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Research Letter

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Acquisition of the yeast *Kluyveromyces marxianus* from unpasteurised milk by a kefir grain enhances kefir quality

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One sentence summary: Cycling kefir grain in raw milk causes it to uptake *Kluyveromyces marxianus* from the environment and changes the resulting kefir's flavour.
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ABSTRACT

Kefir is a fermented milk beverage consumed for nutritional and health tonic benefits in many parts of the world. It is produced by the fermentation of milk with a consortium of bacteria and yeast embedded within a polysaccharide matrix. This consortium is not well defined and can vary substantially between kefir grains. There are little data on the microbial stability of kefir grains, nor on interactions between microbes in the grain and in the milk. To study this, a grain was split, with one half of each stored at −20°C and the other half passaged repeatedly in whole unpasteurised milk. Grains passaged in the unpasteurised milk recovered vigour and acquired the yeast *Kluyveromyces marxainus* from the milk which was confirmed to be the same strain by molecular typing. Furthermore, these passaged grains produced kefir that was distinguished chemically and organoleptically from the stored grains. Some changes in ultrastructure were also observed by scanning electron microscopy. The study showed that kefir grains can acquire yeast from their environment and the final product can be influenced by these newly acquired yeasts. *Kluyveromyces marxianus* is considered to be responsible for some of the most important characteristics of kefir so the finding that this yeast is part of the less stable microbiota is significant.

Keywords: kefir; flavour; yeast; *Kluyveromyces*; fermented beverage; milk

INTRODUCTION

Kefir is a fermented milk beverage often drank as a health tonic in many dairy regions in the world (Kabak and Dobson 2011). Traditionally, it is produced by inoculating milk with a microbial consortia in the form of a kefir 'grain'. The beverage is typically acidic (pH ~4), slightly alcoholic and contains bioactive compounds associated with microbial fermentations such as vitamins, peptides and bacteriocins, which may contribute to the reputed health benefits of kefir (Guzel-Seydim *et al.* 2011; Kabak and Dobson 2011). Microbial metabolites are also the main

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contributors to the complex flavour of kefir, which is generally sharp and sour but with fruity aspects and slight effervescence. Kefir grains themselves are a complex collection of yeast and bacteria housed in a polysaccharide matrix. Bacteria often associated with kefir include lactic acid bacteria and acetic acid bacteria (Mainville *et al.* 2006; Guzel-Seydim *et al.* 2011; Kabak and Dobson 2011; Pogacic *et al.* 2013; Garofalo *et al.* 2015) with common yeasts including *Saccharomyces cerevisiae*, *Kluyveromyces marxianus* and *Pichia fermentans* (Marsh *et al.* 2013; Korsak *et al.* 2015). Although all kefir grains have this symbiotic nature, the types of yeast and bacteria and their relative proportion in the grain vary between grains and geographical regions (Marsh *et al.* 2013). Neither the stability of a grain's microbial composition nor the extent to which this is susceptible to influence from the natural microbial load of its surrounding environment is well understood at this time.

Although kefir-like beverages are produced commercially, the lack of detailed knowledge of the microbial community and interlinked metabolism of the microbial consortia has precluded the development of authentic starter cultures. There is considerable interest in defining the species that contribute most to the qualities of kefir and to developing mixed or pure starter cultures based on knowledge of strains and their metabolism. In an ongoing project exploring yeast diversity and function in kefir grains, we have been studying grains from a collection held at Teagasc, Moorepark, Co. Cork. Two aspects of this analysis caused concern. First, grains that had been stored at −20°C for 6 months under aseptic conditions in 10% reconstituted skim milk (RSM) failed to produce robust kefir on subsequent inoculation and, second, we failed to recover the yeast *K. marxainus* from the grains although this yeast was previously reported to be part of the community of the grains under investigation (Marsh *et al.* 2013). This led us to question whether long-term storage may be damaging the grain and whether recultivation in unpasteurised, whole milk would allow the grain to recover traits associated with robust kefir grains. To address these questions, we took a stored grain, subdivided it and subsequently passaged (cycled) one portion for several fermentations in unpasteurised, whole milk while storing another portion (non-cycled) at −20°C. The cycled and non-cycled versions were then used to produce kefir. The microbial composition of these kefirs and their chemical and organoleptic characteristics were compared to determine whether passaging grains in raw milk led to a change or improvement in the kefir grain.

