The microbial composition of kefir fermented from raw milk

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Abstract:

Aim:
This research aims to elucidate the microbial composition and ratio between myco- and microbiota abundance of two raw milk kefir-style beverages (RMK). Produced by backslopping a traditionally with kefir-grains fermented raw milk kefir or by a commercially available defined starter culture. Additionally, kefir from pasteurized milk (PMK) was analysed to determine specific effects of raw milk on the microbial composition.

Methods:
DNA extracted from kefir beverages was artificially spiked with a microbial standard containing known concentrations of fungal and bacterial genomic DNA. In order to avoid over or under spiking of the samples, a specific amount of spike-in was added in proportion to the sample bacterial genomic DNA content determined by qPCR, followed by metagenomic amplicon sequencing of the bacterial V3-V4 region of the 16S rRNA gene and the fungal ITS1 region.

Results:
Backslopping of traditional kefir changed the microbial composition with a decrease of *Lactobacilli* and an increase of *Lactococcus* species. Defined culture based RMK contained all the myco-microbiota present in the added starter culture and raw milk specific microbes, such as *Galacotomyces, Streptococcus dysgalactiae, Pichia kudriavzevii* and *Staphylococcus sciuri* were detected in end products implicating their contribution in the fermentation process. No differences were observed in bacterial genomic DNA content between PMK or RMK for both style kefir beverages. Higher relative amounts of mycobiotal spike-in were found in both styles of RMK compared to PMK, implicating lower concentrations of fungi in RMK.

Conclusion:
This study indicates that the majority of microbial species found in RMK and PMK are similar albeit in different proportions. Furthermore there appears to be a higher amount of fungi present in PMK. These findings could have implications regarding the observed beneficial health effect of RMK consumption, but future studies are required to determine the exact molecular immunological mechanism.

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Introduction

Raw Milk (RM) is a complex emulsified colloid liquid secreted by the exocrine mammary glands which is not-homogenised nor pasteurized. The composition of RM varies during the lactation period and is species-specific (Pietrzak-Fiećko & Kamelska-Sadowska, 2020). Many milk compounds for example miRNAs are conserved between species (van Herwijnen et al, 2018) and are primarily intended for mammalian infants, as the main source of nutrition promoting infant growth, health and development through a myriad of biological active milk compounds. These active factors can reside in the form of small single soluble compounds, in macromolecular structures and in complex assemblies for example in micelles and vesicles. In addition to these compounds whole pro-eukaryotic cells are present in RM (Fox et al, 2015).

Microbial exposure is essential for microbial colonisation which is believed to influence the immune system in its development and in maintaining immune homeostasis. Altered gut colonisation is associated with increased risk of immune related diseases (Tanaka & Nakayama, 2017). Various studies showed associations with reduced incidence of these non-communicable diseases by children who consumed bovine RM or farm milk (Loss et al, 2011; Illi et al, 2012). Others found farm environmental effects, and even indoor bacterial microbiota in urban areas reassembling a farm environment to be explanatory for the observed protective effects (Kirjavainen et al, 2019). Dietary intervention in the form of RM can potentially prevent or alleviate immunological related diseases. Although some studies claim RM consumption to be beneficial for health, RM consumption is controversial because of potential public health risk posed by the potential presents of zoonotic pathogens causing foodborne illnesses. Improvements in animal husbandry led in several countries to the eradication of: bovine tuberculosis, brucellosis and enzootic-bovine-leukosis, in other regions these zoonotic organisms are still present, complete eradication is unlikely since wild animals can be a reservoir for several pathogens (European commission, 2019*;* Mailles et al, 2012). The risk for illness caused by unpasteurized dairy and its products, is in the United States found to be > 800 times greater compared to pasteurized milk (Costard et al, 2017). 44% of the RM obtained directly from retail sales in England were of unsatisfactory microbial quality and 3.2% were found to be potentially injurious to health (Willis et al, 2018). Even under satisfactory hygiene dairy practices, pathogens such as the *Escherichia coli O157:H7* can reside in RM which was linked to an outbreak in England (Treacy et al, 2017). Safe consumption of RM solely based on proper hygiene is, therefore no guarantee for the complete absence of zoonotic pathogens.

Traditionally, fermentation was used to preserve dairy and to change its functional and sensorial attributes. Kefir is one of these traditional fermented dairy products, originating from the Caucasus and have been produced for centuries. Kefir can be produced by fermenting (raw) milk by addition of kefir-grains (KG), a polysaccharide matrix containing a symbiotic consortium of bacteria and yeast. Variations of kefir beverages are produced for example by addition of freeze-dried starter culture or by backslopping prior fermented dairy beverages.

Earlier research by S. Abbring found that ovalbumin sensitized mice fed RM kefir (RMK) had significant reduced ear-swelling compared to pasteurized milk kefir (PMK). In Dutch adults the consumption of RMK improved the perceived health and several other self-reported health indicators (Baars et al, 2019). The microbial composition of RMK could be a component responsible for the observed health effects.

Knowledge about the microbial composition of RMK is limited. Therefore this study focuses on elucidating the microbial composition of RMK produced by either a commercially defined freeze-dried or an inhouse backslopped kefir-grain based culture. The composition is determined by metagenomic amplicon sequencing of the bacterial v3-v4 region from the 16s rRNA gene and the fungal internal transcribed spacer 1 (ITS). In order to determine the fungal abundance compared to bacteria, a genomic DNA (gDNA) spike-in containing an artificial microbial community composed of micro- and mycobiota is added. The spike-in is added in proportion of the bacterial genomic content quantified by a SyBr based quantitative polymerase chain reaction (qPCR) of the 16S rRNA gene. Additionally PMK of both kefir-style beverages and several shop-bought PMK are analysed and

compared to RMK. After the description of the microbial composition, a molecular mechanisms is proposed that could possibly explain the health benefits of RMK consumption descripted in previous studies.

Materials and Methods:

Samples and Kefir processing:

Samples used in this study were provided by Ton Baars in cooperation with the Raw Milk Company. The two different styles of kefir are produced named Backslopped Kefir-grain based Kefir drink (BKK) and Defined Culture Kefir drink (DCK). DCK is produced by addition of a defined starter culture (DSC) from Chr. Hansen eXact® 2 kefir (Hoersholm, Denmark) to RM which is held at 28°C for 24 hours. Afterwards the product is slowly mixed and chilled to 4°C. The manufacture of DSC declares in the product information (Version: 2 PI EU EN 03-03-2018) the presence of five different bacterial species: *Leuconostoc, Streptococcus thermophilus, Lactococcus lactis* subsps*. cremoris; lactis* and biovar*. Diacetylactis* and the yeast *Debaryomyces hansenii*.

The in-house "Mother Culture" (MC) is used to produce BKK. MC is added to RM held at 24°C for 36 hours afterwards the product is gently mixed and chilled to 4°C. MC is produced by inoculating a small batch of RM with an in-house kept kefir-grain (KG). The RM used to produce kefir originated from two organic dairy farms (E) and (J) at two different timepoints in September 2019 referred as 1 or 2. Samples during the fermentation were taken at different timepoints, including samples during storage of the kefir product in a bottle for up to three weeks. For this study four batches of RM were pasteurized (PM) for the production of PM kefir (PMK) based on BKK for J1, E2 and DCK for E1, J2. A schematic overview of the fermentation process and the analysed samples are displayed in figure 1. Additionally, three commercial PMKs, brand A, DM and K, were analysed.

In order to determine the DNA isolation efficiency, read depth and taxonomic assignment of the amplicons a mock microbial community from zymoBIOMICS™ Microbial community standard II log distribution (Irvine, United States) and the zymoBIOMICS™ Microbial community DNA standard II log distribution (Irvine, United States) referred as spike-in was used. The spike-in was added to determine the proportion between the amplicons of the V3-V4 region of the 16S rRNA gene and the ITS amplicons. Three duplicates of J1, J2 and E2 PMK were used to evaluate the technical reproducibility of this study.

