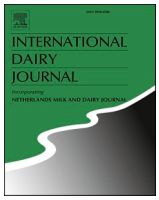
International Dairy Journal 164 (2025) 106202

Contents lists available at ScienceDirect

International Dairy Journal

journal homepage: www.elsevier.com/locate/idairyj



Research paper

Bacterial diversity, bioactive peptides, and enhanced immunomodulatory effects in raw milk kefir made with defined starter cultures versus backslopping

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ARTICLE INFO

Keywords:
Raw milk kefir
Backslopping
Kefir using starter cultures
Microbial composition
Bioactive peptides
Immunomodulation

ABSTRACT

This study compared the microbial composition, peptide profiles, and immunomodulatory effects of raw milk kefir produced using a defined starter culture (RMK-S) versus backslopping (RMK-B). RMK-B exhibited significantly higher microbial loads, with a 10-fold increase in total plate counts and 35-fold increase in lactic acid bacteria compared to RMK-S. This correlated with higher peptide content in RMK-B kefir, though RMK-S displayed higher bacterial diversity and a more diverse, bioactive peptide pool. Microbial analysis revealed RMK-S retained the starter culture's profile, while RMK-B was dominated by *Lactococcus lactis* and consistent yeast species, including *Kazachstania*, *Klyveromyces,* and *Galactomyces*. In a murine food allergy model, RMK-S kefir significantly reduced the allergic skin response and increased IFN-γ production, demonstrating enhanced immune modulation. RMK-B did not exhibit these protective effects. These findings point towards the role of bacterial diversity and peptide composition in kefir's health benefits, favoring defined starter cultures over backslopping.

1. Introduction

Commercial kefir is often produced using processed milk and a defined starter culture (SC), whereas traditional kefir is produced using a Symbiotic Culture of Bacteria and Yeast (SCOBY), also known as kefir grains (Ding, Stoyanova, & Netrusov, 2022; Nejati et al., 2022). The SCOBY multispecies biofilm comprises an undefined natural starter culture with a variety of bacteria and yeasts and a self-aggregated cauliflower-like matrix of the exopolysaccharide kefiran, consisting of glucose, galactose, and proteins (Dong et al., 2017; Nejati et al., 2022). Kefir SCOBY form a resilient ecosystem, with up to 30–50 microbial species colonizing milk in varying amounts (Blasche et al., 2021). Traditional kefir produced using SCOBY harbours different compositions from region to region, resulting from local processing practices (Ding et al., 2022; Garofalo et al., 2020; H. Wang et al., 2021), and fluctuating over time (Nejati et al., 2022).

Several studies have indicated the presence of a bacterial core community in kefir SCOBY (Blasche et al., 2021). DNA sequence analysis showed that kefir SCOBY consist of lactic acid bacteria, including homofermentative species *Lactobacillus kefiranofaciens* ssp. *kefirogranum* and heterofermentative species *Lentilactobacillus kefiri* (basonym: *Lactobacillus kefiri*) and *Lentilactobacillus parakefiri* (basonym: *Lactobacillus parakefiri*), which produce lactic acid, acetic acid, carbon dioxide, and flavour components (Ding et al., 2022; Georgalaki et al., 2021; Nejati et al., 2022). The yeast community can be divided into lactose- and non-lactose-assimilating yeasts (Prado et al., 2015), often with species of the genera *Kazachstania, Saccharomyces,* and *Kluyveromyces* (Blasche et al., 2021; Kazou et al., 2021). Yeasts are responsible for the

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https://doi.org/10.1016/j.idairyj.2025.106202
Received 3 December 2024; Received in revised form 26 January 2025; Accepted 26 January 2025
Available online 5 February 2025

production of carbon oxide and small amounts of ethanol, typical components of SCOBY-based kefir drinks (Oberg et al., 2022).

SCOBY-based kefir is not used on a commercial scale because of limited reproducibility, high costs, and slow rate of processing (Nejati et al., 2022). If kefir SCOBY is used for large-scale kefir production, an additional backslopping step is introduced to mimic traditional kefir production. Backslopping increases kefir yield 50-fold compared to traditional kefir (Kim et al., 2018). After sieving the SCOBY, traditional kefir beverage is used as a mother culture (MC) to produce a larger batch of backslopped kefir (BK) (Gao et al., 2015; Garofalo et al., 2020; Simova et al., 2002). Backslopping is also known as the Russian kefir-method (Prado et al., 2015). Changes in the relative abundance of lactic acid bacteria, acetic acid bacteria, and yeasts in BK vary among studies. However, a common finding is that the abundance of kefiran-producing species decreases and that of lactococci increases during BK production (Garofalo et al., 2020; Kim et al., 2018).

Interest in fermented dairy products, such as yoghurt and kefir, has increased because of their supposed health effects. Kefir, however, cannot be called a 'probiotic product' due to its diverse and undefined microbial composition, and presumed health benefits cannot be ascribed to specific microbial species. Nevertheless, in both animal and human studies, the intake of kefir has been associated with health improvements in terms of immune modulation, allergy, type-2 diabetes, and cancer (Farag et al., 2020; Slattery et al., 2019). The mechanism by which kefir may improve health is not always clear, because of its complex composition. Changes in the gut microbiome (Ding et al., 2022) and epigenetic effects (Gao & Zhang, 2019) have been observed after kefir consumption. Kefir contains a wide range of lactose-fermenting bacteria (Slattery et al., 2019). However, its yeast composition is also associated with health improvement (W. Wang et al., 2018). Another impact of kefir is as a postbiotic, as bioactive peptides are released from kefir proteins that may have an impact on immunomodulation, hypersensitivity, weight control, skin health, and metabolic syndrome (Amorim et al., 2019; Dallas et al., 2016; Ebner et al., 2015).

In our previous study, kefir produced using a defined starter culture was analysed for its microbial composition, bioactive peptides, and immune response in a murine food allergy model (Baars et al., 2023). This study showed that kefir based on a defined freeze-dried starter and prepared from raw cow milk provided protection against food allergy symptoms, which was not observed after kefir was prepared from heated milk. Pasteurization (heat load) of milk affects the allergenicity of the milk as shown in studies where mice were protected against allergic symptoms when treated with raw milk. This was confirmed in a pilot clinical study in food allergic infants (Abbring et al., 2019). In this study, we analysed the impact of two kefir end products commercially available in The Netherlands, both based on raw cow milk, and prepared with either a defined starter culture (RMK-S) or backslopped from SCOBY (RMK-B). We examined the allergy modulatory aspects, the kefir microbiome, and peptide compositions.