MATERIALS AND METHODS

Culture conditions

Kefir grain SP1, which had previously been characterised at the metagenomic level (Marsh *et al.* 2013), was chosen for this study. The grain was stored aseptically in 10% RSM at −20°C. Two grain-forming units (GFUs) (approximately 1 g wet weight) were removed from the grain using a sterile scalpel. A GFU is defined as a portion of the grain of sufficient size to form a new grain. One GFU was put into 10% RSM and stored at −20°C and the other inoculated into unpasteurised whole milk obtained from a local dairy farm in Donoughmore, Co. Cork, Ireland. The whole milk GFU was passaged for >5 cycles in 250 mL at room temperature with each cycle lasting between 5 and 7 days. Each cycling procedure involved recapture of the grain from the fermented milk, two washes with deionised sterile water to remove residual milk and subsequent re-inoculation into fresh whole milk from the same farm. To recover the control stored grain, the frozen GFU was thawed and propagated twice in 250 mL 10% RSM. Kefir was produced by both the cycled and non-cycled GFUs by parallel fermentation in 250 mL pasteurised milk for 24 h at room temperature. 100 μL samples were taken at 3, 6 and 12 h, serially diluted and plated onto Yeast Extract Glucose Chloramphenicol Agar (Merck) and MRS Agar (Sigma) to determine yeast and bacterial numbers respectively during the early stages of fermentation. Plates were routinely incubated at 30°C for 24 h. An additional test was done to test for the presence of thermotolerant yeast—YGC plates were placed at 42°C for 24 h. An enrichment step was required to recover thermotolerant yeast from the whole milk. This involved mixing 1 part whole milk to 10 parts YPD media consisting of yeast extract (10 g L^{-1}), peptone (20 g L^{-1}) and lactose (20 g L^{-1}) and incubating for 24 h at 42°C before plating samples as before on YGC medium. The yeast isolated from the milk was named UCKM1 and the yeast isolated from the cycled kefir grain was named UCKM2.

Scanning electron microscopy (SEM)

The kefir grains were dissected and gently rinsed to remove unattached debris. Small pieces of the grain were fixed in primary fixative which consisted of 2% glutaraldehyde, 2.5% paraformaldehyde in 0.165 M phosphate buffer (pH 7.3). Following primary fixation, specimens were washed in buffer, post-fixed in 2% osmium tetroxide in the same buffer, dehydrated in graded acteones and air-dried from tetramethylsilane. The samples were then mounted onto aluminium stubs using double-sided carbon tape. All samples were sputter coated with a 5 nm layer of gold palladium (80:20) using a Quorum Q150 RES Sputter Coating System (Quorum Technologies, UK) before being examined using a Jeol JSM 5510 Scanning Electron Microscope (Jeol Ltd, Japan). Digital electron micrographs were obtained of representative regions of interest.

Gas chromatography–mass spectrometry (GCMS)

For volatile analysis, a static HS SPME-GCMS method was used. 4 mL of sample was obtained from the cycled and non-cycled versions of the grain after 24 h and was added to an amber 20 mL screw capped La-Pha Pack SPME vial with a silicon/PTFE septum (Apex Scientific, Kildare, Ireland). The vials were equilibrated to 75°C for 5 min with pulsed agitation of 5 s at 400 rpm using a CTC Analytics CombiPalAutosampler. The samples were analysed in duplicate. A single 50/30 μm CarboxenTM/divinylbenzene/polydimethylsiloxane (DVB/CAR/PDMS) fibre was used (Agilent Technologies, Cork, Ireland) and exposed to the headspace above the sample for 20 min at depth of 1 cm at 75°C. The fibre was retracted and injected into the GC inlet and desorbed for 2 min at 250°C. Injections were made on an Agilent 7890A GC with an Agilent DB-5 (60 m × 0.25 mm × 0.25 μm) column using a multipurpose injector with a merlin microseal (Agilent Technologies). The temperature of the column oven was set at 35°C, held for 0.5 min, increased at 6.5°C min^{-1} to 230°C and then increased at 15°C min^{-1} to 325°C, yielding at total GC run time of 36.8 min. The carrier gas was helium held at a constant pressure of 23 psi. The detector was an Agilent 5975C MSD single quadrupole mass spectrometer detector. The ion source temperature was 230°C and the interface temperature were set at 280°C and the MS mode was electronic ionisation (−70 V) with the mass range scanned between 35 and 250 amu. Compounds were identified using mass spectra comparisons to the NIST 2011 mass spectral library and AMDIS using deconvolution reporting software, and from an internal data