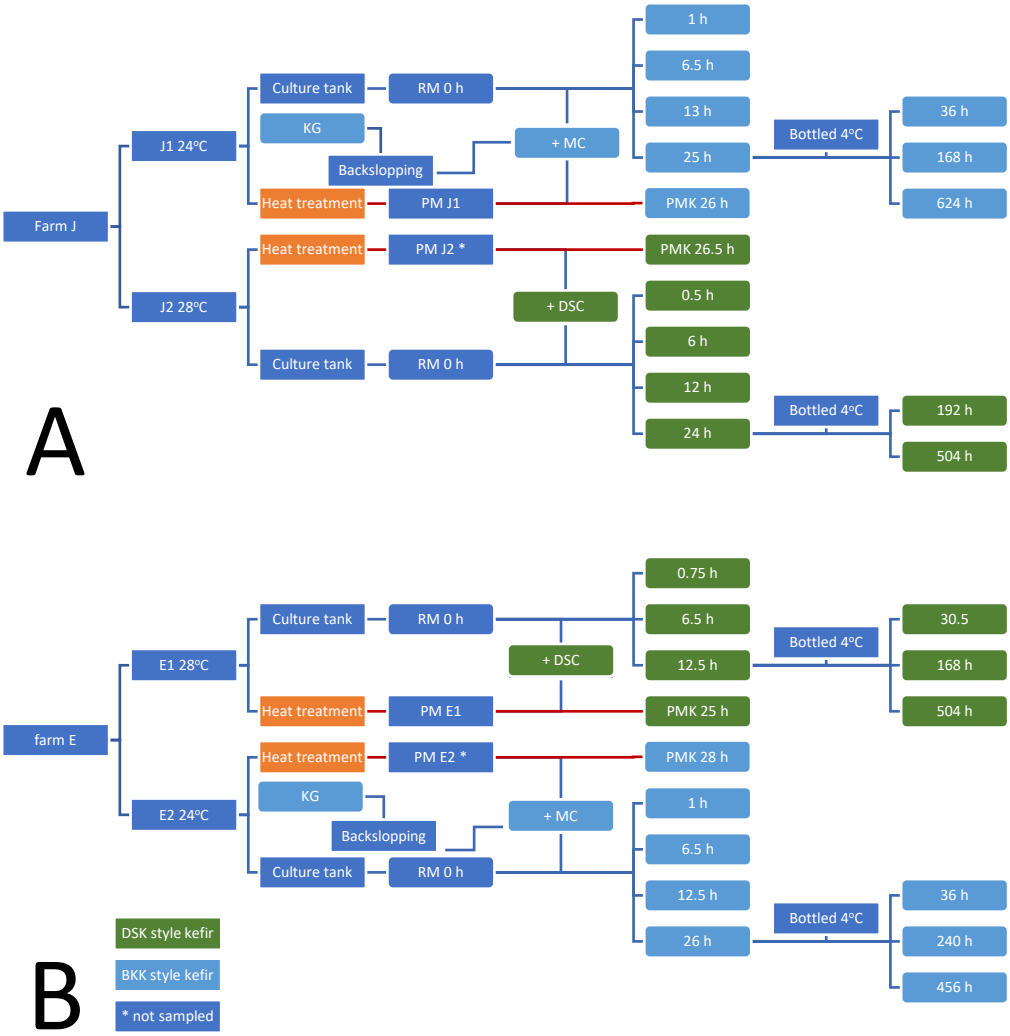

*Figure 1 Schematic representation of the two styles of kefir fermentation for dairy farm J (A) and dairy originated from E (B). Green boxes indicate DSK and light blue BKK style of fermentation. The microbial community of the samples in rounded edged boxes are displayed in this study. The batches of starters cultures are date specific and correspond with the displayed fermentations. Several time points have been translated from weeks to hours. PM with a * were not provided for analysis.*

DNA extraction

The Norgen Biotek Milk bacterial DNA isolation kit (Thorold, Canada) was used with modifications of the manufactures protocol. The following amount of samples were used. For milk 1 ml per sample, for fermented samples 0.2 ml with the addition of 0.8 ml phosphate buffer. For the defined starter culture 20 mg was added to 1 ml phosphate buffered saline solution and vortexed vigorously and 200 mg of frozen KG was added to 0.8 ml phosphate buffer. The manufacturers recommendation of 75 µL mock microbial community suspension was used what is supposed to result in a total of 220ng of genomic DNA. All fermented samples were passed through a 21-gauge syringe to reduce the viscosity. After centrifuging for 2 min at 20.000 G the fat fraction and supernatant was removed. The cell pellet was resuspended with 100 µL resuspension solution A from the DNA isolation kit and was transferred towards a 2 ml screw cap tube preloaded with 0.5 g 0.5 mm acid washed, autoclaved glass beads. The samples were chilled on ice for 1 minute followed by a 10 second bead shaking treatment with the speed set to 6 meters per second on a MPBio Fastprep®-24 5G Instrument (Solon, United States). The cycle of chilling and shaking was performed eight times. After the mechanical cell lysis the manufactures protocol for gram positive and unknown strains of bacteria from the DNA

isolation kit was continued with the addition that only the lysate and not the glass beads were transferred to the binding column.

qPCR based addition of genomic spike-in:

The aimed amount of spike-in derived reads per sample was set on < 10 % of the reads per sample. Therefore, a quantitative SYBR based qPCR on the 16s rRNA gene was deployed on the gDNA from the samples and for the 5 times 10-fold serial diluted spike-in. Per reaction 1 µL gDNA template and 19 µL mastermix was used per reaction, which consists of 10 µL SYBR mastermix from Meridian bioscience™ SensiFast SYBR® Hi-Rox-Kit (London, United Kingdom) 0.8 µL forward F357: "CCTACGGGAGGCAGCAG" (10 µM), 0.8 µL reverse R518 "ATTACCGCGGCTGCTGG" (10 µM) primer and 5 µL nucleotide-DNase free water. The qPCR was performed on an Applied biosystems ™ 7300 RT-PCR system (Waltham, United states) with the amplification setup of an initial start for 10 min at 95°C followed by 40 amplification cycles with denaturation at 95.0 °C for 5s, annealing at 58.0°C for 10s and elongation for 30s at 73.0°C. Results were analysed on a Windows 10 machine running SDS version 1.4 with the cycle threshold (Ct) level set automatically. The Ct values were used under the presumption that all samples had a similar amplification efficiency of $R^2 = 2$. Therefore the Ct of the added dilution of the spike-in was aimed to be 3.3-6.6 Ct higher compared to the sample Ct. What would translate to 1-10 % of the 16S rRNA reads. If a sample Ct was not within this range compared to the highest available concentration of spike-in, a 10 times dilution of the sample was used. The spike-in was added to all samples except for MC, DSC, RM and PM. A complete overview of the sample Ct values and estimated and measured concentration of added spike-in can be found in the supplementary excel file:

DNA Quality control, library preparation and sequencing:

DNA quality control, metagenome amplicons amplification, library preparation and sequencing was performed by Macrogen (Seoul, Korea). The double-stranded DNA binding dye, Picogreen (Invitrogen, cat.#P7589) (Walthan, United States) was added to the samples DNA and was quantified by Victor 3 fluorometry (Waltham, United States). Amplicons of the V3-V4 region of the 16s rRNA gene and the and the ITS1 region and library prep were generated by Herculase II Fusion DNA Polymerase Nextera XT Index Kit V2 (Illumina, San Diego, United states) according to the 16S Metagenomic Sequencing Library Preparation Part # 15044223 Rev. B protocol from Illumina. The products of the library prep were validated by a screentape on a Agilent Tapestation D1000 (Santa Clara, United States). Paired-end sequencing was performed with 2x 300 base pairs (bp) per sequence on the Illumina MiSeq System platform. The bcl2fastq package from Illumina was used to demultiplex the sequence data and to convert the base calls binary into FASTQ files.

Data processing:

Demultiplexed and adapter trimmed sequences were visualized and loaded into Qiime2-2020.2 (Bolyen et al, 2019) through the paired-end manifest method. Based on the quality plots, sequences were truncated at the median nucleotide Phred score of <30. The primers were trimmed of the sequences according to primer length. The trimmed sequences were denoised, paired-end joined and chimeras removed by the DADA2 plugin (Callahan et al, 2016) Taxonomic annotation of the amplicons was performed by a Naive Bayes based model, trained on the 16s V3-V4 region extracted from the SILVA 138 database (Quast et al, 2012). For the ITS classification the model was trained on full length sequences from the dynamic clustered UNITE-V8 database (Nilsson et al, 2019). Taxonomic classification was performed on an Amazon Web Services, AWS EC2 instance: r5ad.xlarge with 100gb attached storage with the Amazon Machine Image, Oregon i-074eb5f908da8ceae QIIME 2 Core - 2020.2 (1583437115). The feature table was exported to a biological observation matrix (.biom) file and converted into a tab separated file which was concatenated with the assigned

taxonomy and amplicon sequence variant (ASV). The spike-in ASV were identified and the total percentage spike-in per sample was calculated, afterwards these ASV were removed. ASV were blasted against the National Centre for Biotechnology Information (NCBI) database and the taxonomy was manually adjusted if there were discrepancies or led to confident taxonomic identification of unknown sequences. The relative abundance (RA) of ASV per sample were calculated by:

reads ASV / total non-spike-in ASV reads per sample

And the ratio ITS/16s is defined by:

Spike-in ITS % / Spike-in 16S%

Visualization were produced by (Graphpad Prism version: 6) (San Diego, United States)

Data availability:

Al raw sequence data generated in this project are available at NCBI under the bioproject number: PRJNA716278. The ASV and additional sample information are present in the supplementary excel file.

Results

DNA extraction efficiency, dynamic detection range and technical variation.

In order to determine the DNA extraction efficiency, detection range, taxonomic classification and the presence of experimental induced microbial contamination DNA from a mock microbial community was isolated and sequenced including the mock microbial DNA standard. The extracted purified DNA of the mock microbial community was 1.909 ng/µL resulting in a total amount of 86 ng what is ~ 36% of the expected total of 220 ng. In both mock communities six of the eight bacteria and both yeasts were detected. The lowest abundant, detected bacteria was *Lactobacillus fermentum* with the abundance of $2.5*10^{-4}$ derived from approximately $1.5*10^{4}$ cells and the most abundant was *Listeria* with an abundance of $9.6*10^{-1}$ with a cellular load of $1.1*10^{8}$. The dynamic detection range is therefore roughly 10^{4}. The genus of all members of the detected mock community were correctly assigned and the correct species annotation were allocated to *Lactobacillus fermentum* and *Saccharomyces cerevisiae*. *Cryptococcus neoformans* is incorrectly classified as *Cryptococcus gatti.* Eight unexpected ASV were detected in the mock microbial community standard and one in the DNA standard. Which could be an indication of contamination.

