. casei subsp. casei* and *L. casei subsp. paracasei* pure cultures followed by amplicon length detection in 1.5% agarose gel with ethidium bromide staining; the obtained amplicon lengths were in accordance with the expected values (the latter being 438 bp for the *L. rhamnosus*, 200 bp for the *L. casei subsp. casei* and 514 bp for the *L. casei subsp. paracasei*). The primers mixture for *L. casei subsp. casei* and *L. casei subsp. paracasei* was used in the analysis.

2.4. DNA extraction and preparation of reference samples for qPCR

The extraction of DNA from the samples was performed for further qPCR and 16S rRNA sequencing. As an alternative to application of commercial kits, to improve robustness and cost-efficiency we established a custom protocol based on the one described previously (Wilson, 2001) using lytic enzymes (lysozyme, proteinase K and RNase A) in combination with treatment with charged (ionic) surfactant (CTAB) and phase separation in the presence of chloroform; CTAB in high concentrations allows to get rid of polysaccharides abundant in the fermented dairy products. The complete DNA extraction protocol is provided in the Supplementary Notes.

The amplicons obtained via PCR from DNA of pure cultures of *L. rhamnosus, L. casei subsp. casei* and *L. casei subsp. paracasei* using species-specific primers were used as calibration samples for quantitative evaluation in qPCR analysis. The DNA of the three cultures was mixed in equal proportions. The concentration of DNA in genome equivalents per μL was pre-calculated.

2.5. Real-time PCR (qPCR)

The real-time PCR was performed on QuantStudio 5 thermal cycler (Thermo Fisher) in 96-well PCR plate for 200 μL (PCR-96-AB-C; Axigen). Amplification for each sample was carried out in two repetitions in a total volume of 25 μL, containing: 100 pM oligonucleotide primers, 1 μL target DNA, 2 μL 10x PCR Turbo buffer with 2.5U hot start Taq polymerase (Evrogen, Russia), 0.4 mM dNTPs (Evrogen, Russia), 0.2 μL 5x SYBR Green I (Evrogen, Russia). Amplification was carried out according to the recommended protocol (Morovic et al., 2016):

1.95 °C - 30 s.
2.95 °C - 30 s.
63 °C - 3 min FAM/Green.
72 °C - 1 min.
Repeat 2 step 34 times.
3.72 °C - 10 min.
4.4 °C - hold.

2.6. Enumeration of viable cells using PMA-qPCR

To measure the levels of viable (intact) cells specifically, PMA-qPCR (qPCR with PMA, propidium monoazide) was used according to the PMA manufacturer's protocol (Biotium, Fremont, CA) similarly to the description of (Scariot et al., 2018). The dye was added to the sample to a final concentration of 50 μM and thoroughly mixed on a vortex. The samples were incubated in the dark for 10 min. Then the samples were placed under the light of a lamp at 4200 Lm for 15 min, mixed with a vortex and proceeded to DNA extraction described above. The PMA-qPCR analysis has been performed only for a part of the samples due to the specifics of reagent and food product shipping and sampling schedules.

2.7. 16S rRNA gene sequencing

Amplification of the V4 region of 16S rRNA gene was performed from the DNA samples as described previously (Volokh et al., 2019). The following modification of 515F/806R primers were used: GTGBCAGCMGCCGCGGTAA and GACTACNVGGGTMTCTAATCC. The read length was 252 bp; the coverage was >100,000 reads per sample. The reads are deposited in the Sequence Read Archive (SRA) under project accession number PRJNA658877.

2.8. Sequencing data processing

The obtained reads were analyzed in Knomics-Biota system (https://biota.knomics.ru/) (Efimova et al., 2018) using "16S dada2 V4" analysis type involving DADA2 algorithm (Callahan et al., 2016) followed by blastn (Altschul et al., 1990) to 16S RefSeq NCBI database (O'Leary et al., 2016). The low-abundant species (<500 reads in each sample), non-relevant species detected in the negative control samples as well as the species reported as likely laboratory contaminants (Park et al., 2019; Salter et al., 2014; Weyrich et al., 2019) or present due to well-to-well contamination from other sample types (Supplementary Table 3) were removed from the analysis.

2.9. Statistical analysis

Statistical analysis was performed in R programming language version 4.0.3 (R Core Team, 2013). The presence of probiotic abundance temporal trends in CFU counting, qPCR and PMA-qPCR analyses was analyzed using Mann-Kendall test. The differences between the recipes with two types of starter cultures were assessed with two-sided Mann-Whitney test with *post hoc* one-sided version of the criterion (all time points were pooled).

For 16S rRNA-seq data, abundance differences between recipes were not analyzed due to the strong overrepresentation of *Streptococcus* and subsequent low relative abundance of the remaining components. To analyze the temporal dynamics of each detected taxa, MaAsLin analysis with Benjamini-Hochberg multiple comparison adjustment was used. To

assess the difference in bacterial abundance between the PMA-treated and non-treated samples independently of the compositionality effect, a linear regression was used. For each sample, we constructed linear models predicting the taxon abundance expected using the PMA-qPCR method basing on the values obtained from the experiments without PMA. For each sample, the outlier taxa were identified as those having absolute studentized regression residuals >2.

3. Results

3.1. Bacterial cultivation results were consistent between different media

Cultivation-based methods used in this study included cultivation on MRS-agar and M-RTLV-agar media. M-RTLV-agar allows targeted quantification of the used probiotics (*L. casei, paracasei* and *rhamnosus*) by inhibiting the growth of the starter culture *Lactobacillus delbrueckii* and *L. acidophilus*. Moreover, it allows the visual differentiation between *L. casei/L.paracasei* and *L. rhamnosus*. Since *L. casei* and *L. paracasei* are not capable of fermenting L-rhamnose, in this selective medium they form dark-crimson colonies (Sakai et al., 2010). *L. rhamnosus* is capable of utilizing L-rhamnose and form colonies with a slightly noticeable crimson color, or with a small point of crimson color only in the center of the colony; at the edges the colony are beige.