base with known target and qualifier ions for each compound. An auto-tune of the GCMS was carried out prior to the analysis to ensure optimal GCMS performance. A set of external standards was also run at the start and end of the sample set and abundances were compared to known amounts to ensure that both the SPME extraction and MS detection were performing within specification.

Sensory evaluation

Twenty sensory panellists were recruited in University College Cork, Ireland. The age range of assessors was between 25 and 45 years old. Selection criteria for panellists were availability and motivation to participate on all days of the experiment and that they were familiar with kefir as a product. All panellists had participated in dairy descriptive profiles in the past and were well versed in the sensory experimental protocol. Panellists were trained using sensory intensity descriptors and ranking descriptive analysis (Dairou and Sieffermann 2002; Richter *et al*. 2010) was carried out in panel booths conforming to international standards (ISO 8589: 2007) on two kefir samples, SP1 cycled and non-cycled. All samples were prepared fresh by inoculation of pasteurised milk the day before sensory analysis and fermentation at room temperature for 24 h. Samples were then held at refrigeration temperatures (4°C), before being presented to the panel at ambient temperatures (21°C) and coded with a randomly selected three digit code. The kefir samples were immediately served to panellists in a monadic fashion. Each assessor was provided with deionised water and instructed to cleanse their palates between tastings. Additionally, each assessor was presented with samples in duplicate and asked to assess the intensity of the attributes, according to a 10 cm line scale ranging from 0 (none) at the left to 10 (extreme) at the right and rating subsequently scored in centimetre from left. The order of the presentation of all samples was randomised to prevent first order and carryover effects. The mean was obtained and significant differences between kefir produced from cycled GFU and the non-cycled GFU were determined using Student's *t*-test with $P < 0.05$ detonating a significant difference.

Molecular identification of *Kluyveromyces marxianus*

Molecular identification of *K. marxainus* was carried out by PCR of the D1D2 region of the 26S rRNA gene using primers NL1 and NL4 as previously described (Kurtzman and Robnett 2003; Lane *et al*. 2011). In order to investigate whether the *K. marxianus* strain isolated from the milk was the same strain that was incorporated into the cycled Kefir grain, strain typing primers were designed for the *IPP1* housekeeping gene. Forward and Reverse primers were designed to amplify all 13 single nucleotide polymorphisms (SNPs) and were located in a conserved region according to a multiple sequence alignment across the five available *K. marxianus* genomes (CCT 7735; DMB1; NBRC 1777; DMKU 3–1042 and KCTC 17555). The sequences of the forward and reverse primers for *IPP1* were as follows: 5′-ATCGGTGCCAAGAACACCTT-3′ and 3′-TTGTCGATTGGCTCGTCTGG-5′ respectively (Fig. S1, Supporting Information).

RESULTS AND DISCUSSION

To address the hypothesis that passaging kefir grains in unpasteurised whole milk would lead of improved growth of grains and more robust, authentic kefir, a kefir grain was subdivided and sections of the grain of sufficient size to grow into a new grain (GFUs), were used for analysis. One was stored at −20°C while the other was passaged for several cycles in unpasteurised milk. These duplicate GFUs are described as 'non-cycled (NC)' or 'cycled (C)' respectively in this study. Characteristics of the kefir produced by the non-cycled (NC) and cycled (C) grains were compared. There were two clear qualitative differences between the two grains. First, the cycled grain showed a significant increase in size during fermentation, suggesting increased polysaccharide production and second, the kefir produced by cycled grains had a noticeable pleasant fruity aroma, in contrast the musty smell produced by non-cycled grains. Thus, the cycled grains exhibited traits typical of healthy, robust grains. To study these differences in greater detail, it was decided to do more detailed analysis between the two grains.