Variation greater than 30% have been detected in the bacterial mock microbial community and DNA standard compared to the expected composition (figure S1). This indicates that alterations in the relative abundance and thus a technical bias is present in the microbial composition. Variation in DNA extraction, library preparation and sequencing was determined by the use of duplicates. This indicates that in the PMK 28 duplicate eighteen additional bacterial ASV were present of which eight were solely found in the duplicate. The duplicate of PMK 26 contained one ASV less and in PMK 26.5 no differences was observed in detected ASV. The bacterial relative composition are similar between the samples and duplicates (figure S2). This could be indicative of a minimal technical induced variation in the detected bacterial composition. The used ITS data analysis is insufficient causing technical induced unique sequences therefore no conclusive assumption can be made regarding ITS ASV. The percentual difference between the samples and duplicates ITS/16S spike-in ratios are 10%, 35% and 49%.

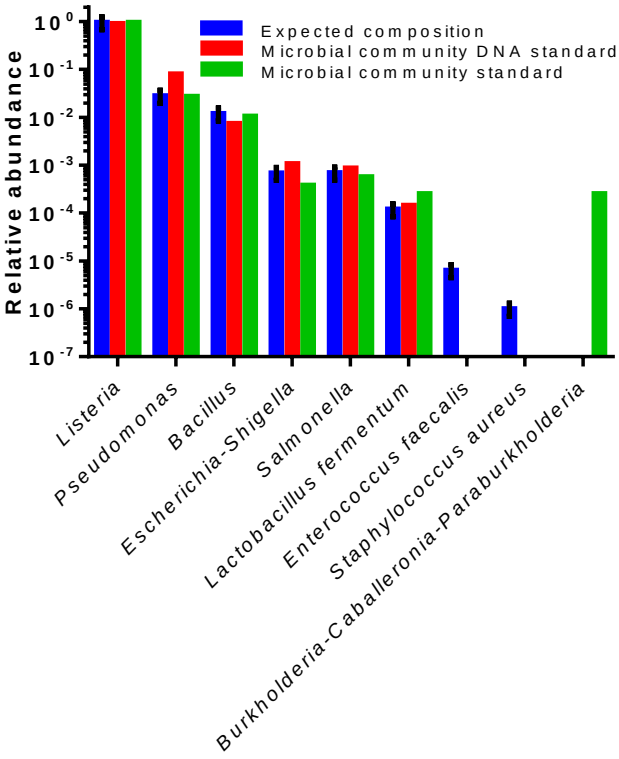

Figure S1: The 16s rRNA V3-V4 region based bacterial composition of the mock microbial community in green, microbial DNA standard in red and the expected composition in blue the wishkers indicate 30% deviation in the relative abundance as indicated by the manifacturer.

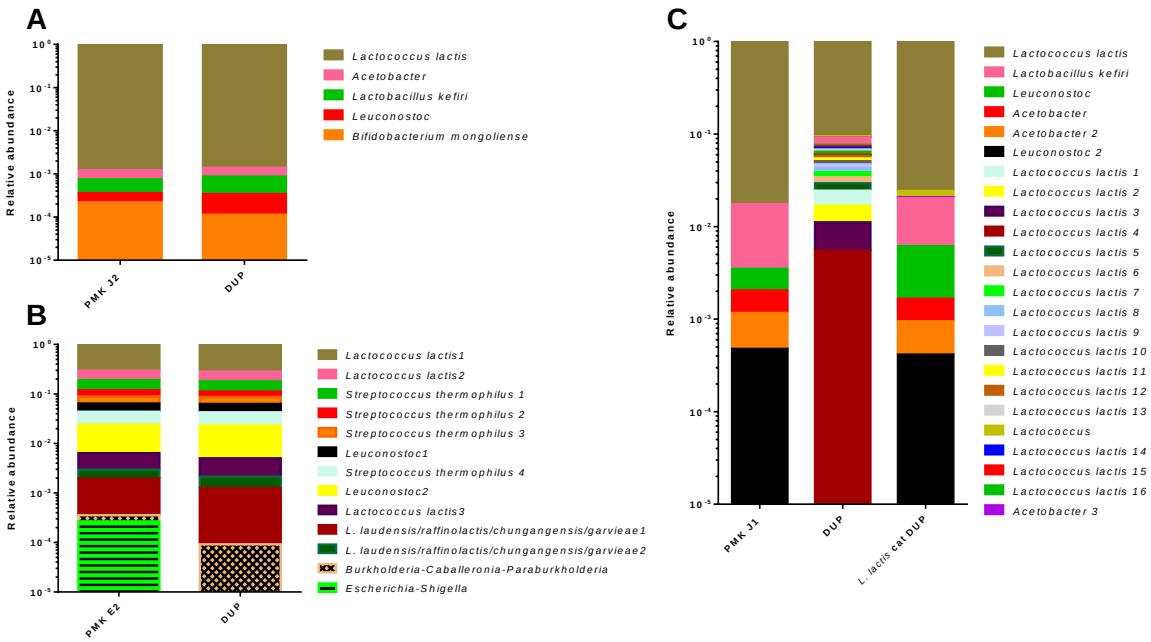

Figure S2: The bacterial composition of three PMK and the corresponding duplicate displayed in ASV. A J2, B E2 and C J1 the additional bar includes concatenated L. lactis ASVs.

Similar bacterial load in different styles of kefir beverages

Samples obtained at several time points during fermentation were analysed for pH, culture dependent microbial cell counts and the 16S rRNA gene based qPCR was used as an culture independent method to determine the bacterial concentration. BKK based fermentation of RM (Figure 2AB) seems to acidify more rapidly and the added starters culture contained a higher concentration of bacteria determined by Ct values compared to DSK style fermentation (figure 2CD). BKK fermentation with dairy from J1 (Figure 2A) contained after one hour a total plate count of $5.5*10^3$ CFUs and dairy from E2 $4.0*10^7$ CFUs (Figure 2B). The 16S rRNA gene based Ct value, however did not showed a similar difference. Samples taken after 12 hours during the fermentation process had an average Ct value of 10.81. BKK and DSK from raw or pasteurized milk does not seem to differ in Ct values, what is indicative of similar concentration of bacterial genomic content. Commercial raw milk yoghurt and PMK from brand K had similar Ct values (Supplementary excel file). Commercial pasteurized milk kefir A, DM and pasteurized milk yoghurt had higher Ct values what implies lower bacterial abundance (Supplementary excel file). Interestingly the total plate count shows a multitude of 10-100 times more colony forming units (CFU) per ml for BKK compared with DCK, this difference is not found in total DNA (Supplementary excel file) or bacterial genomic content, where a minimum reduction of 3.3 in Ct value would be expected. The pH of both kefir-styles is within 24 hours below 4.5. During storage of bottled RMK a reduction of CFUs grown on lactic acid bacterial media is found, although no apparent differences were observed in total plate counts. Yeast counts seem to be stable or are increased during storage.

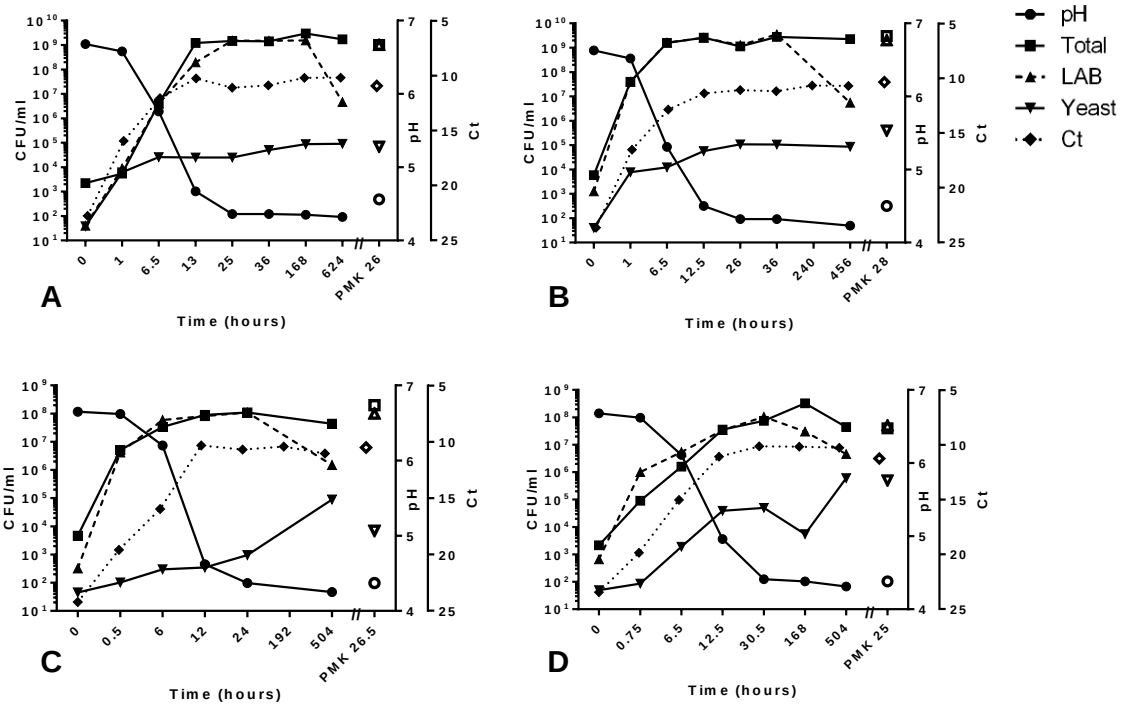

Figure 2: Microbial plate counts, 16S rRNA gene qPCR Ct values and pH during fermentation. The total plate count is displayed by squares, yeast count by downwards triangles, LAB count by upwards triangles on the left y-axis in in CFU per ml. On the right y-axis PH is indicated by circles and on the inversed right y-axis the Ct value by diamonds. The X-axis displays the time in hours after addition of the starter culture during RM kefir fermentation, of A,B) Dairy from J1 and E2 during BKK fermentation. C,D) Dairy from J2 and E1 during DCK fermentation. PMK symbols are similar as RMK but are unfilled. Raw milk is represented by 0.