2. Materials and methods

2.1. Kefir production and sampling

Based on two different milk batches on successive days from the same farm, either RMK-B or RMK-S was produced at a commercial dairy plant, the Raw Milk Company (De Lutte, The Netherlands). Each batch of kefir was produced from bulk tank milk of one morning milking. Raw bulk milk was cooled to approximately 25 °C in a transport tank (1000 L). The time between the end of milking and the start of fermentation was less than 2 h. The plant is under control of The Netherlands Food and Consumer Product Safety Authority according to the Regulation (EC) No 852/2004 of the European Parliament and of the Council of April 29, 2004 on the hygiene of foodstuffs. Two types of raw milk kefir were prepared, based either on SCOBY in a backslopping process (RMK-B) or on a freeze-dried defined starter culture (RMK-S). Raw milk was delivered by two organic farms. For research purpose one of the farms was selected, and the RMK-B and RMK-S were made on successive days. The changes in the microbiotoa composition during kefir production were determined from milk originating from both farms and showed similar outcomes with limited impact of the farm.

To control the fermentation process, the pH was measured every 1–3 h. The sampling time points were set at 0 h (raw milk) and after inoculation (1 h, 6 h, 12 h, 24 h, 1 week, 3 weeks). The RMK-B was also sampled at 36 h. Kefir end products were defined for RMK-B at 36 h and RMK-S at 24 h, based on the processing protocol in the plant. For the outgrowth of the yeast, RMK-B was fermented for an extra 12 h.

The RMK-B kefir is based on backslopping of an in-house produced culture from SCOBY, see also Garofalo et al. (2020). Every day, 20 L of kefir-culture was made from SCOBY at 24 °C, to be used as a mother culture (MC) to produce RMK-B. Before further use of the kefir-culture, SCOBY was sieved for reuse. RMK-B was produced by adding 2% MC to a fermentation tank (1000 L) filled with fresh, warm raw milk. Fermentation was performed at 24 °C for 36 h, after which RMK-B was filled in bottles, which were stored at 4 °C. The RMK-S was produced by adding 2% freeze-dried starter culture 'eXact® 1 Kefir' from the company Christian Hansen (Hoersholm, Denmark) in a fermentation tank (1000 L). Fermentation was performed at 28 °C for 24 h. The kefir was bottled and stored at 4 °C. The company 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* subsp. *cremoris, L. lactis* subsp. *lactis, L. lactis* biovar. *diacetylactis,* and the yeast *Debaryomyces hansenii*.

2.2. Enumeration of total bacteria, lactic acid bacteria and yeasts

Agar plates were used to determine total bacterial counts on Tryptic Soy Agar (TSA), lactic acid bacteria on Man, Rogosa, and Sharpe (MRS) agar, and yeasts on Sabouraud Dextrose Agar (SDA) agar with chloramphenicol. Petri dishes with agar media and NaCl solutions (0.9%, pH = 7.0) were delivered by Biotrading Benelux BV (Mijdrecht, The Netherlands). After incubation at 30 °C, colony forming units of selected dilutions were determined after 24–30 h.

2.3. 16S rRNA and ITS amplicon sequencing

Samples were collected during milk fermentation, stored on dry ice, and kept at −80 °C prior to analysis. Microbiota composition was determined by sequencing the 16S rRNA gene (V3-V4 regions) and fungal ITS1 region, as described in detail (Baars et al., 2023). In summary, DNA isolation efficiency and taxonomic assignment were assessed using a mock microbial community and genomic spike-in control. Samples were processed with a specific milk bacterial DNA isolation kit (Norgen Biotek, Thorold, Canada). Mechanical cell lysis involved bead shaking according to the manufacturer's protocol (Fastprep®-24 5G Instrument; MP Biomedicals, Solon, OH, United States). DNA quality control, library preparation, and sequencing were conducted by Macrogen (Seoul, Korea). Sequencing involved the V3-V4 region of the 16S rRNA gene and ITS1 region on the Illumina MiSeq platform (Illumina, San Diego, CA, USA). Sequence data processing included demultiplexing, trimming, denoising, and taxonomic classification using Qiime2 (Bolyen et al., 2019). The final data, excluding spike-in sequences, was visualized by a heat map made with the multi-experiment viewer (MeV) (Saeed et al., 2003). All raw DNA sequence data generated in this project are available at NCBI under the bio-project number PRJNA716278. The amplicon sequence variants and metadata are presented in Supplemental Excel File 1.

2.4. Kefir peptidomics

Peptide samples were analysed using nano LC-MS/MS as described previously (Baars et al., 2023). In summary, samples were thawed and

centrifuged to remove caseins and fat, followed by protein precipitation by adding 200 g/L trichloroacetic acid. The peptide fraction in the supernatant was cleaned by solid-phase extraction. Then, 4 μL of peptide solution was separated onto a ReproSil-Pur analytical column in a gradient of 9–34% acetonitrile in water (Thermo nLC1000). Full-scan FTMS spectra were obtained using a Q-Exactive HFX (Thermo Electron, San Jose, CA, USA). The 25 most abundant positively charged peaks in the MS scan were fragmented (HCD). The resulting LC-MS/MS data files were processed using MaxQuant v1.6.1.0, using a database comprising only the proteins of which peptides were identified in an initial search. Oxidation of methionine, N-terminal acetylation, deamidation of asparagine and glutamine, and phosphorylation of serine and threonine were set as variable modifications. Peptides were filtered based on score (>80). The bioactivity of the identified peptide sequences were predicted using the Milk Bioactive Peptide Database (MBPDB), using 'precursor' with a similarity threshold of 100%.