In our experiments, both dark pink colonies - *L. casei/L.paracasei* - and colonies with a red dot in the center - *L. rhamnosus* - were observed (Fig. 2A and B). Species identification was successfully confirmed by

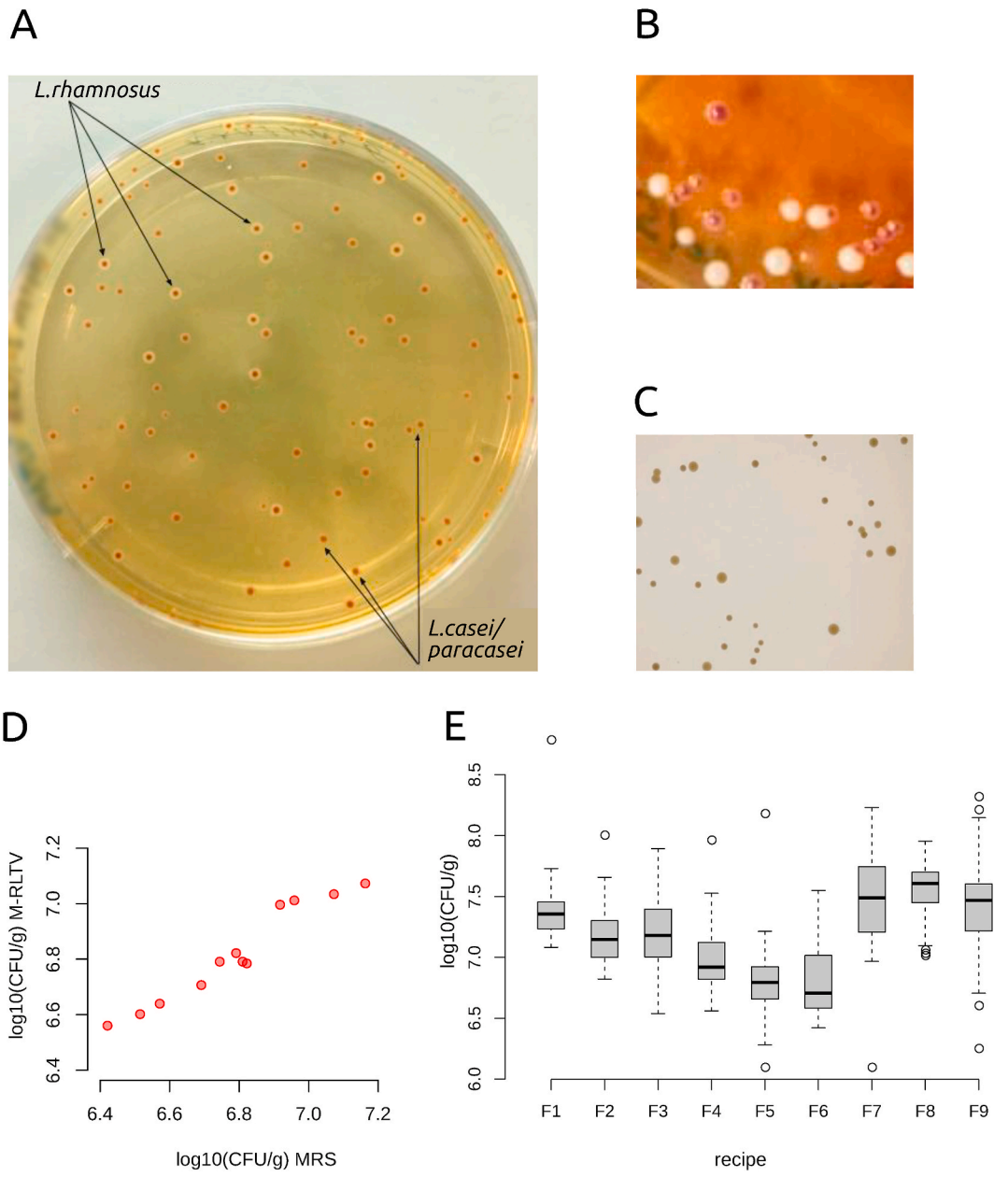

Fig. 2. Cultivation of the fermented dairy product sample. A, B - in a dilution of 1×10^{-6} on M-RTLV agar medium. The colonies of *L. casei/L. paracasei* are dark-crimson and the colonies of *L. rhamnosus* are pale, with crimson color only in the center of the colony. C - on MRS agar medium. The large colonies on MRS are *L. casei, L.paracasei* and *L. rhamnosus*, and small colonies - other lactic-acid bacteria from the starter culture. D - Comparison of the colony counts (log-scale) obtained on MRS medium (only large colonies counted) and on M-RLTV medium ($p = 1.28 \times 10^{-7}$, linear regression). E - The large colony counts (log-scale) in the investigated product pooled across all time points during the shelf-life (on MRS medium). (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

PCR of single colonies. On MRS, small (~0.5 mm diameter) and large (>1 mm) colonies were observed. PCR-genotyping of single colonies showed that large colonies on the MRS were the probiotics, while the small colonies belonged to starter culture microorganisms (Fig. 2C; Supplementary Fig. 1).