Raw milk derived myco-microbiota in defined culture based kefir.

In low relative abundance several raw milk derived myco-microbiota ASVs are detected in DSK style raw milk kefir which has not been detected in the added DSC or in pasteurized milk kefir.
RM harbours a diverse microbial community albeit in low absolute concentrations indicated by colony counts and 16S rRNA gene qPCR Ct values (Figure 2). E1 contained a higher bacterial richness, defined as amount of taxa and or ASVs compared to J2 and vice versa for fungal richness (Supplementary excel file). E1 and J2 share 19 identical bacterial ASVs of which 11 were found in all RM samples. Including the in DSC identical ASV *Lactococcus lactis 1.* The top 80 % of the relative abundant RM ASVs and ASV which are detected during the fermentation process are displayed in the microbial composition (Figure 3).
The DSC which was added to RM and PM is composed of eleven bacterial ASVs identified as four different bacteria: *Streptococcus thermophilus* '1, 2, 3, 4', *Lactococcus lactis* '1, 2', *Lactococcus* '2, 4' and *Leuconostoc* '1, 2' (Figure 3AB). Besides bacteria DSC contains fungal ASV annotated as the yeast *Debaromyces 1* (Figure 3 CD). In low relative abundance were *Kazachstania turicensis, Cladosporium 3, Erythrobasidium hasegawianum* and *Saccharomyces cerevisiae cat* detected in the DSCs and flagged as potential contaminants.
During fermentation a decrease in richness is observed resulting in a microbial composition of in DSC detected ASV, with a total RA > 0.9 in all DSC based samples. For E1 after 30 hours of fermentation three additional bacterial ASVs: *Lactococcus lactis 3* (6.1 x 10^{-3} RA) *Lactococcus 7* (7.60 x 10^{-3} RA) and *Streptococcus dysgalactiae* (7.89 x 10^{-5} RA) were identified compared to PMK after 25 hours of fermentation or the added DSC (Figure 3A). The composition of PMK 25 seems to be more similar to the DSC composition in terms of ASV and their relative abundance compared to RMK E1. One additional bacterial ASV: *Dadabacteriales* (6.17 x 10^{-5} RA) was detected in PMK 25. *Lactococcus lactis 3* (3.68 x 10^{-3} RA) and *Lactococcus 7* (5.61 x 10^{-3} RA) were additionally present in J2 24h compared to the added DSC. However in PMK 26.5h *Lactococcus lactis 3* (3.40 x10^{-3} RA) was similarly present and additionally the not in spike-in detected ASV *Escherichia-Shigella 1* (2.66 x 10^{-4} RA). The solely in dairy from J detected *Staphylococcus sciuri* was interestingly, present in J2 0, 0.5, 6 and 504 hours after addition of DSC. The overall bacterial composition is remarkable stable after 24 hours of fermentation for both DSC fermentations.
Several fungal ASVs were unique for each sample but had similar associated taxonomic classifications and where concatenated which is indicated in the annotation as *cat. Galactomyces* was used to annotated and concatenate the unique ASV which were identified as *Geotrichum candidum, Galactomyces geotrichum, Galactomyces candidum. Pichia kudriavzevii cat* and *Galactomyces* are detected in all samples of RM E1 fermentation is but not in the PMK 25 or the added DSC (Figure 3C). PMK 25 contains besides in the DSC detected *Debaromyces 1,2* several other fungal ASV annotated as *Aspergillus 1*, *Malassezia restricta cat*, *Neodevriesia* fraserae and *Pichia fermentans cat*. The fungal composition during the J2 fermentation does not show conserved RM derived specific ASVs in a similar manner as during E1 fermentation (Figure 3D). For example *Galactomyces* is present in PMK 26.5 hours, but is not detected in the RMK 12, 24 or 504 hours after the addition of DSC. Strangely RMK 12 seems to contain solely DSC derived fungi including the not in other samples detected ASV *Cladosporium 3*. The relative abundance of *Debaromyces* is observed to be higher in J2 fermentation compared to E1.

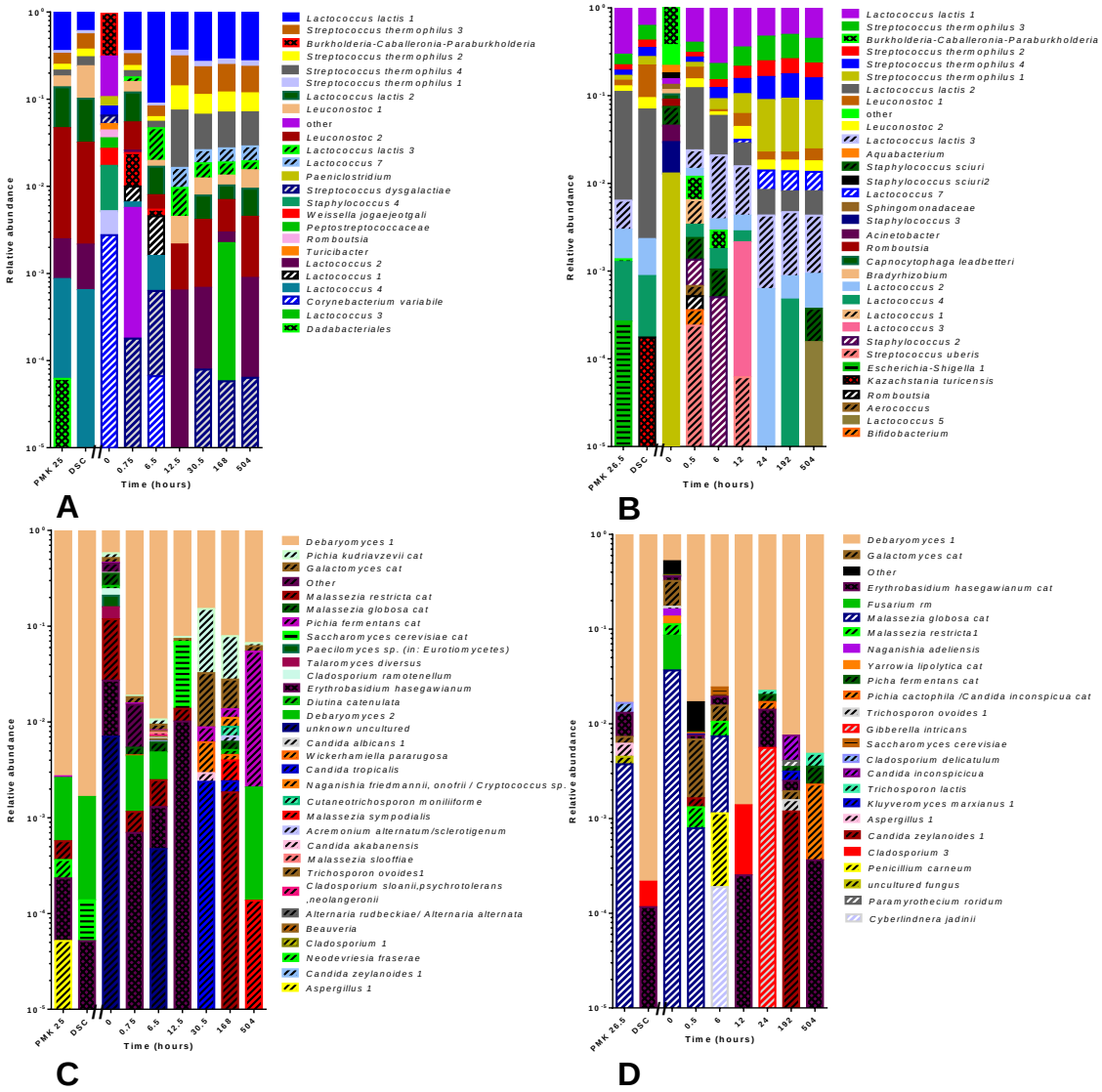

Figure 3: The microbial composition during DSK fermentation and storage. Identical annotation indicates identical ASV, cat is used for multiple ASV concatenated to one annotation. The bacterial composition determined by the v3-v4 region of the 16S rRNA gene amplicons are displayed in (A) for DSK with dairy source E1 and (B) for J2. The fungal composition is displayed in (C) for E1 and (D) J2. DSC per graph corresponds to the used batch of DSC during fermentation. Y-axis displays relative abundance in logarithmic order. X-axis displays samples with time after addition of the starters culture. Dashed lines indicate ASVs which not present in the inoculum. Horizontal lines are taxa similar to the spike-in but these specific ASVs were not present in the spike-in. Checkered bars indicate potential technical contaminants. Raw milk is represented by t= 0.

Backslopping of kefirgrain kefir increases *L. lactis* and decreases *Lactobacillus* abundance.