To do this, grains were inoculated into pasteurised milk, kefir was produced over 24 h and microbiological, sensory and chemical tests were carried out. To determine if the change in the cycled grain was due to an alteration in the numbers of yeast or bacteria actively fermenting the milk, 100 μL kefir samples were obtained at 3, 6 and 12 h time points and serial dilutions were plated on YGC and MRS media to quantify yeast and bacteria, respectively. In general, there were no major differences in microbial load with both grains showing the same general trend (Fig. 1). In both cases, the yeast and bacterial numbers in the fermented milk increased rapidly between 3 and 6 h and continued to increase at a slower rate thereafter. Yeast levels were in the range of $0.5–1 \times 10^6$ CFUs mL^{-1} and bacterial levels up to two to three orders of magnitude higher. The amount of polysaccharide in the grain may be a factor in release of microbes to the fermenting milk as has been suggested previously that this matrix acts as a permeable membrane allowing free flow of substrate and product but restricting the movement of microbes into the fermenting milk itself (Lu *et al*. 2014). It was also assessed whether the thermotolerant yeast *Kluyveromyces marxianus* was present in the kefir. *Kluyveromyces marxianus* is desirable as a starter culture due to its fast growth rate and attractive organoleptic profile, in particular fruity ester production. In addition *K. marxianus* also has good acid tolerance and produces low amounts of ethanol. It is therefore an important species for both food and biotechnology (Fabre, Blanc and Goma 1998; Lane and Morrissey 2010; Lane *et al*. 2011; Gethins *et al*. 2015; Morrissey *et al*. 2015). Since *K. marxianus* is thermotolerant, this provided an easy screen to investigate whether this yeast was present (Marsh *et al*. 2013; Lu *et al*. 2014; Korsak *et al*. 2015). Interestingly the non-cycled grain had no thermotolerant yeast, yet a thermotolerant yeast was readily recovered from the kefir (~1% of total yeasts in kefir at 12 h). This yeast was capable of growth on lactose at 42°C and molecular analysis using the 26S rRNA gene (D1D2 region) confirmed it to be *K. marxianus* (data not shown) (Kurtzman and Robnett 2003). As *K. marxianus* is frequently associated with kefir grains (Gao *et al*. 2015) it could be a dormant yeast that became activated after the repeated cycling or it could be a fresh acquisition from the milk. To determine which, we cultured thermotolerant yeast from the unpasteurised milk samples. Direct plating yielded no colonies but overnight pre-enrichment and incubation at 42°C did yield a small number of yeast colonies, also confirmed to be *K. marxianus*. To investigate whether the *K. marxianus* from the milk (UCKM1) was indeed the same strain that was incorporated into the cycled grain (UCKM2), a method to distinguish strains based on SNPs in a conserved gene was developed. Eight housekeeping genes were identified in *K. marxianus* genomes of sequenced strains, and a multiple sequence alignment was carried out to identify SNPs. One of these, IPP1, showed good SNP coverage and was used in this study. A total