Cultivation on M-RTLV medium (N = 12) was conducted for a subset of samples for validation of the less specific cultivation on MRS agar medium (N = 264). The comparison of the two methods showed a high correlation between the number of large colonies (>1 mm) on the MRS medium and the number of colonies on the M-RTLV medium (Pearson correlation coefficient in log scale r = 0.69) (Fig. 2D).

The cultivation on MRS agar with the counting of large colonies was applied for probiotic quantification in all 264 samples. Overall, the values were in the range of 10^6–10^9 CFU/g (Fig. 2E) - on average 7.2 ± 0.4 log10 CFU/g. Thus, the CFU counts were not lower than the values declared by the manufacturer for total probiotic count at the end of shelf-life (10^6 CFU/g). The fluctuations were moderate (mostly around 0.8 log10 CFU/g); most recipes did not show temporal trends. One recipe - F3 - showed slightly negative and one - F2 - slightly positive trend (Fig. 3 and Supplementary Table 2).

Interestingly, the probiotic counts were higher in the recipes with 3-component starter culture of *S. thermophilus*, *L. acidophilus* and *Bifidobacterium animalis* (F7, F8, F9) than in the other recipes based on 2-component starters (F1-F6). The log10(CFU/g) were 7.48 ± 0.39 for the recipes F7-F9 vs 7.06 ± 0.37 for the recipes F1-F6 (Mann-Whitney test, $p = 4 \times 10^{-16}$, n = 264).

Species-specific qPCR analysis of *Lacticaseibacillus* coupled with viability evaluation using PMA during the shelf-life.

Each of the probiotic species, *L. casei* and *L. rhamnosus*, was quantified in all DNA samples, including those isolated with PMA, via a taxon-specific qPCR (the analytical characteristics of qPCR were previously described in (Morovic et al., 2016)). For constructing the calibration curves, dilutions of DNA isolated from pure cultures at concentrations from 7×10^6 to 7×10^2 genome equivalents per µl were used. Each sample was analyzed twice in one amplification run.

3.2. *L. casei* and *L. rhamnosus* levels according to qPCR

The qPCR results for *L. casei* and *L. rhamnosus* showed that each of the species was present in all formulations and persisted over the product shelf life (N = 270). The average values for each recipe were 4.4 ± 0.5, 4.7 ± 0.7 and 5.1 ± 0.6 log10 PCR quantity for *L. casei*, *L. rhamnosus* and their sum, respectively (Fig. 4A).

Similarly to the cultivation results, the qPCR showed moderate fluctuations of the probiotic abundances during shelf life (<1.6 log10 PCR quantity). Slight temporal trends were observed for some of the recipes: negative - for *L. casei* in F4, F5 and F9; positive - for *L. rhamnosus* in F8 recipe (Fig. 3, Supplementary Table 2). As seen, the trends were different from ones observed with CFU-based approach, possibly due to overall low variability of the probiotics abundance (Fig. 3).

According to qPCR, the DNA levels of each probiotic species were higher in the recipes with 3-component starter culture (F7-F9) than in the 2-component ones (F1-F6). For *L. casei*, the log10 values of abundance were 4.65 ± 0.45 for the recipes F7-F9 vs. 4.35 ± 0.64 for the recipes F1-F6 (Mann-Whitney test $p = 9 \times 1\ 10^{-6}$, n = 265). For the *L. rhamnosus*, they were 4.86 ± 0.81 vs 4.67 ± 0.70, respectively (p = 0.0047, n = 269). Although a similar effect was observed during cultivation analysis, the overall correlation between CFU counts and qPCR quantity was not significant: Pearson's r = 0.03 for sum of two probiotics (p = 0.5995).