The microbial composition changes during the several stages involved with the production BKK style kefir. From a *Lactobacillus (Lb) kefiranofaciens* with *Kazachstania (K.) turicensis, K. unispora* dominated composition in kefir grains towards a *Lactococcus lactis* with *Galactomyces* dominated composition in BKK style kefir.

The bacterial ASV found in the KG used in the J1 BKK fermentation process were: *Lb. kefiranofaciens 1, Acetobacter 3, Bifidobacterium mongoliense, Lb. kefiri* and *Lactococcus lactis 1* (Figure 4A). The KG used for MC production in the E2 fermentation had several additional ASVs: *Lb. kefiranofaciens 2,*

Acetobacter 1, Lb. parakefiri. *Acetobacter 3* and *Bifidobacterium mongoliense* where absent (Figure 4B). The relative abundance of *Lactococcus lactis 1* increased almost 20 times, *Acetobacter* was reduced by half, *Lb. kefiranofaciens* by >2000 times, 2-25 times less *Lb. kefiri* and *Lb. parakefiri* was complete undetected in MC compared to KG. Furthermore *Leuconostoc 2* and *Bifidobacterium mongoliense* was also detected in MC for RMK E2 (Figure 4B). In RMK J1 *Bifidobacterium mongoliense* was found at 36h and at 168h.
Leuconostoc 1 ASV was not detected in MC or KG but is present during the fermentation of RMK J1, E2 after 26h and in PMK 26. *Leuconostoc 2,* however is found in both MCs and in the majority of the samples during fermentation. *Acetobacter 1, 4* was present in PMK J1, E2 and not in RMK samples after >12.5h. *Staphylococcus 1* is found in RMK E2 at 1, 6.5, 12.5 and 240 hours after addition of MC. *Lb. Kefiri* resides in all BKK samples.
The fungal composition of KG consists of *K. turicensis 1, K. unispora1, Kluyveromyces marxianus cat* and *Galactomyces cat.* The KG used for MC in E2 fermentation had the additional ASVs: the not in spike-in found *Saccharomyces cerevisiae* and *Pichia* kudriavzevii (Figure 4D). The relative abundance of *Galactomyces cat* increased by ~ 40-55 times. *K. unispora1* and *Kluyveromyces marxianus cat* abundance remained similar in MC compared to KG. Additionally *Debaryomyces 1* was present in MC used for J1 fermentation. PMK 26 and RMK J1 samples contained all the ASV found in MC except for the low abundant *Naganishia diffluens1 (*$5.11*10^{-5}$ RA*)* and *Pichia kudriavzevii* ($6.81*10^{-5}$ RA). *Debaryomyces 1* was absent from MC used in E2 fermentation, but present in all RMK E2 samples. *Cladisporum 3* is observed in MC (figure 4D), RMK E2 1, 456h and RMK J1 25, 36, 168h. However, not present in MC used in J1. *Trichosporon lactis cat* is found at 12, 25 hours of RMK J1. In addition *Malassezia restricta1* is detected in PMK J1 and RMK J1 1, 6.5, 36, 168h. Although all spike-in ASVs were removed, ASVs annotated as *Saccharomyces cerevisiae* were found in RMK J1 36, 168, 624, RMK E2 240, 456 and KG used in E2.

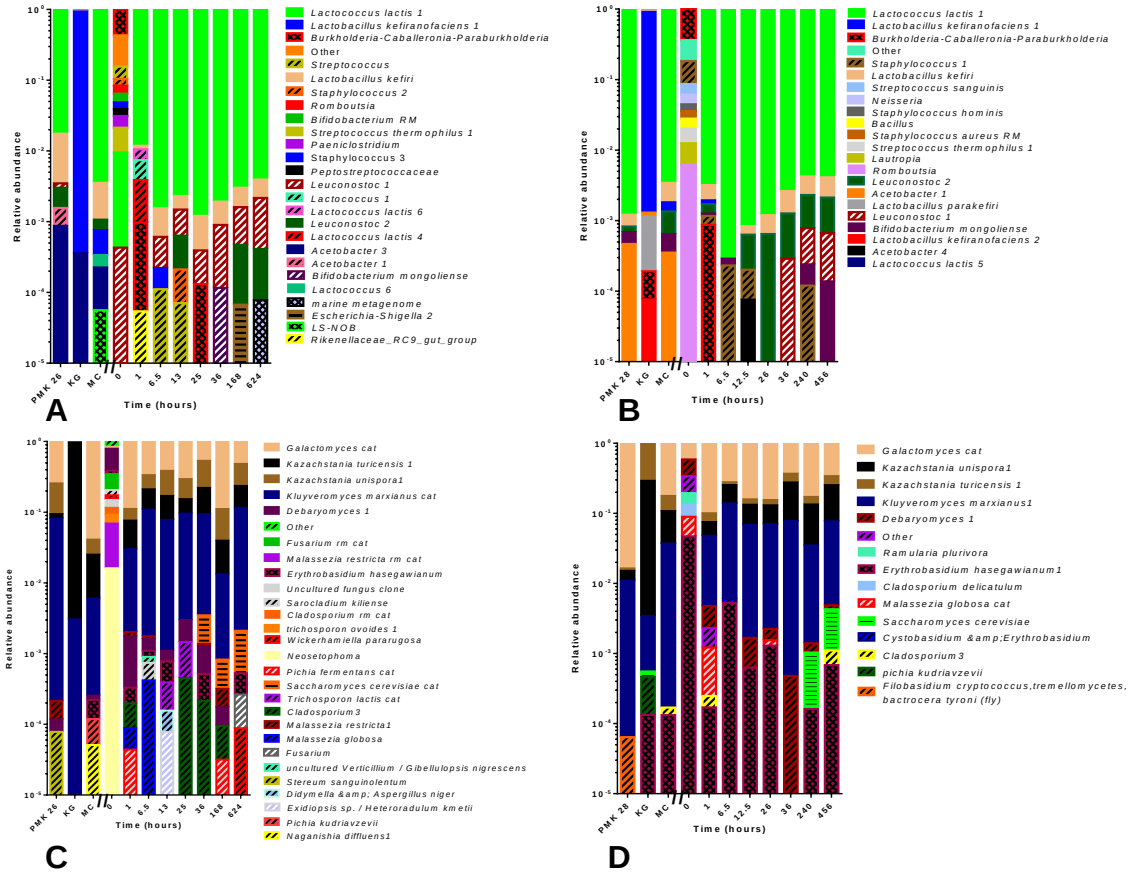

Figure 4: The microbial composition of backslopped kefir grain based kefir, identical annotation indicates identical ASV, cat means multiple ASVs are concatenated to one annotation. The bacterial composition determined by the v3-v4 region of the 16S rRNA gene amplicons are displayed in (A) for BKK with dairy source J1 and (B) for E2. The fungal composition is displayed in (C) for E1 and (D) J2. KG and MC corresponds to the used batch for the production of BKK. Y-axis displays relative abundance in logarithmic order. X-axis displays samples with time after addition of the starters culture. Dashed lines indicate ASV not present in the starters culture. Horizontal lines are taxa similar to the spike-in but these specific ASVs were not present in the spike-in. Checkered bars indicate potential technical induced contaminants. Raw milk is represented by t = 0.

Lower fungal abundance in raw milk compared to pasteurized milk kefir.

The fungal abundance of BKK and DSK style RM fermentation is higher compared to PMK. This abundance is determined by dividing the relative abundance of the in spike-in detected 16S rRNA and ITS ASV. The bacterial to fungal ratio for BKK style fermentation is lower compare to DSC style fermentation. Implying a higher fungal concentration in BKK.

The micro- mycobiota spike-in ratio for DSK fermentation is 8.2 and 11.5 for RMK J2 at 12h and 24h and 6.3 for PMK J2 at 26.5h after addition of DSC. For E1 a bigger difference is observed with a ratio of 9.1 and 8.6 for the time points: 12.5h and 30.5h compared to 1.4 in PMK 25h. For BKK fermentation the spike-in ratio for PMK 26h and 28h was 0.20 and 0.19 compared to RMK J1 25h 3.2 and E2 26h 3.8.

An interesting observation is the total amount of ITS amplicons correlates with higher fungal abundance. The amount of ITS reads for BKK style PMK were 52155 and 63463 compared to 44646 and 48305 reads in RMK. For E1 used in DSK style fermentation 48611 reads were present in PMK 25h compared to 37298 and 40820 reads in RMK 12.5h and 30.5h. This difference has not been

observed between RMK and PMK J2.

Reduced microbial abundance for several commercial milk kefirs.

The microbial abundance of two of the three shop bought kefirs harbour lower myco-microbial abundance compared to BKK or DSK style kefir.
In order to determine the microbial difference between kefir produced in this study and other commercially available kefirs three shop-bought kefirs were studied.
The lowest 16S rRNA Ct value was 10.67 for brand K where brand A and DM had Ct value of 14.31 and 15.21 (supplementary excel file). This indicates lower bacterial abundance in brand DM and A compared to the other studied kefir samples. All shop bought kefirs contained *Lactococcus lactis 1*, *Lactococcus 2* and *Leuconostoc 1,2. Streptococcus thermophilus 1* is present in PMK K and DM. *Ruminococcus* only in DM in a similar abundance as the systemic contaminant identified as *Burkholderia-Caballeronia-Paraburkholderia. Lactococcus lactis C* is unique for PMK A and K. *Lactococcus 4* and the unique *Lactococcus lactis C2* were only present in PMK A (figure 5A). *Debaryomyces 1* and the as contaminant identified *Erythrobasidium hasegawianum 1* were detected in all three shop-bought PMK with a RA of for A: $3.34*10^{-2}$, K: $1.52*10^{-4}$(K) and DM: $5.75*10^{-1}$. PMK A contained ASVs annotated as *Magnusiomyces capitatus*, *Galactomyces*, *Malassezia restricta 1* and *K. unispora 1*. The majority of the reads with 0.997 abundance accounts for *Debaryomyces 1* in PMK K, the other reads were identified as *Debaryomyces 2, Candida albicans* and the non-fungal amplicon *Lactococcus raffinolactis*. While no fungal CFUs were present, PMK DM contained reads annotated as *Geotrichum sp., Cutaneotrichosporon cutaneum, Aspergillus1* and *Pichia fermentans* (figure 5b). The amount of ITS reads and ratio micro- microbiota spike-in where for PMK A 28681; 13.5, K 55290; 1.31 and DM 22939; 11.3.