2.5. Murine food allergy model

This murine food allergy model has been described in detail previously (Baars et al., 2023). In short, three-week-old, specific pathogen-free female C3H/HeOuJ mice were obtained from The Jackson Laboratory (Bar Harbor, ME, USA) and housed at Utrecht University (Utrecht, The Netherlands). The mice were maintained in filter-topped makrolon cages under a 12-h light/dark cycle, with unrestricted access to food and water. Following a 6-day habituation period, the mice were randomly assigned to four experimental groups: PBS/PBS ($n = 6$), PBS/OVA (allergic control, ($n = 8$)), RMK-S/OVA ($n = 8$), and RMK-B/OVA ($n = 8$). Sensitization was conducted on experimental days 0, 7, 14, 21, and 28 using oral administration of ovalbumin (OVA) with cholera toxin (CT) as an adjuvant. The mice received treatments of PBS, RMK-S, or RMK-B three times a week from day −1 to 32 via oral gavage. Kefir end products were used after 24 h of fermentation for RMK-S and 26 h of fermentation for RMK-B (Table 1). The batches of kefir end products were divided over multiple samples and frozen immediately after fermentation at −18 °C. Kefir samples were thawed at the day of treatment. Mice received kefir end products from the same batch from day −1 to 32. On day 33, an intradermal OVA challenge was performed to evaluate acute allergic responses, including the acute allergic skin response (measured as ear swelling), anaphylactic shock symptoms, and body temperature changes. Blood samples were collected on day 34 to measure OVA-specific IgE levels using ELISA. Additionally, splenocytes were harvested and stimulated ex vivo with OVA for cytokine production assessment of IL-5 and IFNg by ELISA following manufacturing protocol (Ebioscience, Inc). Statistical analyses included one-way ANOVA with Bonferroni's multiple comparison test for preselected groups and the Kruskal-Wallis test with Dunn's multiple comparisons test for non-parametric data, with significance set at $p < 0.05$. This study was approved by the Ethical Committee for Animal Research of Utrecht University and adhered to the European Directive 2010/63/EU on the protection of animals used for scientific purposes (AVD108002015346).

3. Results

3.1. High bacterial and fungal load in RMK-B

We compared the total plate count (TPC), total lactic acid bacteria count (LAB), and total yeast and mold count (TYMC) of RMK-B with RMK-S. The milk used at successive days to produce RMK-S and RMK-B showed very similar TPC counts ($2.2x10^3$ and 5.8×10^3 CFU/ml respectively). TPC was more than 10-fold higher in the RMK-B at 36 h. Even though the inoculum had a higher TPC in the SCOBY culture, RMK-B kefir reached values of approximately 10^9 CFU/ml. This was also reflected in the LAB counts with a 35-fold difference in the final product. For both types of kefirs, LAB counts decreased to approximately 5×10^6 CFU/ml at the end of the shelf life (3 weeks). The difference in TYMC counts was more subtle, with a 2-fold difference in the end product. However, both types of kefirs comply with the values recommended by the FAO and WHO for kefir products containing TYMC counts of at least 10^4 CFU/ml (Dimidi et al., 2019).

3.2. Distinct microbial populations in RMK-B and RMK-S

Analysis of the freeze-dried SC led to the identification of *Streptococcus thermophilus, Lactococcus lactis*, *Leuconostoc,* and the yeast *Debaryomyces* (Fig. 1), exactly matching the microbial genera reported on the label of the SC (Baars et al., 2023). After inoculation with the SC, the microbial richness observed in raw milk itself (at 0 h) decreased and the species present in the starter culture became dominant. Several species were identified in the final product at 24 h, which may have originated from the raw milk. These include additional sequence variants of *Lactococcus* (*lactis*), and the yeasts *Pichia* and *Galactomyces,* as reported previously (Baars et al., 2023).

The bacterial population in SCOBY consist predominantly of three species: *Lactobacillus kefiranofaciens* (90.8%), *Lactococcus lactis* (4.7%) and *Lentilactobacillus kefiri* (4.2%); the four predominant fungal species in the raw milk kefir backslopped fromSCOBY are *Kazachstania turicensis* (68.8%), *Kazachstania unispora* (28.7%), *Galactomyces* (2.1%), and *Kluyveromyces marxianus* (0.3%) (Fig. 1). In the mother culture (MC) made from SCOBY all these species could be identified, but with a drastic change in relative abundance. The bacterial composition changes to *Lactococcus lactis* (99.7%), and the yeast composition to *Galactomyces* (82.3%) and *Kazachstania* spp (13.9%), and *Kluyveromyces marxianus* (3.7%). This retained the composition of the kefir end product at 36 h. However, relatively small amounts of *Lentilactobacillus kefiri* and *Debaromyces* end up in the final kefir product, most likely originating from MC starter and raw milk, respectively.

3.3. Peptide profiles

3.3.1. Peptide diversity and length distribution in kefir samples

We compared the peptides in the two raw milk samples and the two kefir end products. The differences between RMK-B and RMK-S on

Table 1
Sample characteristics of raw milk kefir produced using a defined starter culture (RMK-S) and backslopping with SCOBY (RMK-B) during production and storage. TPC: total plate counts of mesophilic bacteria; LAB: total lactic acid bacteria count; TYMC: total yeast and mold counts. Fermentation was performed for 24 or 36 h, followed by storage at 4 °C for three weeks. In bold, the kefir end products; (n.d., not determined).

	RMK-S (CFU/ml)				RMK-B (CFU/ml)			
Time	pH	TPC	LAB	TYMC	pH	TPC	LAB	TYMC
0 h	6.7	$2.2x10^3$	$6.6x10^2$	$5.0x10^1$	6.6	$5.8x10^3$	$1.3x10^3$	$4.0x10^1$
1 h	6.6	$9.1x10^4$	$1.0x10^6$	$8.6x10^1$	6.5	$4.0x10^7$	$4.0x10^7$	$7.7x10^3$
6 h	6.1	$1.6x10^6$	$5.4x10^6$	$1.9x10^3$	5.3	$1.6x10^9$	$1.6x10^9$	$1.2x10^4$
12 h	5.0	$3.6x10^7$	$3.4x10^7$	$3.9x10^4$	4.5	$2.6 x10^9$	$2.6x10^9$	$5.7x10^4$
24h	**4.4**	**$7.5x10^7$**	**$1.0x10^8$**	**$5.0x10^4$**	4.3	$1.1x10^9$	$1.3x10^9$	$1.1x10^5$
36h	n.d	n.d.	n.d.	n.d.	**4.3**	**$2.8x10^9$**	**$3.5x10^9$**	**$1.1x10^5$**
1w	4.4	$3.3x10^8$	$3.0x10^7$	n.d.	n.d.	n.d.	n.d.	n.d.
3w	4.3	$4.4x10^7$	$4.6x10^6$	$6.1x10^5$	4.2	$2.3 x10^9$	$5.5x10^6$	$8.6x10^4$