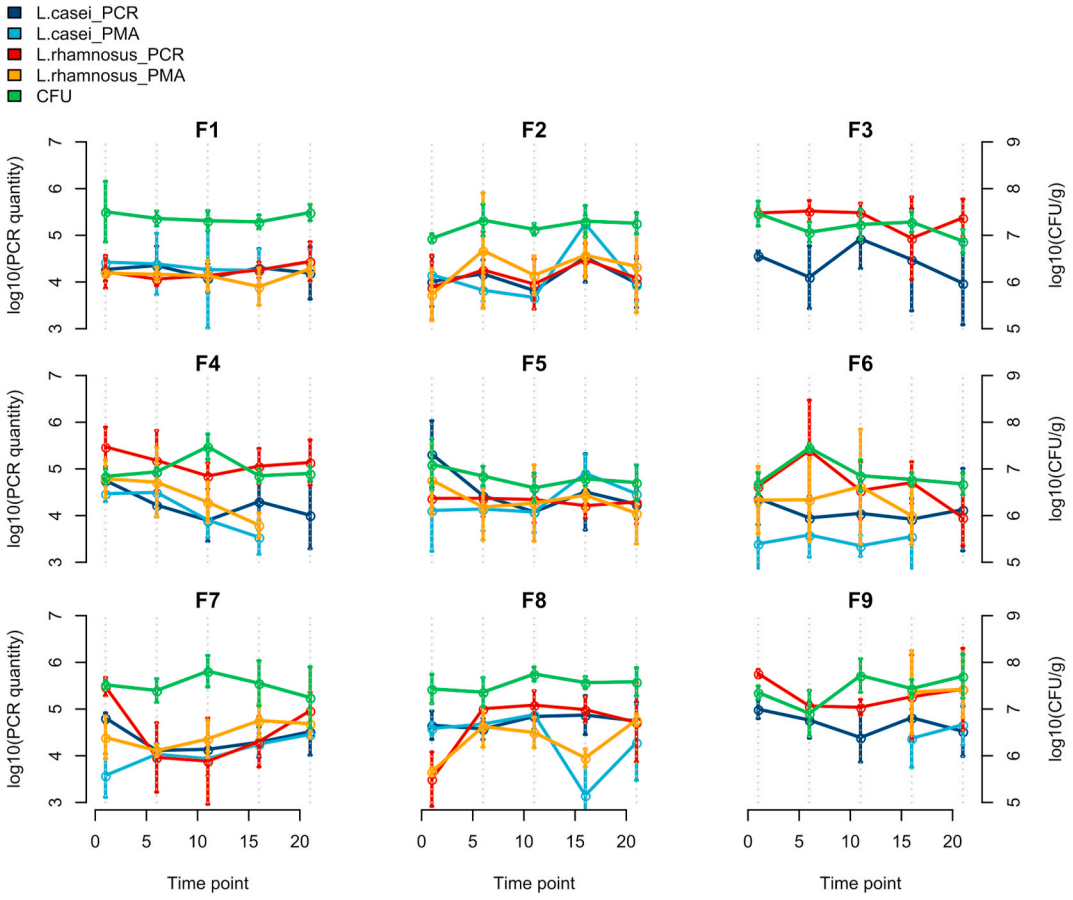

Fig. 3. Probiotic abundance during the shelf-life averaged by the replicates.

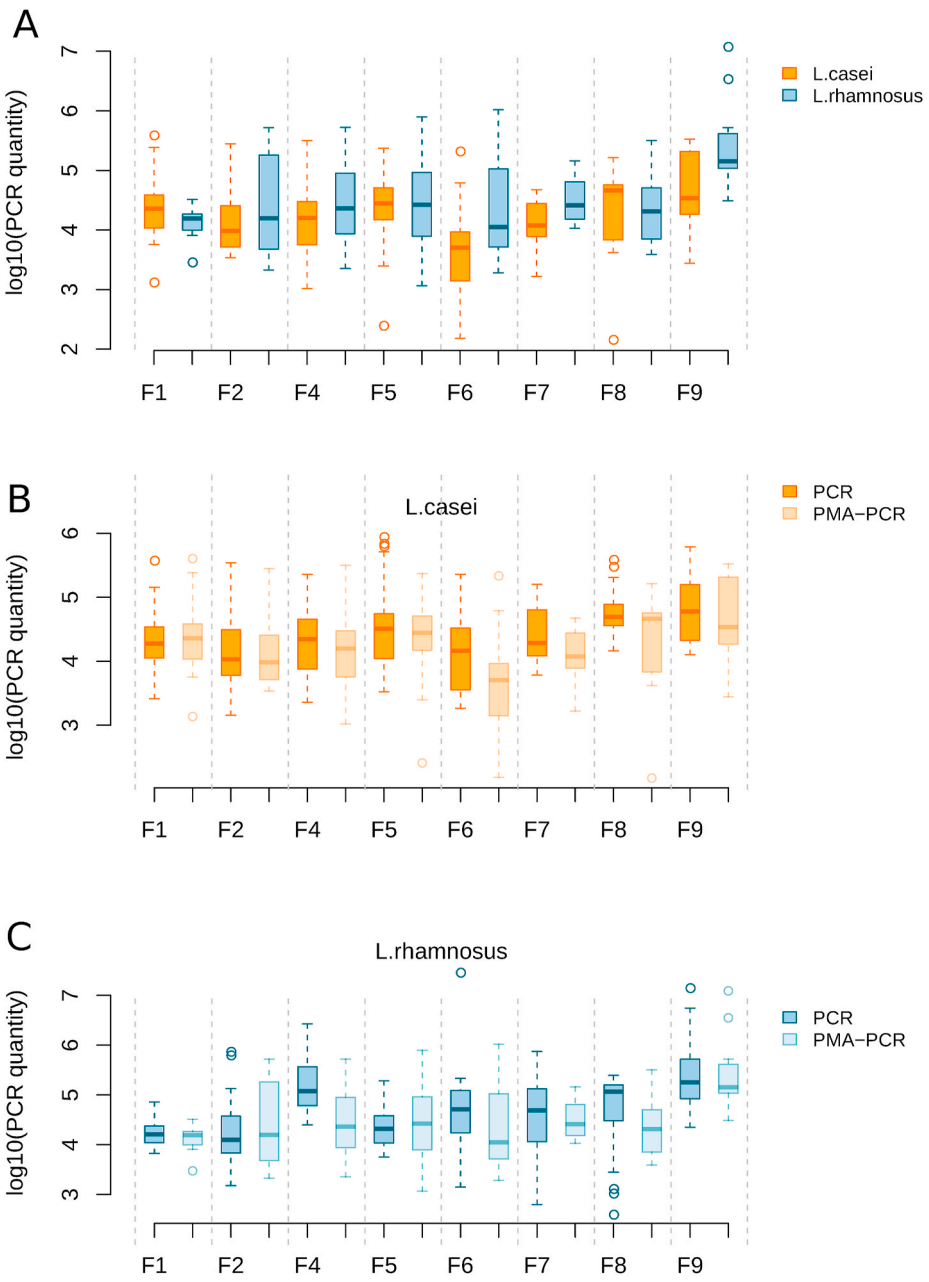

Fig. 4. The levels of probiotic species according to qPCR with and without PMA during the shelf-life. A - The level of *L. rhamnosus* and *L. casei* according to qPCR. B - The level of *L. casei* according to qPCR and PMA-qPCR. C - The level of *L. rhamnosus* according to qPCR and PMA-qPCR.