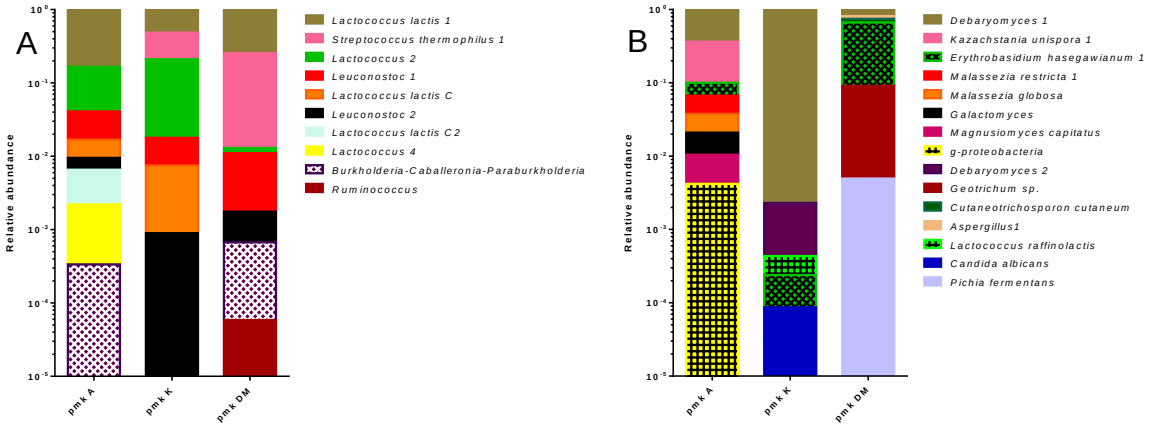

Figure 5: The microbial composition of three different brands shop-bought PMK. A is available in ordinary grocery shops, K bought at a small local dairy shop. DM is available in organic supermarkets similar as where RMK DSK and BKK is sold. (A) Displays the bacterial composition determined by the v3-v4 region of the 16S rRNA gene (B) ITS based fungal composition. Identical annotation indicates identical ASV. Y-axis displays relative abundance (Log) X-axis displays samples PMK A, K and DM. Checkered bars indicate potential contaminants, in B) squares are used to indicate bacteria where fungi was expected.

Discussion:

The aim of this study is to give an inside in the microbial composition of kefir derived from raw milk. By comparing RM samples of different origin, during fermentation and storage up to three weeks of two different fermentation methods based on an inhouse developed kefir grain backslopping method or by addition of a commercially available defined starters culture. Pasteurized milk kefir was produced for the above mentioned type of fermentation and three additionally commercially available shop-bought PMK were studied. The samples were analysed for pH, myco-microbial cell counts by culture depended quantification methods and culture independent techniques where bacterial abundance was determined through 16S rRNA specific qPCR. Amplicon sequencing of the v3-v4 region of the 16S rRNA gene and the ITS region was used to elucidate the micro-mycobiota composition. The fungal abundance was determined by the ratio of bacterial and fungal amplicons of the added spike-in. Where a lower ratio indicates higher concentrations of fungi.
This harvested some interesting observations regarding RMK composition. First of all, a decreased fungal abundance presence was observed in RMK compared to PMK in terms of spike-in ratio and total amount of reads. This effect has been found in both styles of kefir where BKK had a higher amount of fungal abundance compared to DSC, indicating an inherent effect of fermenting RM with a microbial consortium containing myco- and microbiota. Second, although RMK and PMK are largely similar in microbiota composition, some RM derived micro-organisms were detected in RMK and not in PMK. Besides the difference in pasteurization status, dairy source specific microorganisms were detected in RM and in RMK.
Increased fungal abundance in PMK compared to RMK had not been described for BKK or DSC style of kefir fermentation. However with KG based fermentation it had been found that PMK had higher fungal and bacterial cell counts accompanied with higher ethanol concentrations (Moreno & Emata, 2020). Compared to this results study no differences were found in the bacterial abundance between PMK and RMK. Several factors could explain the altered fungal abundance. Certain species for example *Lactobacillus subspecies* derived from RM show antifungal activity (Afzali et al, 2020), although the concentration of the *Lactobacillus subspecies* was in their study considerably higher compared to the low RM microbial concentration in this study. Additional RM factors could be present and aid in the observed altered fungal bacteria ratios for example by specific bacterial growth stimulating compounds (Yu et al, 2019). Unfortunately it is unknow what the fermentation speed in terms of microbial growth and pH decrease is of PMK since only one sample per fermentation was analysed. The antifungal activity of Debaromyces hansennii might explain the additional fungal reduction of DSC compared to BKK style of fermentation (Huang et al, 2021).
Potential specific RM derived microbes in RM DSK are: *Lactococcus lactis 3, Lactococcus 7* and *Galactomyces.* Specifically for RMK E1 *Streptococcus dysgalactiae* and *Pichia kudriavzevii cat. Staphylococcus sciuri* was found in PM J2, RM J1 and with a higher abundance J2 what might be the result for detectable levels in some RMK J2 samples. *Pichia cactophila/Candida inconspicua cat,* was detected in RM J1, 2 and in RMK J2 but not in RM E1, 2 or RMK E2. This might indicate that this could be specifically derived from dairy farm J. It is described that in other RM dairy products for example the spontaneous fermented RM product called Nunu contained several similar fungi for example *Pichia kudriavzevii, G. geotrichum* and three other candida yeasts (Akabanda et al, 2013)
BKK is based on a KG which ferments raw milk what is backslopped towards a bigger batch, this altered the microbial composition considerably from a *LB. kefiranofaciens* dominated abundance towards a *Lactococcus lactis*. *LB. kefiranofaciens* is not found in BKK, *LB. Kefiri* and *Leuconostoc* are. The fungal composition does alter less drastically, *K. turicensis, Kazachstania unispora, Kluyveromyces marxianus cat* and *Galactomyces cat* are present in KG, MC and BKK although in different relative abundances. Microbial composition shift has been described by Garofalo et al, (2020), what might be related towards the yeast bacteria interaction. Interestingly, *Acetobacter* is present in KG and MC but seems to be only detectable in PMK.

Due to the experimental study design several considerations regarding to the results of this study are present. For this study several types of kefir were analysed to elucidate the microbial composition and to assess the microbial differences of RM based kefir compared to PM kefir. Fermentation of the kefirs was not performed under laboratorial conditions and possibly different sizes/types of containers were used, what could lead to microbial alterations (Kebede et al, 2007). Therefore, biases regarding the microbial composition could have been introduced. For example, by cross- or environmental contamination. However, the results do represent the store bought RMK available to the general public. One of the unique traits of RMK is the use of non-homogenised RM, which might influence the spatial distribution of microbes. It is unknown if 1 ml of sample is representable for the total microbial community during the fermentation, although no apparent difference were observed for samples taken from the fermentation tank at ~ 24h compared to bottled RMK >30 H. The difference observed for cell counts in BKK at t=1, which has not been observed in Ct value, might suggests variation in sampling.

The DNA extraction regarding the total amount of DNA concentration was not found to be optimal and not assessed in the milk kefir matrix and could therefore be potential be lower. For example, microbes which adhere towards the milkfat fraction, or which were lysed during the freeze thaw cycle and centrifugation were discarded during the natant removal (Cheema et al, 2021; Aphale & Kulkarni, 2018). Cell lysis was performed by chemical and mechanical cell lysis. The bead diameter was selected to perform optimal lysis in *Saccharomyces cerevisiae* cells. Therefore, other fungal or hard to lyse bacterial cells, such as *Staphylococcus,* lysozyme resistant species could have had an un or less than optimal disrupted microbial membrane and where therefor not detected (Vandeventer et al, 2011). Although an additional mechanical lysis procedure was added, it could be that other DNA extraction methods tailored specific for fungi could improve the extraction efficiency**.**

Addition of the spike-in was based on 16S qPCR values, due to study constrains the qPCR was not optimized, performed in triplicates or was the actual efficiency determined. In combination with estimated Ct values of diluted samples and spike-in dilutions might be the cause for the difference in estimated and measured amount of spike-in (Ruijter et al, 2021). The Ct values does seem to correlate till a certain extend towards the amount of total CFUs with the exception that no difference was found between BKK and DSK after 12 hours, although a 10- 100-fold difference of CFUs were observed. Additionally no difference was observed for the total DNA concentration. An explanation could be that BKK bacteria were more viable and therefore caused the observed difference. It is found that bacteria in mixed communities containing *Galactomyces* had an 130-fold increase in proliferation compared to single species communities, in addition to this it was found that volatile organic compound produced by *Galactomyces* had the ability to increase bacterial proliferation in terms of CFU of harvested cells which were resuspended on culturing pates (Chaves-López et al, 2017; Cosetta et al, 2020). Culture plates including antifungals could exclude this potential effect of fungi. It is unlikely that the maximum binding capacity of the DNA purification column was exceeded, since DSC and several not in this study included samples had lower Ct values and higher DNA concentrations.