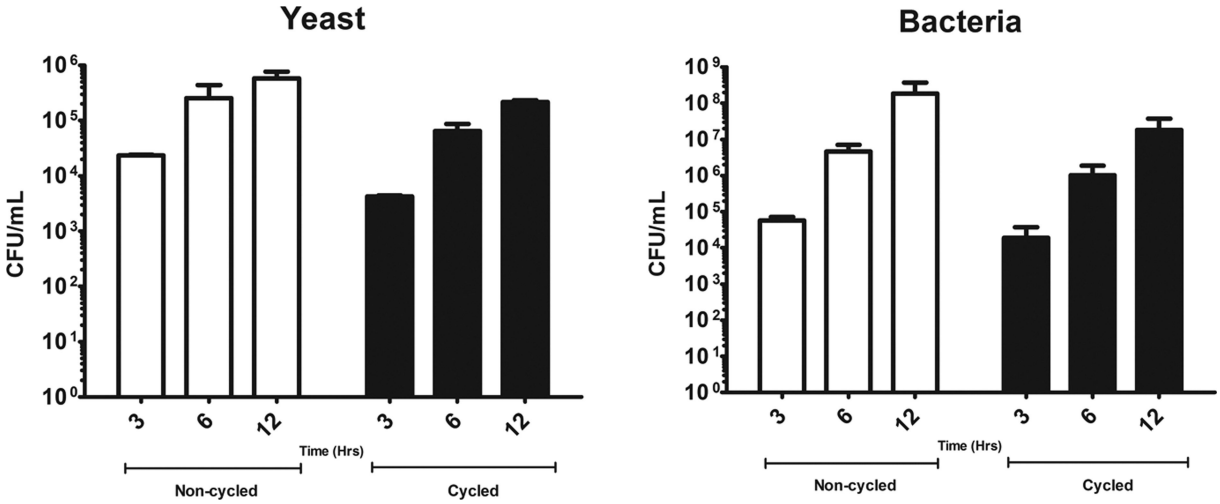

Figure 1. Quantification of yeast and bacteria during fermentation of kefir. Non-cycled (open bars) and cycled (solid bars) versions of the kefir grain SP1 were used to ferment pasteurised milk to produce kefir. Yeast and bacteria in the milk were quantified (CFU mL^{-1} milk) at 3, 6 and 12 h after inoculation.

Table 1. SNPs in gene *IPP1* in the 5 available *K. marxianus* genomes, *K. lactis*, *K. marxianus* isolated from the cycled grain (UCKM1) and the *K. marxianus* isolated from the milk (UCKM2). 13 SNPs were identified and their location within *IPP1* is indicated across the top.

	Base Pair Number												
Strain	219	246	348	408	429	501	555	618	846	861	891	894	900
Kluyveromyces lactis	C	T	T	C	T	G	A	T	C	T	A	T	T
CCT 7735	T	C	C	C	C	G	C	C	T	T	A	N	A
DMB1	C	T	T	C	T	A	C	C	T	G	G	A	T
NBRC 1777	C	T	T	T	T	A	T	C	T	G	A	T	T
DMKU 3–1042	C	T	T	T	C	A	T	T	T	G	A	T	T
KCTC 17555	C	C	T	T	C	A	C	C	T	G	A	T	T
UCKM1	T	C	C	C	C	G	C	C	A	T	A	T	A
UCKM2	T	C	C	C	C	G	C	C	A	T	A	T	A

of 13 SNPs were identified (Table 1). Primers were designed for this region (Fig. S1, Supporting Information) and PCRs were performed on genomic DNA extracted from the *K. marxianus* isolated from the grain and the unpasteurised milk. It was found that both strains were identical (Table 1). These data show that very low numbers of the yeast *K. marxianus* were present in the raw milk and that the kefir grains captured this yeast and allowed it to grow within the kefir grain. This is an intriguing and significant finding since it suggests that a grain can readily acquire a new yeast from its growth medium, and it is especially notable that *K. marxianus* was acquired, given the important role that this yeast is believed to play in kefir. A second grain passaged in milk at the same time also incorporated the same *K. marxianus* strain though that grain was not as thoroughly analysed (data not shown).