3.3. Viable *L. casei* and *L. rhamnosus* levels according to PMA-qPCR

Overall, the numbers of viable cells were slightly lower but close to the ones obtained without PMA (Pearson's r = 0.21 for log10 *L. casei* level and 0.16 for log10 *L. rhamnosus* level, N = 150). Unlike in the case of qPCR without PMA, the correlation with cultivation results was significant (r = 0.18 for sum of two probiotics, p = 0.0297). The PMA-qPCR levels were 4.2 ± 0.7 log10 for the *L. casei* strains, 4.5 ± 0.7 log10 - for *L. rhamnosus* and 4.8 ± 0.6 log10 PCR quantity - for the sum of two probiotic species (Fig. 4B and C).

The *L. casei* abundance slightly increased in the F7 recipe and decreased - in F4 recipe. For *L. rhamnosus*, the trends were observed in two of the other recipes: positive - in F8 - and negative - in F5 (Supplementary Table 2, Fig. 3). The trends for F4 and F8 were the same as in PMA-free qPCR but none of these trends were observed in CFU data. For some recipes, the estimated log10 values were decreasing with time for PMA-qPCR analysis, while the common qPCR values were more constant (F4, F8) (Fig. 3) - apparently, reflecting loss of viability by a part of the bacterial population.

Similarly to the results of qPCR and cultivation analyses, the PMA-qPCR levels of probiotics were higher in the recipes with 3-component starter culture (F7-F9). The log10 values of *L. casei* abundance were 4.34 ± 0.66 for the recipes F7-F9 vs 4.15 ± 0.67 for the recipes F1-F6 (Mann-Whitney test, p = 0.0740, n = 149); for *L. rhamnosus* - 4.72 ± 0.71 vs 4.37 ± 0.71 (p = 0.0073, n = 146).

3.4. 16S rRNA gene sequencing

High-throughput 16S rRNA gene sequencing was performed for all recipes (except for F3 due to technical reasons) at the beginning of the shelf life (day 1, with replicates, n = 47) and at the expiry date (day 21, no replicates, n = 15).

3.5. 16S rRNA-seq of starter cultures and probiotic additives

To obtain the exact 16S rRNA gene region sequence for the probiotic and starter culture strains used in the study, these samples were sequenced. The results were in concordance with the microbial composition provided by manufacturers (Table 1 and Supplementary Fig. 2). Particularly, the taxonomic identity of probiotic strains was as claimed. The *L. casei* strains Lc1 and Lc3 had identical sequences of V4 region of 16S rRNA gene region, so could not be distinguished in food samples with this type of assay. However, interestingly, the sequence of *L. casei* strain Lc2 was different from that of Lc1 and Lc3 by 7 nucleotides and thus could be distinguished. According to the information on the label, the S4, S5 and S6 starter cultures should have contained *Streptococcus thermophilus*, *Lactobacillus acidophilus* and *Bifidobacterium animalis*; and the S1, S2 and S3 - *Streptococcus thermophilus* and *Lactobacillus delbrueckii*. These were exactly the bacterial taxa that were identified using the 16S rRNA-seq.

3.6. 16S rRNA-seq of food product

Overall, identification of the NGS reads of the yoghurt showed that *S. thermophilus* dominated all samples (85.38 ± 18.76%). The remaining part of the community was mostly represented by the other starter culture microbes (*L. delbrueckii*, *L. acidophilus* and *Bifidobacterium*): their total proportion was 12.32 ± 15.77%. The relative abundance of probiotics (*L. casei* and *L. rhamnosus*) was significantly lower than of the species from starter culture in total (the average sum of abundance 2.64 ± 6.51%). The sequencing depth of 100,000 reads was insufficient to detect *L. rhamnosus* in most samples (Fig. 5A), so this method did not allow robust analysis of temporal dynamics of the probiotic strains at the provided sequencing coverage.

Compositional nature of the data and high *Streptococcus* relative abundance complicated quantitative comparison of different recipes. However, Fig. 5 suggests that for the recipes with 3-component starter cultures (F7-F9), the total proportion of probiotic species was higher than for the recipes with 2-component starter culture (F1-F6).

3.7. Dynamics of bacterial community identified using PMA-augmented 16S rRNA-seq

In addition to common NGS, the 16S rRNA-seq was also performed after PMA treatment for 6 recipes (F1, F2, F4, F6, F7 and F8) (Fig. 4B, n = 10 samples). Paired comparison of the profiles with and without PMA allowed us to evaluate the contribution of DNA originating from non-viable microbial cells to the total microbiome composition.

According to the linear regression analysis (see Methods), in 5 of these recipes (all but F4) the relative abundance of *S. thermophilus* tended to be lower for the PMA-treated samples compared to untreated samples (FDR-adjusted p = 0.0889). However, even in the PMA-treated samples, the *S. thermophilus* proportion was very high (>40% for all but one sample vs >70% in the non-treated ones) (Supplementary Fig. 3). These results suggest that the major dominance of this microorganism in untreated samples was largely due to the DNA originating from non-viable cells.