It is unknown if the same amount of genomic DNA or volume per sample is used for the libprep. The amplicon-library prep involves an eight-step PCR therefore bias towards PCR efficiency per sequence could occur due to variation in gDNA content (Laursen et al, 2017). Additional variation could occur by the addition of the spike-in which DNA quality might differ. In a prior small subset of samples not used in this study it was found that the percentage of filtered reads was ~ 90 % in the biological samples compared to ~ 20 % for the spike-in. For ITS at least five sizes of amplicons were observed by screentape analysis it might be that this causes additional biases towards the abundance of amplicons. Decisions in data analysis pipelines are known to introduce biases regarding detection of the microbial composition (Prodan et al, 2020). It would be preferred to not truncate sequences

based on the average phred score per nucleotide, but to use the individual phred score per nucleotide to trim sequences. Furthermore a total sum of phred scores per amplicon could perhaps be used to determine the validity of the ASV potentially with additional factors which includes the evolutionary conservation of nucleotides. In this study multiple *Lactococcus lactis,* and many unique ITS ASVs can be indicative for technical derived alterations of ASVs. Improved analysis may circumvent these biases. The abundance of an ASV does not necessarily equal the quantity of a single strain, multiple strains can harbour similar ASVs and multiple ASVs could be derived of a single species. Furthermore micro-organisms can harbour different amount of ribosomal DNA copy numbers, in fungi it ranges from 14 -1442 (Lofgren et al, 2019). Another matter is the lack of consensus regarding the annotation of micro-organisms, for some species multiple synonyms exist. The *Magnusiomyces capitatus* is named as anamorph *Saprochaete capitata* and has the following synonyms: *Trichosporon capitatum, Geotrichum capitatum, Ascotrichosporon capitatum, BIastoschizomyces capitatus, Dipodascus capitatus, Sporotrichum spicatum, Geotrichum linkii, Blastoschizomyces pseudotrichosporon* (de Hoog & Smith, 2011). Taxonomic annotation can therefore be to some extent arbitrary. Standardized systemic annotation of ASV could therefore improve the reliability, objectivity and comparability of future amplicon studies. The spike-in and the mock community was detected at a similar detection level compared to the manufacture and was on genius level correctly identified. Several additional unexpected ASVs were detected and were used to identify potential contaminants. Contaminants were not removed in this study but were flagged. What could be regarded as a more transparent method, since it cannot be verified that a contaminant is an actual contaminant in this study. Furthermore, no filtering was performed on low abundant reads. The mock community, spike-in and the three duplicates indicates that certain microorganisms could be present in the samples on the verge of the detection limit**.** These low abundance ASV could be RM derived or where post milking and or post- fermentation introduced**.** Therefore the absence of specific ASVs does not imply that certain microbes were absent in samples and vice versa. Comparing the presence of low abundant ASV in several consecutive RMK samples with one PMK sample could imply the that certain microbes are present in RMK although it is not detected in all consecutive samples. Comparing one RMK to PMK would therefore be preferable, if it was sampled at a similar fermentation time point. Conclusive statements on the presents or absence of low abundant microbes should consequently not be exclusively based on amplicon sequencing, additional techniques such as species specific qPCR, cell sorting, fluorescence in situ hybridization or culture depended techniques on selective media could be deployed to elucidate the presence of low abundant species. In order to determine the ratio between bacteria and fungi a spike-in containing gDNA from both was added. This method is not widely used. It does however seems to reveal a certain indication of absolute proportions. Where the ratio ITS/16S spike-in is low a higher amount of fungi would be expected. Unfortunately the spike-in was composed of widely present micro-organisms such as *Saccharomyces cerevisiae*. If these are present in a sample with identical ASVs, could lead to under estimation of fungal abundance and removal of biological relevant data. It was observed that in samples where no spike-in was added for example in KG, DSC and milk samples *Saccharomyces cerevisiae* ASV were present. In other samples different ASVs were found compared to the spike-in which were similarly annotated. Therefore *Saccharomyces cerevisiae* might be a part of the fungal composition of the analysed kefirs in a lesser extend this effect can also be present for the bacteria *Escherichia-Shigella*. Besides these limitations a background contaminant was found in the majority of the samples. It could be that the absolute amount of *Erythrobasidium hasegawianum 1* is equally distributed across all samples. If the ITS amplicon of *Erythrobasidium hasegawianum 1* is present in similar proportions could suggest similar levels of fungi, higher abundance could be suggestive of lower amounts of fungi. In addition a higher amount of ITS reads were found in samples with low ITS/16S spike-in ratio what could be further indicative of a higher fungal abundance.

Some inconsistencies were found in the microbial composition of PMK E2. The duplicate of PMK E2 does not seem to differ in composition therefore it is less likely that a DNA isolation specific bias is responsible for the observed irregularities. Where the three other PMK controls showed a lower ITS/16S spike-in and a higher amount of ITS reads, PMK E2 did not. Abundance of *Erythrobasidium hasegawianum 1* was higher in PMK E2 and was in certain PMKs even absent. Additionally potential RM specific ASVs: *Lactococcus lactis 3* and *Galactomyces* are present. In contrast *Aspergillus 1* had been detected, what could be PM specific since it was solely found in PMK E1, DM, PM yoghurt and in PMK E2. This might be due to the presence of heat resistance *Aspergillus* spores (Dijksterhuis, 2019). For the sake of simplicity PMK E2 was discarded in the conclusive statements.
Another interesting discrepancy is present in shop-bought PMK A. The manufacturer of shop-bought kefir A mentioned in their personal correspondence that their kefir product contains 30 different bacterial species and several fungi such as: *Kluveromyces marxianus, Brettanomyces, Saccaromyces, Debaromyces and Pichia*. This study did not detect *Kluveromyces marxianus, Pichia* and *Brettanomyces* in PMK A. In total eight 16S and eleven ITS (including spike-in *S. cerevisiae*) ASVs were found it could be that different species share the same ASV or the abundance was low compared to other spieces and could therefore not be detected. Another possibility could be that the used DNA extraction method was not sufficient enough to lyse the cells or that lysed cells were removed during prior to DNA binding to the column.
It is observed that the concentration of PMK A DNA is at least 10 times less with a corresponding higher 16s qPCR Ct value compared to DSK, BKK and PMK K kefir. Even lower values for shop-bought PM yoghurt and kefir DM were found. When the Ct values are compared to DSC fermentation, it could be suggested that shop-bought PMK A and DM were fermented for 6 till 12 hours. This was confirmed for PMK DM which according to Reeb, (2019) is fermented for 8 till 12 hours. The potential lower fermentation time of these products might reduce potential beneficial health effects. Vieira et al, (2021) found that extended fermentation time with a KG based PMK was associated with significant higher increase in anti-inflammatory activity.

Safety and health implications

This chapter will discuss the potential safety and health implications, concerns and provide future suggestions regarding the microbial observations in this study.
The majority of the detected microbial amplicons are identical to or are derived from the added starters culture and could be considered as safe or even beneficial for consumption. Although human infections with food derived yeasts harbouring virulence factors such as seen in some food derived *Kluyveromyces marxianus* is potentially possible(Peréz-Través et al, 2021). Several ASV were not found in the starters cultures but had similar taxonomic classification and might therefore be considered similar in terms of health implications.
Several additional microbes are observed in RMK in concentrations lower than the starters culture abundance and are accessible for consumers which may have a health implications.
Streptococcus dysgalactiae was detected in RMK J2. Further amplicon analysis by BLAST revealed a 100% and 99,77% identity with the subspecies dysgalactiae and equisimillis. The subspecies dysgalactiae is considered a generally non-human pathogen but is the third most mastitis causing bacteria in Norwegian dairy cows. (Porcellato, et al, 2021).
According to (von Mutius & Smits 2020) nasal exposure to probiotic farm bacteria for example the in RMK E1 detected Staphylococcus sciuri protects against airway inflammation in a mouse model. Besides potential beneficial effects several infections in humans with Staphylococcus sciuri are reported but in general it is considered a harmless bacteria (Chen et al, 2007; Nemeghaire et al, 2014).
Staphylococcus1 was found in several RMK E2 samples with a 100% identity with multiple uncultured