Kefir produced in parallel over the course of 24 h from both the non-cycled and cycled versions of the grain had noticeably different aromas. To study these differences, HS SPME-GCMS was conducted to measure individual volatiles and a trained tasting panel was used to compare organoleptic descriptors. In the HS SPME-GCMS analysis, a total of 42 volatile compounds were identified showing that kefir is indeed quite a complex food product. These volatiles consisted of 10 alcohols, 8 ketones, 8 aldehydes, 7 esters, 5 acids and a furan, lactone, benzene and a sulphur compound (Table S1, Supporting Information). To assess whether kefir produced by the cycled and non-cycled grains could be differentiated based on volatile patterns, a principle component analysis was carried out (Fig. 2A). There was a strong separation of the three non-cycled replicates (SP1NC-1, SP1NC-2, SP1NC-3) from the three cycled replicates (SP1C-1, SP1C-2, SP1C-3) demonstrating that the kefir produced by the two grains is quite different. To assess which particular volatiles distinguished the grains, statistical analysis was performed on the 42 volatiles detected in the kefir (Table S1, Supporting Information), with those showing significant differences (as determined by Student's *t*-test) presented in Fig. 2B. It is seen that the main metabolites responsible for the difference are various aldehydes; the ketone, diacetyl; and the ester, ethyl acetate, with some at higher levels and others at lower levels in the kefir made from cycled grains. To determine whether these volatiles changed the overall flavour of the product, a tasting panel of 20 individuals was recruited, trained and provided with relevant descriptors to carry out a taste analysis (Fig. 3). Kefir produced from the cycled and non-cycled versions of the grains were compared to each other using Student's *t*-test. There were several differences recorded for the cycled version: a reduction in aftertaste; and increases in fruity estery flavour, prickling texture and viscous texture. The fruity esters and the prickling texture of CO_2 (from fermentation of pyruvate to ethanol) are interesting as they are yeast-associated traits consistent with a change in the yeast composition of this grain.

The cycled grains were larger with more polysaccharide so the ultrastructure was visualised using SEM. Both the exterior and interior of each grain were examined with representative