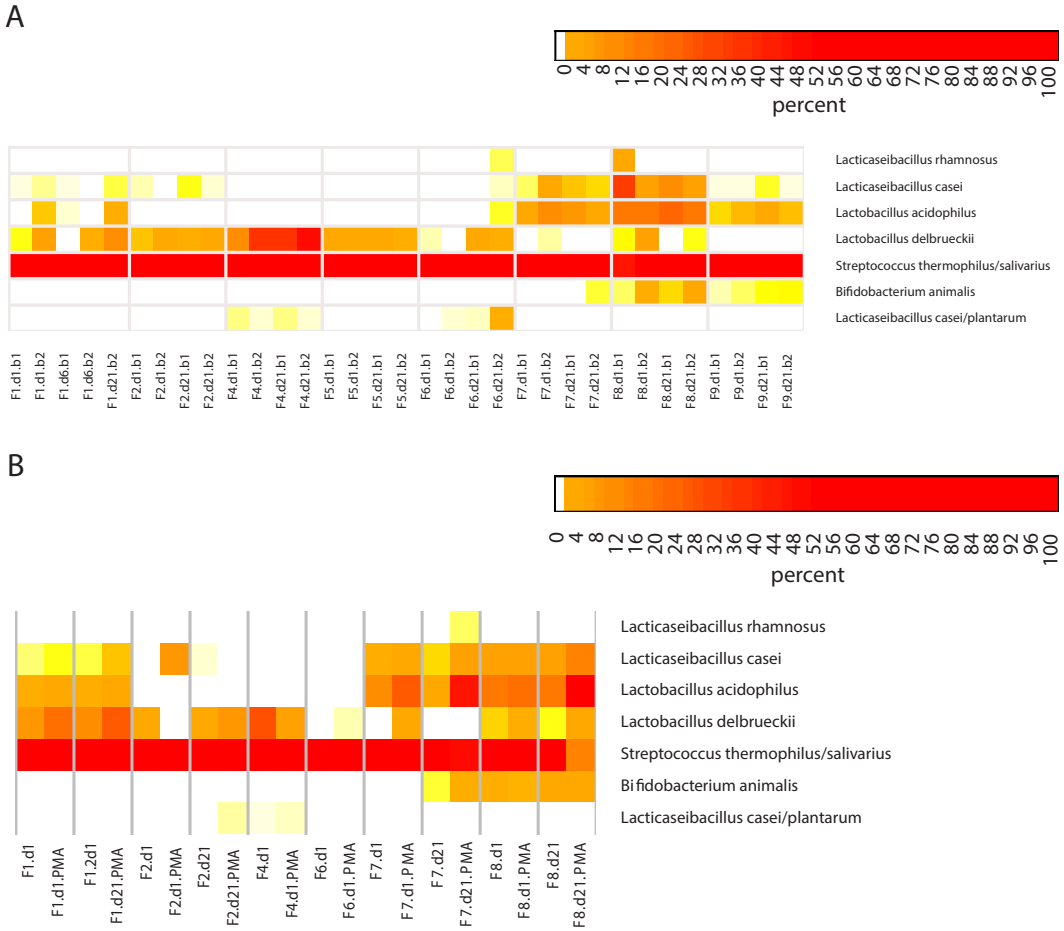

Fig. 5. Taxonomic composition of investigated food products according to 16S rRNA gene sequencing. A - Temporal dynamics: microbiome composition on days 1–21. B - Paired comparison of microbiome composition obtained with and without PMA treatment. In the sample names, the "d …" denotes the days after manufacture and "b …" - the production batch.

An opposite tendency was observed for some taxa from starter culture and probiotic additives. The *L. delbrueckii* relative abundance tended to be higher in PMA-treated samples from F1, *L. acidophilus* - in F7 and F8, *L. casei* - in F2 recipe (FDR-adjusted $p < 0.1$). It suggests that the detected DNA of this taxa was mostly contributed by viable cells. Interestingly, for a single recipe (F4), the *S. thermophilus* was higher in the PMA-treated samples and *L. delbrueckii* - in the untreated.

3.8. Links of product microbiome to the technological factors

To investigate the consistency of formulations, we assessed the contribution of several technological factors to the total microbial composition of the product: batch, time from manufacture date and recipe. As the formulations originally form 2 groups distinct by their targeted species-level bacterial composition (recipes F1-F6 and recipes F7-F9), this analysis was performed in a stratified way within each group. Therefore, the pairwise dissimilarity (Bray-Curtis distance on the level of species) was calculated between the samples of several groups (Fig. 6; see Supplementary notes).

Among the factors that were not nested (starter culture, probiotic and time point), choice of starter culture and probiotic additive contributed most (by explaining 23% and 11% of variability for the recipes F1-F6 and 73% - for F7-F9). Compared to these two factors, the effect of the time point was low (1% for the recipes F1-F6 and 0.3% for - F6-F9; (PERMANOVA analysis)). This suggests that the slight difference in microbial composition of the products inoculated by the same species but different strains persists during the shelf-life.

4. Discussion

There are multiple methods for enumerating probiotics that allow advanced quality control of fortified food products. In the present work, we compared their efficacy on the example of multiple samples of a single product, a drinkable yogurt containing *L. casei* and *rhamnosus*. One of the common methods is cultivation followed by CFU counting. We have validated MRS agar-based large colony counting for probiotic *L. casei* and *rhamnosus* via a PCR analysis of single colonies and cultivation on an alternative medium (M-RTLV agar). Overall for the cultivation results, we discovered that the summary levels of both probiotic species were highly consistent during the shelf life, across the batches and starter cultures; the CFU counts were according to the claims of the manufacturer (on the order of 10^6 CFU/g or higher). Even though the method can be partly automatized by the means of image recognition software, as performed here, it is still rather time-consuming. Moreover, it does not allow to distinguish the probiotic species with standard media. In this regard, new high-throughput and rapid methods for studying the composition of fermented products are of increasing interest.