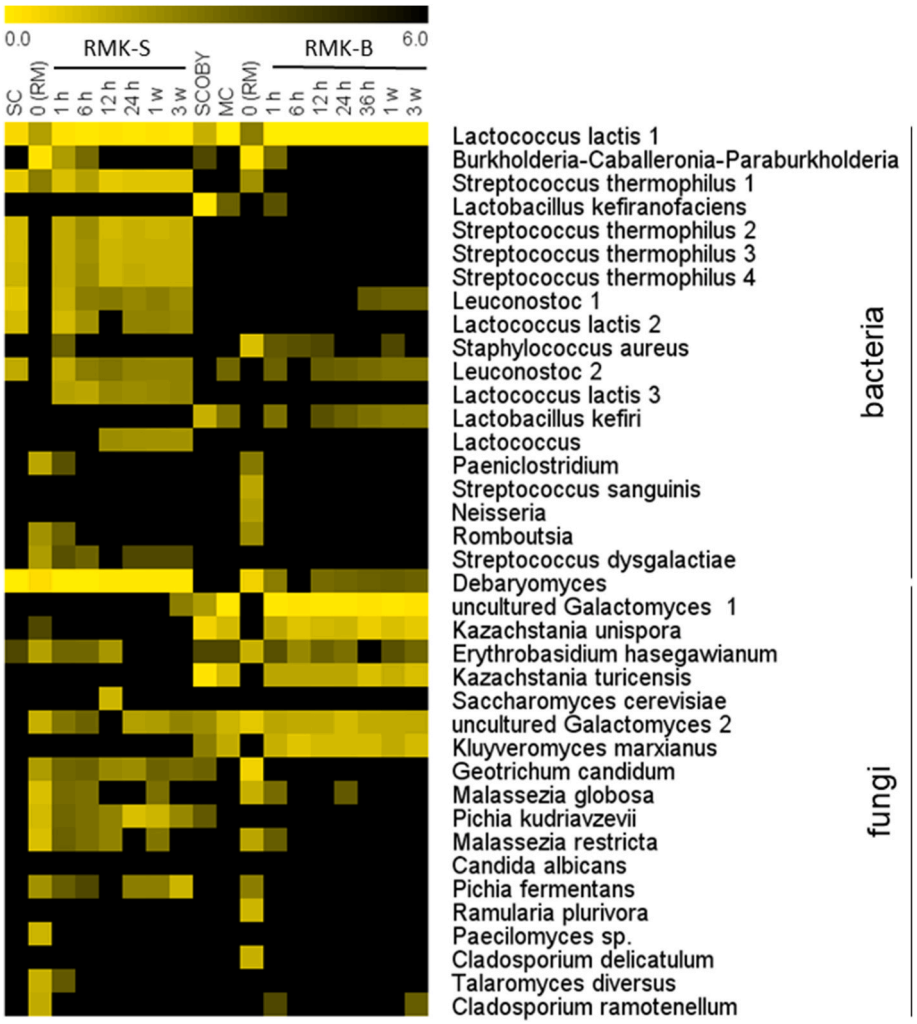

Fig. 1. Heat map of the bacterial and fungal populations in raw milk kefir obtained from a defined starter culture (RMK-S) and backslopping (RMK-B). Values for the -log10 fractions were plotted on a scale from 0 (yellow, 100%) to 6 (black, 0%). Numbers behind identical genus and species names indicate distinct amplicon sequence variants. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

peptide diversity, both in number and length, is shown in Fig. 2. Many peptides (926) were found in both kefir samples, but were absent in raw milk. The second-largest group of peptides was present in all four samples (519), whereas the third-largest group was specific to the RMK-S sample. This shows that the peptide diversity of the RMK-S sample was greater than that of the other samples. When examining the peptide length distribution, we observed that differences were relatively minor among the different combinations of samples (Fig. 2).

3.3.2. *RMK-B shows a higher peptide content*

After determining the qualitative differences among the samples, quantitative differences were determined, as shown in Fig. 3. This figure shows that kefir production leads to a large increase in peptide intensity, with an even larger increase for the RMK-B sample than for the RMK-S sample. Combined with the qualitative data, this shows that RMK-S has a more diverse peptide profile but at lower peptide levels, compared to RMK-B.

3.3.3. *Predicted bioactivity of unique peptides in RMK-S higher than RMK-B*

To determine the potential bioactivity of the peptides, both the bioactive peptides and their precursors were determined in the two kefir samples, as shown in Fig. 4. Many different potential bioactivities were found, with the highest summed peptide intensities for DPP-IV inhibitory, ACE-inhibitory, antioxidant, and antimicrobial activities. For all these bioactivities that had the highest summed peptides intensities, but also for almost all bioactivities detected, the RMK-S sample showed a higher potential bioactivity than the RMK-B sample. When comparing the level of individual potentially bioactive peptides, it was found that for peptides detected in both kefir samples, RMK-B more frequently contained a higher level of the respective peptide, whereas RMK-S contained more unique peptides that were not detected in RMK-B (Fig. 5). This again emphasizes that RMK-S has a more diverse peptide profile with more unique peptides, albeit at lower peptide intensities.

3.4. *Allergic symptoms and immune modulation in murine food allergy model*

3.4.1. *Treatment with RMK-S suppressed the acute allergic skin response*

Sensitization of control mice to OVA (PBS/OVA) results in an acute allergic skin response. Anaphylactic shock scores and drop in body temperature upon intradermal challenge with OVA were higher than those in PBS/PBS mice, but the differences were not significant (Fig. 6). The intervention with RMK-S (RMK-S/OVA) showed reduced acute allergic symptoms, measured as an allergic skin response, compared to PBS/OVA control mice. No effects were observed on the anaphylactic shock score and body temperature compared to the PBS/OVA mice (Fig. 7C and D). The effect on the allergic response was limited to RMK-