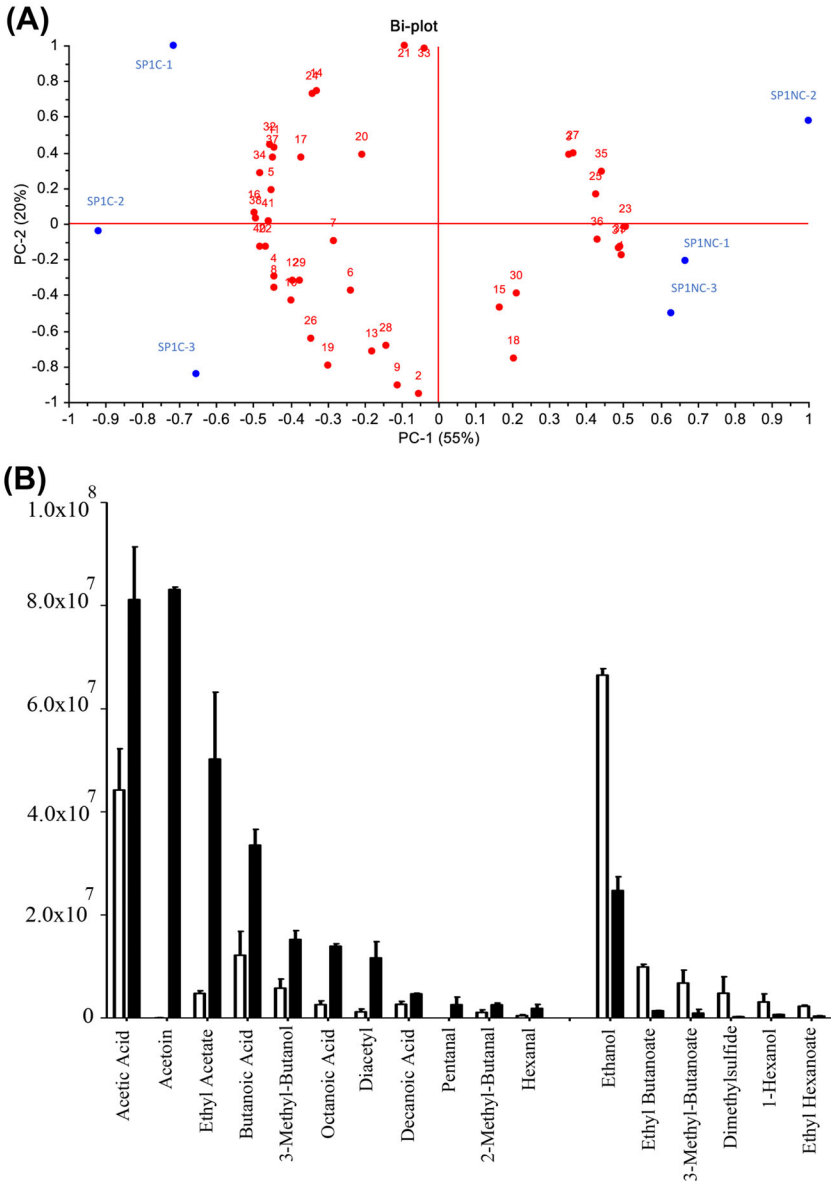

Figure 2. Volatile metabolites in kefir. (**A**) HS SPME-GCMS analysis conducted on kefir produced by cycled (SP1C) and non-cycled (SP1NC) versions of the in triplicate. A biplot showing the 42 volatiles measured and their relationship to the samples is shown. The corresponding number for each volatile can be found in (Table S1, Supporting Information). (**B**) The volatiles with significant differences (Student's *t*-test) are shown in bar chart format. Open bars represent the non-cycled (NC) grain and closed bars represent the cycled grain (C).

images presented (Fig. 4). The main features of bacterial cells, yeast cells and matrix are readily discernible, with arrows showing examples. Long and short bacterial rods are evident (blue arrow), as well as individual, clusters and chains of yeast cells (red arrow). The matrix is also visible (green arrow). Microbes are frequently in close proximity with each other and typically associated with matrix. In both the non-cycled (NC) and cycled (C) versions of the grain, clearly visible clusters of bacterial rods and yeast can be seen. The most striking feature is the copious polysaccharide production on the exterior of the cycled grain (panel D). This is very noticeably absent in the non-cycled grain (panel C).

In summary, culturing kefir grains in unpasteurised whole milk led to the reestablishment of kefir vigour as evidenced by increased growth and EPS production and changes in the yeast composition. The EPS production is likely to be linked to the fat composition of the milk, whereas the changes in microbiota result from a combination of nutrient change and microbial seeding of the grain from the unpasteurised milk. This highlights how different environmental factors can influence kefir since both the milk composition and the microbia will vary. Indeed, given that the fat composition of milk varies over the dairy season, there may also be seasonal variations, though this was not assessed. The changes that were seen, in particular the acquisition of *K. marxianus*, demonstrate an intrinsic fluidity in the grain microbiota, though at the same time, the rejuvenation of grains that lacked vigour indicates an underlying resilience at the core of the grains. It is notable that a previous metagenomic study by Marsh *et al.* (2013) showed a higher level microbial diversity compared to our study. The decrease in diversity indicates that

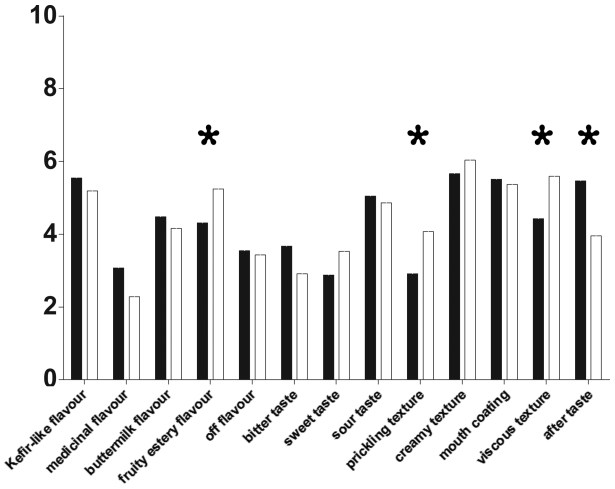

Figure 3. Sensory analysis of kefir produced by non-cycled and cycled SP1 grains. Ranked descriptive tasting analysis of kefir made by fermentation of pasteurised milk was conducted by a trained tasting panel of 20 members. Descriptors are listed along the x-axis with scores on the y axis. Data for SP1 are shown with solid bars representing non-cycled grains and open bars, cycled grains. Statistically significant differences as determined by Student's *t*-test between non-cycled and cycled version are marked with an asterisk.