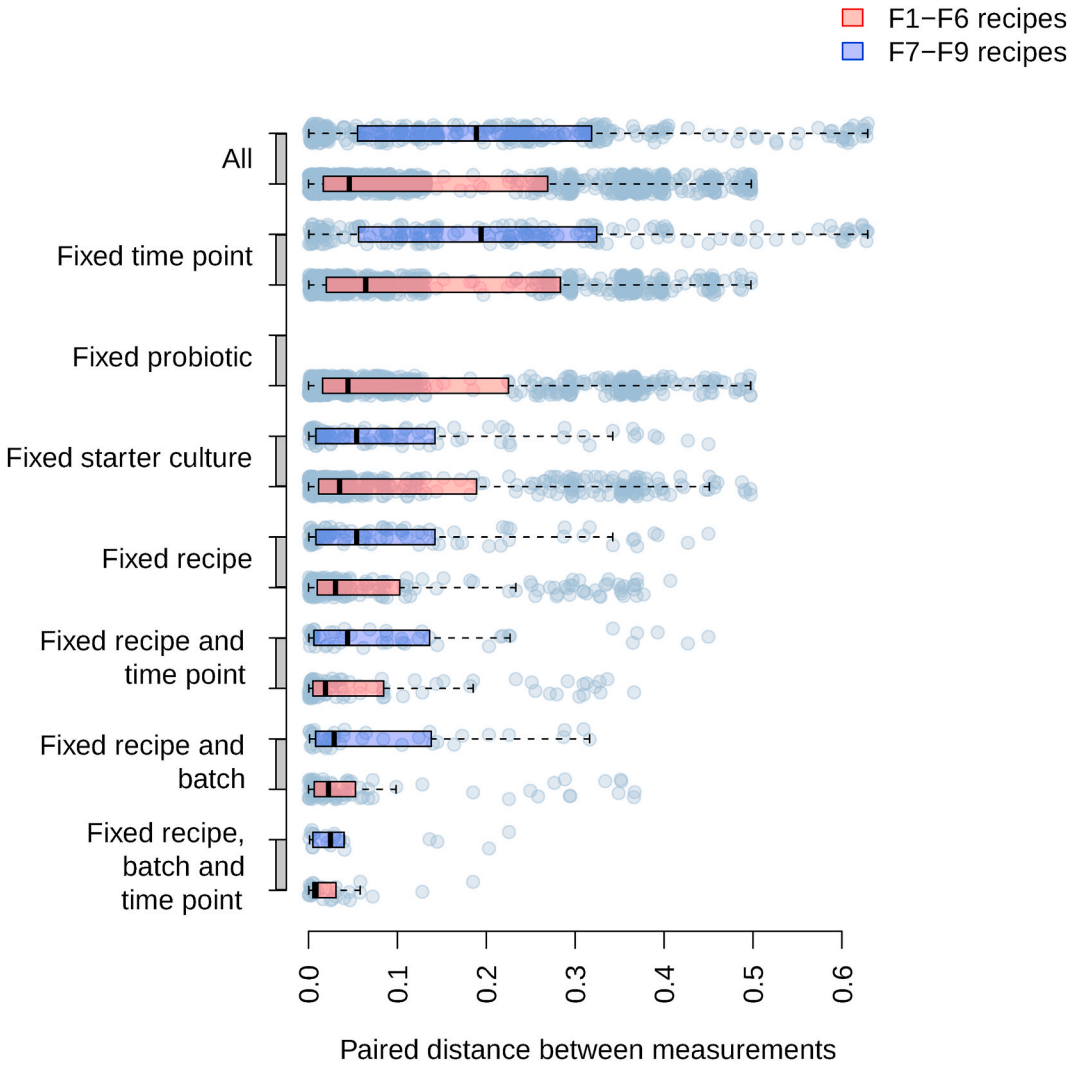

Fig. 6. Contribution of various technological factors to the dairy product microbiome. The variability in composition is assessed via distribution of Bray-Curtis distance between all pairs of samples within the respective groups: F1-F6 recipes (red) and F7-F9 recipes (blue).

One of such methods is taxon-specific qPCR. After validation on pure cultures, we applied it to the product samples. The differences between the formulations were concordant with the results of CFU counting. However, the overall correlation between the CFU counts and qPCR levels was low. We suggest it is due to the overall stability (and hence low variability) of probiotic abundance in the explored samples representing the same product, as well as to the inability of qPCR method to distinguish between viable and non-viable microbes. Overall, the scalability and robustness of the qPCR show it is a good alternative to cultivation for routine application that is more precise by allowing to distinguish between the species of probiotic lactobacilli.

Augmentation of conventional qPCR with PMA pre-treatment yielded highly correlated but slightly lower levels of probiotic DNA suggesting that most probiotic cells in the investigated product were consistently intact during the shelf life and independent of the starter culture choice. Although the PMA-qPCR method combines the advantages of qPCR and CFU counting, use of the PMA increases the cost of the analysis and makes it more vendor-dependent, which is not desired for routine application. Testing a more diverse selection of dairy products with wider varying levels of probiotics is required to make more sound conclusions about the applicability of PMA-qPCR.

As a complement to the above-mentioned targeted methods, a total microbial analysis is sometimes required in the manufacture, for example, for identifying possible food spoilage agents or exploring spontaneous fermentations. The 16S rRNA-seq can be used for such tasks. Due to the compositionality of the microbiome profiles obtained using this method, it does not allow to evaluate the absolute abundance of probiotic and starter culture species making comparative analysis of multiple samples complicated. Augmentation with universal qPCR or flow cytometry can help alleviate this issue. Nevertheless, the method allows to assess the complete diversity of bacterial species in an untargeted way and identify novel candidate targets for qPCR or advanced cultivation techniques.

Our findings showed that the total bacterial content of investigated product, starter cultures and probiotics was in general accordance with the manufacturer's claims. However, 16S rRNA-seq does not perform well for quantifying the probiotics - at least in the cases when their targeted concentration is orders of magnitude lower than one of the starter cultures (which is often the case in the dairy). Certain taxa may appear to be below the threshold of detection despite a high sequencing depth. We observed this effect for *L. rhamnosus* abundance in a number of samples: apparently, due to the dominance of *S. thermophilus* and compositionality, this probiotic was not detectable even with sequencing depth of 100,000 reads and higher.