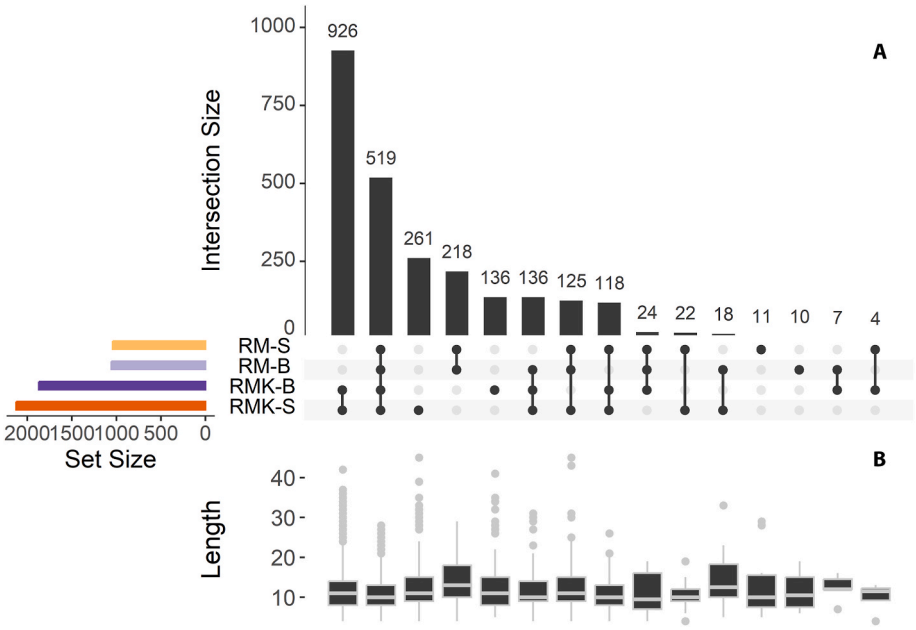

Fig. 2. Diversity of peptide profiles of the raw milk samples used for starter culture kefir (RM-S), backslopped kefir (RM-B), and the respective kefir end products (RMK-S and RMK-B). Above: Number of peptides in different sample combinations. In this visualization, vertical bars represent the number of unique peptides identified in the intersection of samples shown underneath each bar. Horizontal bars represent the total number of peptides identified in each sample. Below: peptide lengths in each sample combination.

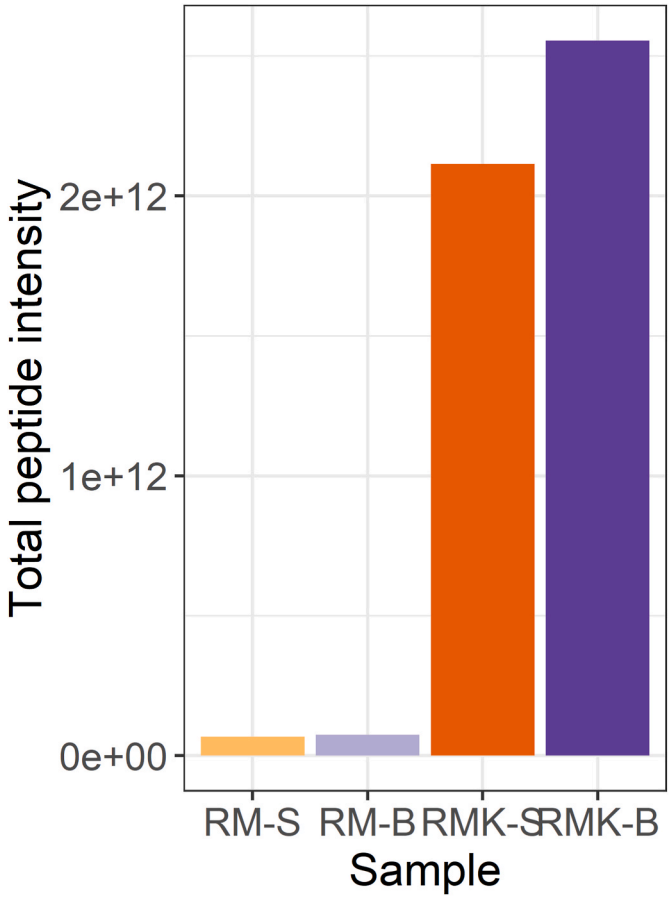

Fig. 3. Summed peptide intensities of raw milk samples (RM-S and RM-B), and the respective kefir end products (RMK-S and RMK-B).

S, as mice treated with RMK-B (RMK-B/OVA) showed no effect on the acute allergic skin response. Allergic symptoms are predominantly mediated by allergen-specific specific IgE). The average OVA-specific IgE levels was high in the PBS/OVA allergic control mice compared to the PBS/PBS sham control mice, but values did not reach significance. Although the acute allergic skin response was reduced in mice treated with RMK-S, no effects were observed on OVA-IgE in mice treated with either RMK-S or RMK-B compared to PBS/OVA allergic mice.

3.4.2. Increased IFNg-production in ex-vivo OVA stimulated splenocytes of mice treated with RMK-S

To determine the local immunomodulatory effects of RMK-S and RMK-B treatments, cytokine production in OVA-stimulated splenocytes was studied. IFNg-concentrations, as produced by splenocytes after ex vivo medium and OVA stimulation, were increased in mice treated with RMK-S (RMK-S/OVA). RMK-S and RMK-B did not affect IL-5, IL10 (Fig. 7), or IL-13 (below detection level).

4. Discussion

In this study, we compared the impact of two types of commercially available kefirs on its microbial composition, peptide composition, and immune modulation in a mouse model for food allergy. Both types of kefir were made from raw, organic and antibiotic free cow milk. We could show that in the process of backslopping of kefir (RMK-B) a loss of the bacterial diversity was found, which could be explained by the processing in the plant. One single species, *Lactococcus lactis,* dominated (>99%) the RMK-B bacterial composition. In contrast, the three yeast species found in the SCOBY itself were still present in RMK-B. The composition of RMK-S, however, completely reflected the composition of the freeze-dried starter culture. The number of bacteria and yeasts was 10-fold higher in RMK-B compared to RMK-S. There was no impact from the raw milk composition itself, probably due to the low bacterial load of the raw milk (around 10^3 cfu/ml). The peptide diversity of RMK-S was higher than from RMK-B, but the peptide intensity was higher in RMK-B compared to RMK-S. RMK-S contained more unique bioactive peptides, whereas RMK-B on average contained higher levels of