Staphylococcus in the NCBI database which was found in a human skin microbiome study (Accession number NCBI: JF204698.1) and 99,77% with multiple *Staphylococcus aureus, argenteus* from a food-borne outbreak study (Accession number NCBI: CP075519.1). *S. aureus* is a gram positive commensal bacteria but can be an opportunistic pathogen causing a range of diseases including food poisoning. Colonization with *S. aureus* in the first days of new-borns with *S. aureus* is associated with increased levels of Treg cells and lower incidence of food allergies and is considered a keystone species in upper respiratory tract microbiota development (Calder et al, 2006; Bosch et al, 2016). The presence of *Staphylococcus* in RMK E2 is not unique, McLauchlin et al, (2020) analysed 24 RMK samples of which 36% were microbial unsatisfactory caused by *Staphylococci* and Enterobacteriaceae. 7% of the samples were found potentially injurious to health due to the presence of coagulase positive *Staphylococci* in a concentration above 10^4 CFU/g. The mere present of *Staphylococcus* concentrations above 10^4 CFU/g does not directly translate to negative health effects, since it is unknown if for example *Staphylococcal* enterotoxins are produced. In Italian RM cheeses for example, of the 245 cheeses investigated, 42,86% had *S. aureus* concentrations above EU standards of which three samples contained enterotoxins (Costanzo et al, 2020). Enterotoxins production might be inhibited by *Lactococcus lactis* which is known to strongly reduce the transcription of several *S. aureus* enterotoxins (Schelin et al, 2011).
Various methods could be deployed to control *S. aureus* Howard RMK. Kivanc & Yapici (2018), produced kefir with KG and was found to be effective in eradication of *S. aureus,* which was spiked (10^5 CFU/ml) pre and post fermentation implying that also post fermentation contamination of *S. aureus* can be neutralized. Angelidis et al, (2020) did not detect enterotoxins in kefir if the initial *S. aureus* concentration was low or a higher KG to milk ratio was used, additionally one type of KG did inhibited enterotoxin production in all of the investigated fermentation conditions.
It is unknow if the *Staphylococci* found in RMK E2 is RM derived or was post milking introduced. RM monitoring for potential pathogens could be feasible. For example trough pathogen specific qPCR or by the use of other identifiable biomarkers such as the volatile organic compound: 3-Methyl-butanoic acid, by which *S. aureus* could be detected in a mixed culture when present in RM (Chen, et al., 2018). Furthermore the spatial distribution of micro-organisms during a milking session is unknown. Perhaps by separating the first quantities of milk if the microbial concentration is higher herein higher could result in lower colony counts in bulk RM tanks. Improvements of (sub) clinical mastitis detection could in theory cut the amount of undesirable micro-organisms since several .
Besides monitoring preventative options could be deployed. The *S. aureus* and *Streptococcus dysgalactiae* can be transmitted by flies which might be a vector causing summer mastitis (Chirico et al, 1997). Insect control may reduce the transmission of these bacteria. A biological farming practice utilizing the parasitic effect of the fungi *Beauveria* can be used to reduce flies in the dairy environment (Lohmeyer & Miller, 2006). Several RM derived fungi were present in RMK. Galactomyces was used in this study to describe the presence of the species *Geotrichum candidum, Galactomyces geotrichum or Galactomyces candidum.* Geotrichum candidum is considered safe and is proposed by Pottier et al, (2008) to gain qualified presumption of safety (QPS) status. *Galactomyces geotrichum* is able to produce omega-3 fatty acids, what could be regarded as beneficial for health (Grygier et al, 2020). Omega-3 fatty acids in RM are believe to contribute in reduction of asthma incidence in children (Brick et al, 2016).
Pichia kudriavzevii formerly named *Issatchenkia orientalis* or in the anamorphic state *Candida krusei* is a yeast found in RMK E1 and E2 and is widely distributed and isolated from soil, fruits and natural fermentations. Candida krusei is considered a potential pathogenic yeast since many clinical isolates exist and are described in hundreds of studies (Kurtzman, 2011). However *Pichia kudriavzevii* is generally recognised as safe by the FDA but shows no genomic distinction towards *Candida krusei* (Douglass et al, 2018). Exopolysaccharides produced by *Pichia kudriavzevii* even had an apoptotic

effect on human colon cancers cells trough reduction of the mammalian target of rapamycin (mTOR)(Saadat et al, 2020). The differences in ratio bacteria and fungi as observed between PMK and RMK might have health implications the next chapter proposes a potential molecular mechanism which might explain the observed health effects from Baars et al, (2019) and in the unpublished results of S. Abbring.

Proposed immunomodulatory molecular mechanism of raw milk kefir

Plethora of biological active compounds are present in RM and kefir which are beyond the scope of this article to discuss. However, one intriguing hypothesis will be reviewed regarding a potential molecular immunological mechanism, in which RMK could contribute to reduction of non-communicable diseases by cross communication between bacteria and fungi leading to reduction of mTOR activity.

mTOR is a core kinase included in the mtorc1 and mtorc2 protein complexes involved in protein and lipid synthesis, regulating cellular growth, survival and proliferation. Many external and internal cues influence these regulatory networks for instance growth hormones, cues from nutrient sensing, amino acids and stress (Liu & Sabatini, 2020). Dysregulation of mTOR is associated with several non-communicable diseases. In a mice model of food allergies sensitized with ovalbumin, less symptoms were observed when preventive rapamycin reducing mTOR activity was administered. In addition recovery was observed when mice who developed symptoms of allergy where therapeutically treated with daily rapamycin. Single dose of rapamycin did not reduce symptoms. It is therefore suggested that mTOR inhibitors could be a good candidate to treat food allergies(Yamaki & Yoshino 2012). Similar effects were found for models in: contact dermatitis by measuring ear-swelling, allergic conjunctivitis and allergic airway inflammation (Hua et al, 2015; Shin et al, 2018; Balmert et al, 2017). Melnik & Schmitz, (2021) argues that pasteurized milk but not fermented milk consumption can initiates mTOR overactivation through a numerous factors and compounds. For example, via milk derived extracellular vesicles (EV), named exosomes with micro interfering RNA (MIR) cargo. One of these, MIR-21 downregulate the phosphatase and tensin homolog (PTEN) which is an inhibitor of mTOR. Milk contains 245 different MIRs, of which 71.4% are predicted to target 11 000 humane transcripts, RM processing has been found to alter the MIR composition (Howard et al, 2015). Disruption of EVs led to a significantly reduction of immune-modulatory activity. It is probably essential for effective and cell type specific transfer of small molecules into the cell (Sun et al, 2013). Pasteurization therefore alters potentially the biological active properties of EVs. Milk-derived EVs can also promote specific microbial growth for example *Lactobacillus plantarum* (Yu et al, 2019). Furthermore mice fed milk exosomes showed changes in bacterial communities and in short chain fatty acids (SCFA) such as: acetate, propionate, and butyrate. (Zhou et al, 2019; Tong et al., 2020) SCFA's induces Treg and T effector cells trough regulation of MTor-s6k pathway (Park et al, 2015). Fermentation of milk is found to change the exosome profile and diminished MIR-21, resulting in lower cell growth when fermentation derived exosomes were added (Yu et al, 2017). Consumption of beverages enriched with *Leuconostoc holzapfelii* led to alterations of the microbiota and microbial EV composition in Korean adults (Yang et al, 2019).

During milk fermentation with yeast and bacteria interdependence for nutrient exchange could exists. Inhibition of the TOR complex 1 (homolog of the mammalian target of rapamycin) by rapamycin treatment in yeast resulted in secretion of amino acids which enhanced proliferation for *Lactococcus lactis* and for *Lactobacillus plantarum* up to a 5 fold (Ponomarova et al, 2017). Microbial metabolome derived from the human gut has found to influence fungal growth through TOR signalling (García et al, 2017). Additionally Bourrie et al, (2021) observed that kefir product lacking either yeast or *Lactobacillus* did not result in lower cholesterol levels in an obese mice model. These studies suggest that the interaction between (raw)milk factors and mutualistic fermentation by

bacteria and yeast could possibly result in factors which could alter mTOR complexes direct and indirectly (Cao et al, 2019). It could be possible that lower fungal abundance in RMK is the result of lower yeast proliferation due to inhibition of the TOR pathway, or that higher abundance of yeasts subsequently led to a higher total amount of secreted amino acids, and therefore less factors are produced by bacteria inhibiting TOR to increase fungal amino acids secretion. Subsequently lower levels of mTOR influencing bacterial derived factors could be present in the kefir product what might explain the observed health benefits of RMK.
In order to study this proposed hypothesis several aspects needs to be examined. First of all the potential inherent effect of RM on the fungal abundance needs to be reliable reproduced preferably by a fungal specific qPCR. Followed by an experimental setup in order to determine the potential health effects. Tissue specific determination of phosphorylated mTOR abundance trough specific antibodies based quantitative detection methods can be a powerful method to asses any involvement of mTOR in the RMK health effects.

Conclusion:

This study indicates that the majority of microbial species found in RMK and PMK are similar albeit in different proportions. Certain microorganism for example *Galacotomyces, Streptococcus dysgalactiae, Pichia kudriavzevii* and *Staphylococcus sciuri* detected in RMK could be RM derived. Furthermore there appears to be a higher amount of fungi present in PMK. These findings could have implications regarding the observed beneficial health effect of RMK consumption, but future studies are warranted to determine the exact molecular immunological mechanism.

Abbreviations:

ASV	Amplicon Sequence Variant
BKK	Backslopped Kefirgrain Kefir
cat	Concatenated
CFU	Colony Forming Unit
Ct	Cycle threshold
DCK	Defined Starter Culture
DSK	Defined Starter culture Kefir
EV	Extracellular Vesicles
ITS	Internal Transcribed Spacer
KG	Kefir Grain
MC	Mother Culture
mTOR	mammalian Target Of Rapamycin
NCBI	National Center for Biotechnology Information
PBS	Phosphate Buffered Saline solution
PM	Pasteurized Milk
PMK	Pasteurized Milk Kefir
qPCR	Quantitative real-time polymerase chain reaction
RA	Relative Abundance
RM	Raw Milk
RMK	Raw Milk Kefir

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