Some of the original sequencing datasets contained reads matching to negative control samples reads that were excluded. After such filtering, the composition profiles still included low levels of bacterial species not expected from the formulation and likely corresponding to reagent or laboratory microbiome as from the literature (Park et al., 2019; Salter et al., 2014; Weyrich et al., 2019) or present due to well-to-well contamination from other sample types. Investigation of their presence and viability in further studies should be performed based on taxon-specific assays like qPCR. The results suggest that the 16S rRNA-seq is an appropriate method for detecting high-abundance potential contaminants.

The comparison of 16S rRNA-seq with and without the PMA suggested that in most formulations, the proportion of *S. thermophilus* was largely contributed by DNA from the non-viable cells. At the same time, the probiotic lactobacilli and starter culture bacteria had higher relative abundance after the PMA treatment in most of the recipes. This suggests that these taxa were represented by viable cells to a higher extent compared to *S. thermophilus*. However, the effect of the PMA treatment on the results obtained via 16S rRNA-seq was moderate - similarly to qPCR case. We suggest that a more promising application of PMA 16S-rRNA seq is, for example, the investigation of viability of novel taxa detected in complex spontaneous fermentation products.

The accurate identification and verification of the strains declared by the manufacturers was outside the scope of this work. For such tasks, polyphasic methods are often used (Shane et al., 2010), with "shotgun" metagenomics representing a powerful method. Rather, our task was to compare methods suitable for a routine as well as advanced enumeration of defined probiotic species in fermented dairy products. The enumeration of each probiotic species in a multistrain probiotic is important, as some bacteria can be suppressed by others or, on the contrary, acquire an enhanced probiotic potential in the mixture (Mikelsaar et al., 2011). In our study, high abundance of both *L. casei* and *rhamnosus* in investigated products was confirmed culture-dependent and independent methods. The qPCR analysis and cultivation on M-RTLV medium revealed that the levels of each of the *L. casei* and *L. rhamnosus* are maintained high during the shelf life. The results of PMA-qPCR show that the probiotic bacteria cells are mostly viable. Our results suggest that there was no competition between *L. casei* and *L. rhamnosus* strains in the analyzed product.

Finally, we assessed the contribution of technological factors to the microbial composition of the product. The choice of starter culture and probiotic additive had higher considerably effect than the time point suggesting that the formulations remain distinct throughout the shelf life. One of the hypotheses of our study was that the choice of a starter culture can affect the levels of probiotic in a product. The results showed that overall the respective variability was low, but, interestingly, the 3-component starter cultures performed better than the 2-component cultures in terms of probiotic levels maintenance, as confirmed by multiple methods (CFU counting, qPCR and PMA-qPCR, 16S rRNA-seq). For *L. casei*, it could have been biased by the strain difference since the 3- and 2-component formulations contained different strains of the probiotic (Lc3 vs. Lc1 or Lc2, respectively). But at least for the *L. rhamnosus*, we can suggest possible superiority of a more diverse starter culture for the probiotics survival.

5. Conclusions

General stability of microbial composition in drinkable yogurt during the shelf life and across formulations was confirmed by cultivation, qPCR analysis and 16S rRNA gene sequencing. The summary as well as individual levels of probiotic *L. casei* and *rhamnosus* remained high during the shelf life. The PMA-qPCR suggests that most of their cells were viable. The differences in total microbial composition between the formulations persist through the shelf life, while the survival of added probiotics appears to be dependent on the taxonomic richness of the starter culture.

The taxon-specific qPCR is a promising method for routine use as a complement to cultivation. Although our study showed that the use of PMA is not necessary, further studies are required to evaluate if this observation is generalizable for other *Lacticaseibacillus* strains, other species and product types. The high-throughput 16S rRNA-seq was not efficient for tracking temporal dynamics of probiotics, due to their low levels compared to other microorganisms and inherent compositionality of the microbiome sequencing data.

Due to overall low variability of the microbiome content of investigated product, further studies of an extended set of fermented dairy products with wider quantitative and qualitative microbial diversity will be required in order to come to more generalizable conclusions on methods' application, including use of 16S rRNA-seq for detecting contaminants and PMA - for quantifying viable probiotics.

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CRediT authorship contribution statement

Yulia Berezhnaya: Conceptualization, Methodology, Project

administration, Funding acquisition, Writing – original draft. **Irina Bikaeva:** Investigation, Formal analysis, Writing – original draft. **Anastasiia Gachkovskaia:** Investigation, Visualization. **Artem Demidenko:** Methodology, Investigation, Writing – review & editing. **Natalia Klimenko:** Software, Formal analysis, Data curation, Visualization, Writing – original draft, Writing – review & editing. **Alexander Tyakht:** Conceptualization, Methodology, Project administration, Writing – original draft, Writing – review & editing. **Olesya Volokh:** Conceptualization, Methodology, Project administration. **Dmitry Alexeev:** Conceptualization, Methodology, Project administration.

Declaration of competing interest

The study was conducted during a commercial contract research by Knomics LLC for PepsiCo R&D. Yulia Berezhnaya is an employee of PepsiCo, Inc. The views expressed in this paper are those of the authors and do not necessarily reflect the position or policy of PepsiCo, Inc. Natalia Klimenko and Alexander Tyakht are employees of Knomics LLC. At the time of the study, Irina Bikaeva, Anastasiia Gachkovskaia, Artem Demidenko and Dmitry Alexeev were employees of Knomics LLC. At the time of the study, Olesya Volokh was an employee of PepsiCo, Inc.; at the moment of article preparation, Olesya Volokh is affiliated to Lomonosov Moscow State University.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.lwt.2021.111750.

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