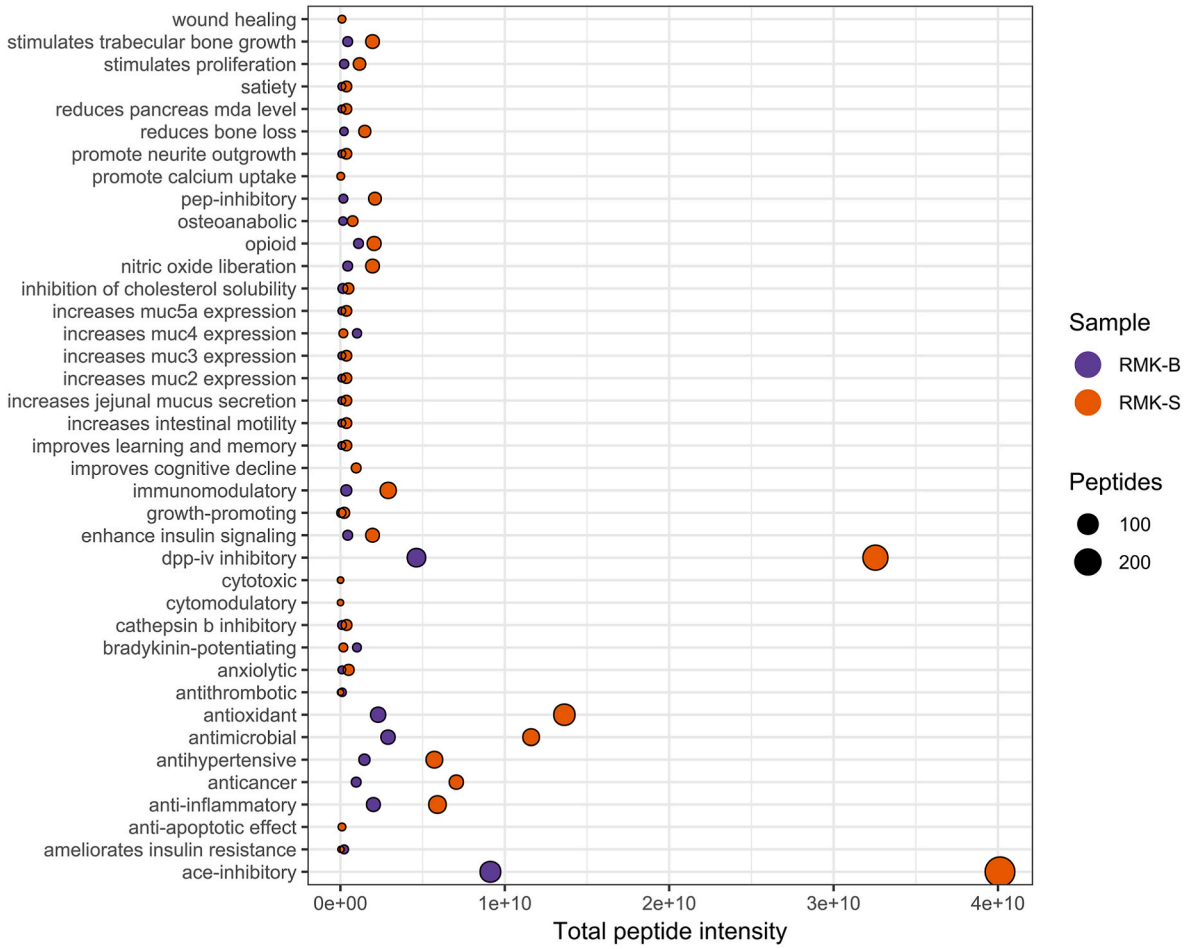

Fig. 4. Summed peptide intensities of the potential bioactive peptides detected in the defined starter kefir (RMK-S) and backslopped kefir (RMK-B). Size of the dots indicates the number of unique potentially bioactive peptides detected.

bioactive peptides. Measured as the changed ear swelling, only the RMK-S suppressed the acute allergic symptoms, not RMK-B. These outcomes correspond with an increased IFNg concentration in the spleen. Other cytokines were not affected. The reduction in allergic outcomes after treatment with RMK-S may have been caused by the other species of bacteria and yeasts in the freeze-dried SC, which caused a wider microbial diversity in this sample. This higher microbial diversity may have caused the higher numbers of bioactive peptides in RMK-S compared to RMK-B, which in turn may also be an explanation for the different outcomes in allergy.

The research described shows remarkable differences in the microbial populations between RMK-S and RMK-B. In our previous study, we only focused on kefir obtained from a defined starter culture and compared the microbial population, peptides, and immune responses of only starter culture-based kefir, either made from raw milk or heated milk (Baars et al., 2023). We showed that starter culture-based kefir predominantly contains a microbial population as present in the defined starter culture, with several bacterial and fungal species taking part in the fermentation process that originates from raw milk. The role, potential risks, and benefits of these species have been described in our previous study (Baars et al., 2023). The microbial composition of SCOBY-based kefir is highly variable due to several factors, such as the origin of the kefir SCOBY, characteristics of the milk, and processing conditions (in-between washing of grains), including the grain-to-milk ratio and temperature (Garrote et al., 1998; Nielsen et al., 2014). Most kefir SCOBY are dominated by either *Kazachstania* or *Saccharomyces cerevisiae, Lentilactobacillus kefiri*, and *Lactobacillus kefirofaciens;* RMK-B typically contains the *Kazachstania* type, for example, found in SCOBY originating from Tibet (Prado et al., 2015). The mother culture (MC) used for production of RMK-B was dominated by *Lactococcus lactis* (>99%), which can be explained by the process of backslopping in the plant. Gao and Zhang (2019) compared the microbiomes of kefir SCOBY and kefir beverages produced using SCOBY, which showed that three out of the five kefir SCOBY origins were dominated by *Lb. kefiranofaciens* (70–80%) and *L. lactis* (20–30%), which were transformed into backslopped kefir beverages dominated by *L. lactis* (75–100%). The main yeast in the three kefir SCOBY was *K. unispora* (45–98%). The kefir beverages made from different SCOBY reflected the yeast composition ratio in the SCOBY. In another study, Gao et al. (2015) showed a gradual change in the microbial diversity in the 2nd-8th generation of sub-cultivation. *Lb. helveticus* was the dominant species in the backslopped generations, reaching levels of approximately 40%, but was not present in traditional, 1st generation kefir. The yeast population was dominated by *K. unispora* in both traditional and backslopped kefir (40–65%). *Saccharomyces cerevisiae* was reduced in subsequent backslopping steps, reaching levels <1% after the 3rd generation. The dominance of *Lb. kefiranofaciens* followed by *Lentilactobacillus kefiri* in SCOBY was exchanged by *L. lactis* in the successive generations of backslopping via the MC to the backslopped kefir. The decrease of *Lb. kefiranofaciens* in the mother culture made from SCOBY, and consequently in RMK-B, could be influenced by a number of factors such as the specific microaerophilic environment, nutrient requirements within the kefir grain interior, and tendency to thrive in close microbial association (Georgalaki et al., 2021). For the fungal composition, the changes in composition were less pronounced than for the bacterial composition. The microbial composition of our mother culture (MC) is comparable to