long-term cold storage negatively impacts the grain microbiota. From our work, it is not possible to say how quickly this loss of diversity occurs (following cold storage/freezing) and addressing this could form the basis of a follow-up study. Taste often arises from a combination of metabolites, and thresholds for different metabolites vary so it is difficult to make conclusions based on increases in individual volatiles. Nonetheless, it was striking to see that passaging in unpasteurised milk and the acquisition of *K. marxianus* led to increased production of known yeast volatiles and perception of yeast-like tastes (fruity and prickling). It is tempting to speculate that these are all linked but further experimentation of the activity of *K. marxianus* in consortia with Lactobacilli in milk will be required. Bacteria, of course, play a fundamental part in the production of kefir and understanding the interactions will be very important for developing applications. Although this paper focuses on the yeast component, studying the fluidity of the bacterial communities using a similar passaging approach would be quite interesting. In addition a metagenomic approach to study bacteria and yeast that may be missed by a traditional culturing approach could also be very enlightening as to the synergistic nature of these complex microbial communities. Marsh *et al.* (2013) previously conducted a metagenomics analysis of the bacteria in this grain and they found that the predominant bacterial species was *Lactococcus*. As *Lactococcus* species are often found in milk it was quite possible that bacterial composition may have undergone a similar effect to the yeast in this study. It would also be interesting to study these interactions on metabolic scale using metabolic modelling. It is quite possible that some of the bacterial and yeast species have developed a metabolic inter-dependency on one another in the grain community.

SIGNIFICANCE AND IMPACT OF THE STUDY

Kefir is a traditional beverage produced by fermentation of milk with a consortium of bacteria and yeast that are embedded in a polysaccharide matrix. We found that stored kefir grains had poor functionality but recovered activity after several passages in unpasteurised milk. The most notable finding was that grains acquired and incorporated the yeast *Kluyveromyces marxianus* from the unpasteurised milk. This is significant as *K. marxianus* is associated with many of the beneficial characteristics of kefir. The study highlights the potential variability of the microbial composition of kefir grains and the relationship between the characteristics of kefir and the source of the milk for fermentation.

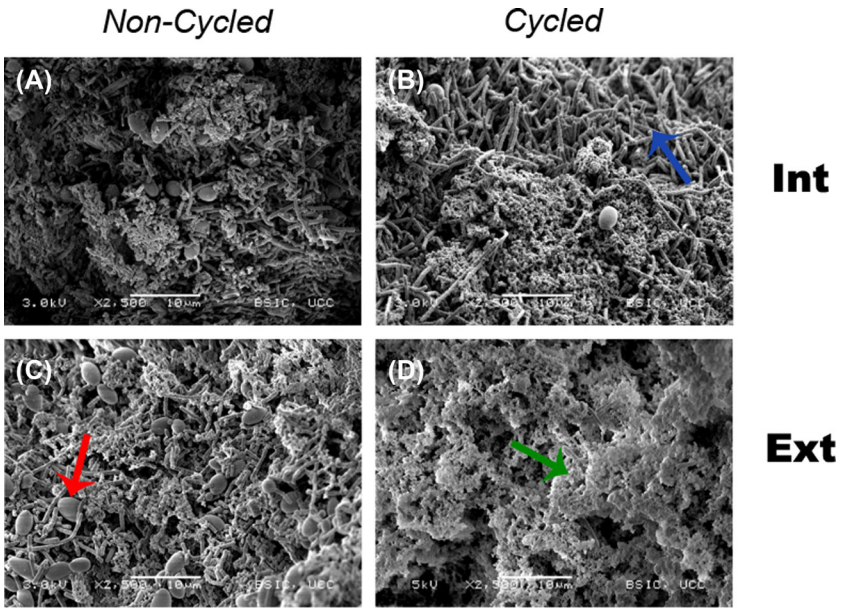

Figure 4. SEMs of the interior and exterior of SP1 cycled and non-cycled. SEMs were conducted to visualise the yeast, bacteria and ultrastructure of the grain. The interior (Int) and exterior (Ext) of both the non-cycled (**A** and **C**) and cycled (**B** and **D**) versions of the grain are shown. An example of a yeast cell (red), a lactobacillus cell (blue) and the polysaccharide matrix (green) are shown with the corresponding arrows.

SUPPLEMENTARY DATA

Supplementary data are available at FEMSLE online.

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Conflict of interest. None declared.

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