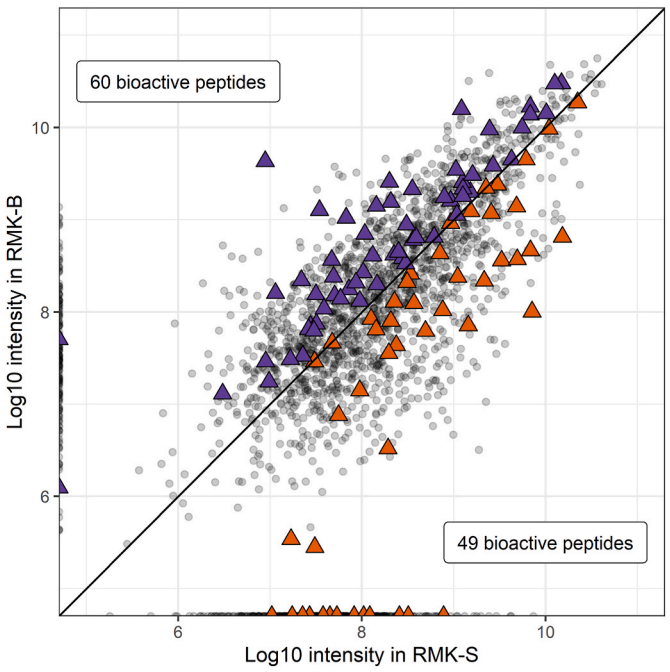

Fig. 5. Comparison of log10 intensity of all peptides in the defined starter kefir (RMK-S) and backslopped kefir (RMK-B) with the potentially bioactive peptides indicated by triangles (purple: higher level or unique in RMK-B, orange: higher level or unique in RMK-S). (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

the kefir beverages mentioned in Gao and Zhang (2019), being dominated by *Lactococcus lactis* (>99%). Due to the protocol of the commercial plant, SCOBY was not washed before further use. If SCOBY is not washed and/or small amounts of MC are inoculated, larger amounts of *Lactococcus lactis* are inoculated at the start, and other species, like *Lb. kefiranofaciens* or *Lentilactobacillus kefiri* could not compete with the *Lactococci*. However, in our findings there is a difference between the microbial composition for bacteria and yeasts. The two *Kazachstania* species present in the SCOBY are still the most abundant species in RMK-B. This means, that a change in the outcome of the kefir processing is crucial to maintain species like *Lb. kefiranofaciens* in the final kefir product.

The peptide profiles of both the milk and kefir samples were compared qualitatively and quantitatively. As the microbial diversity in RMK-B was lower, the range of proteolytic enzymes was also expected to be lower, resulting in a less diverse peptide profile of this sample. At the same time, the limited number of microbial species found in RMK-B, especially the most abundant strain, *Lactococcus lactis* being present at >99% in this sample, is known to be highly proteolytic (Tjwan Tan et al., 1993). This may explain the relatively high peptide intensities observed in RMK-B. At the same time, many different potential bioactive peptides showed increased abundance in RMK-S. Some of these peptides, for example those that are known to be immunomodulatory, may underpin the differences in the immune response, as shown in the murine model. However, the exact relationship between the individual peptides and the immune response in the murine model is difficult to determine. Follow-up studies on either the unique bioactive sequences or sequences present at higher levels, especially those associated with immunomodulation, either in vitro or in vivo, could provide more specific evidence of peptide functionality and shed more light on the potential underlying mechanisms.

The experimental data showed an allergy-protective effect and immune modulation of RMK-S, not RMK-B in a murine food allergy model. There is limited evidence regarding the causal relationship between kefir consumption and its modulating effect on allergic diseases. Mendes et al. (2021) demonstrated protection against lung inflammation and lung function in a murine allergic asthma model using pasteurized milk fermented by a defined starter culture. In a previous study, we described the allergy protective and immunomodulatory effects of kefir based on raw milk when the same starter culture was used, but not after heating of the raw milk (Baars et al., 2023). In humans, the consumption of raw milk kefir was associated with increased perceived immune fitness, as

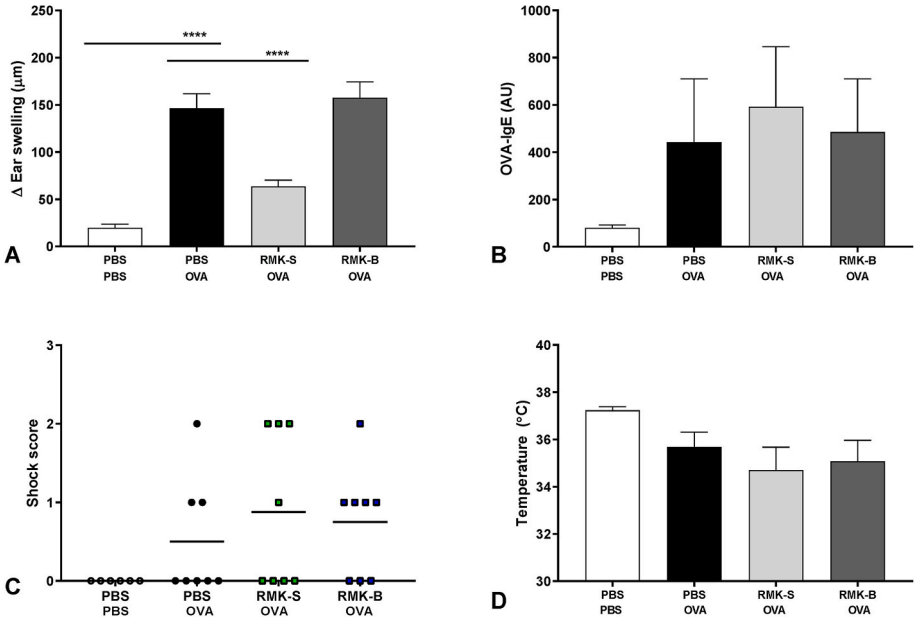

Fig. 6. Reduced acute allergic skin response upon OVA challenge in RMK-S-treated mice. (A) The acute allergic skin response measured as Δ ear swelling 1 h after i. d. challenge, (B) OVA-specific IgE, (C) anaphylactic shock score and (D) drop in body temperature. Data are presented as mean ± SEM (A, B, D) and as individual data points for anaphylactic shock scores (n = 6 in the PBS group and n = 8 in all other groups). ****P < 0.0001, as analysed by one-way ANOVA followed by Bonferroni's multiple comparisons test for preselected groups (A) or Kruskal-Wallis test for non-parametric data followed by Dunn's multiple comparisons test for pre-selected groups (B, C, D). OVA, ovalbumin; RMK-S, raw milk kefir from defined starter culture; RMK-B, raw milk kefir based on backslopped SCOBY grains; i. d., intradermal.

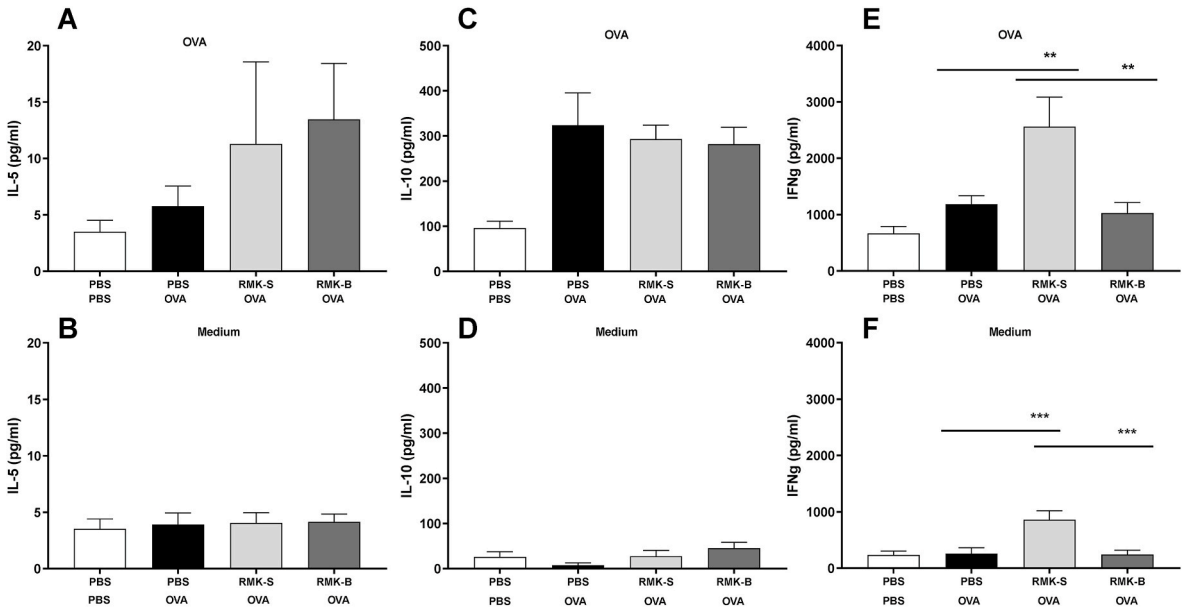

Fig. 7. Increased IFNg production in RMK-S-treated mice. Il-5 (A,B), IL-10 (C.D), and IFNg (E,F)were measured in the supernatant of non-stimulated (B,D,F) and ex vivo OVA-stimulated (A,C,F) splenocytes. Data are presented as mean ± SEM, n = 6 in the PBS group and n = 8 in all other groups. **P < 0.01, ***P < 0.001 as analysed with one-way ANOVA followed by Bonferroni's multiple comparisons test for preselected groups. RMK-S: raw milk kefir from a defined starter culture; RMK-B: raw milk kefir based on SCOBY grains.

measured by validated questionnaires (Baars et al., 2019; Wilod Versprille et al., 2019).

Recently, a new way to prepare kefir using a starter, opened the possibility of integrating selected kefir microorganisms with a known health impact (Bourrie et al., 2020). This kefir improved metabolic health in both mice (Bourrie et al., 2020) and humans (Bourrie et al., 2023), and could play a role in metabolic health. This implies that health outcomes could depend on the species present in the kefir end product. Probably not all kefir species have probiotic characteristics, which implies that further selection of the wild kefir starters, like SCOBY, could be important. Since we only could show an impact of RMK-S on the immune modulation, but not of RMK-B, it could also be crucial to control the processing of the backslopped kefir.

5. Conclusions

In this study, we compared two types of commercial kefirs made from raw cow milk, either based on a starter culture or backslopping from SCOBY. The allergy-protective effect of RMK-S was most likely related to a change in the T-cell compartment, as shown by an allergen induced increase in splenic IFNg production in RMK-S-treated mice. Kefir produced via backslopping showed reduced microbial richness, reduced number of bioactive peptides, despite the higher microbial loads and peptide intensity. The lack of outcome of RMK-B on immune modulation could be due to the specific processing of this kefir in the plant, favouring the predominance of a single bacterial species of *Lactococcus lactis*.

CRediT authorship contribution statement

Ton Baars: Writing – review & editing, Writing – original draft, Methodology, Investigation, Funding acquisition, Formal analysis, Conceptualization. **Betty van Esch:** Writing – original draft, Methodology, Investigation, Formal analysis, Conceptualization. **Mara Diks:** Formal analysis. **Luuk van Ooijen:** Formal analysis. **Zuomin Zhang:** Formal analysis. **Pieter Dekker:** Formal analysis. **Sjef Boeren:** Formal analysis. **Johan Garssen:** Supervision. **Kasper Hettinga:** Writing – original draft, Methodology, Conceptualization. **Remco Kort:** Writing – review & editing, Writing – original draft, Methodology, Formal analysis, Conceptualization.

Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: Ton Baars B was sponsored by the Raw Milk Company. The other authors declare no conflicts of interest.

Acknowledgments

Software AG Stiftung, Darmstadt, Germany (grant ID P12642) for funding the murine allergy study. Raw Milk Company, De Lutte, The Netherlands, for providing the milk and kefir samples in this study, as well as funding for microbial enumeration experiments.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.idairyj.2025.106202.

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