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Development and Nutritional Assessment of Potentially Probiotic Non-Dairy Product – *In vitro* Research

Opracowanie i ocena wartości odżywczej potencjalnie probiotycznego produktu niemlecznego – badania *in vitro*

Doctoral thesis

Rozprawa doktorska

Doctoral thesis prepared under the supervision of

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Table of content

Abstract 6
The list of publications making up the doctoral thesis 9
1. Introduction 10
2. Purpose and scope of the research 13
3. Materials and sample preparation 15
4. Research methods 16
5. Results and discussion 19
5.1 Development of non-dairy potentially probiotic products 19
5.2 Sensory analysis of the developed non-dairy potentially probiotic products 21
5.3 Survival analysis of the probiotic strains 23
5.4 Nutritional value of the developed non-dairy potential probiotic products 27
5.5 Gut microbiota composition and functionality effects of beetroot ketchup 34
6. Conclusions 43
7. References 44
8. Copies of the articles making up the doctoral thesis and statements of co-authors 49

Abstract

The dissertation aimed to assess the possibility of using non-dairy food matrices as carriers of probiotic bacteria and to evaluate the nutritional value of the obtained products based on an *in vitro* model of the human gastrointestinal tract. The influence of the composition on the growth stimulation and viability of probiotic strains, stabilization of pH change during storage and improvement of sensory properties of fermented products were analysed. In addition, the survival of selected potentially probiotic strains during *in vitro* digestion was studied. Furthermore, the impact of the designed products on the composition and functionality of the intestinal microbiota was assessed. In the work, products based on apple puree and ketchup with the addition of red beet were developed; both mixtures were fermented by the selected strains with probiotic properties.

The composition of the two dairy-free matrices was as follows: (1) apple puree, chia seeds and oat bran and (2) ketchup with beetroot, which included tomato paste, beetroot and spices. *Lacticaseibacillus rhamnosus* K3 or *Lactobacillus johnsonii* K4 strains were used for fermentation. The food matrix significantly affected probiotics' viability; in apple puree, the initial number of probiotic bacteria exceeded 9 log CFU/g and remained stable during storage. In fermented ketchup with beetroot, a high number of microorganisms was also found during storage. Sensory analysis of the products showed that fermentation improved the flavour, texture, and odour of both products tested.

Fermented products were characterized by increased amounts of organic acids, sugars and antioxidant activity, which emphasizes the importance of fermentation in improving nutritional value. In the experiment of probiotic survival during *in vitro* digestion, stability of survival was observed in the intestinal phases despite a decrease in their number after the gastric digestion phase.

Finally, i*n vitro* colon fermentation studies showed that unfermented and fermented ketchup with beetroot promoted the growth of beneficial intestinal bacteria while limiting the number of potential pathogens. Increased production of short-chain fatty acids and phenolic metabolites was observed, which indicates complex interactions between food components and intestinal microbiota. These studies prove that dairy-free plant foods have the potential to act as probiotics and support gut health.

Keywords: non-dairy food, digestion, fermentation, gut microbiota, probiotics

Streszczenie

Celem rozprawy doktorskiej była ocena możliwości wykorzystania niemlecznych matryc żywnościowych jako nośników bakterii probiotycznych oraz ocena wartości odżywczej otrzymanych produktów w oparciu o model *in vitro* przewodu pokarmowego człowieka. Analizowano wpływ składu na stymulację wzrostu i żywotności szczepów probiotycznych, stabilizację pH podczas przechowywania oraz poprawę właściwości sensorycznych produktów fermentowanych. Ponadto, badano przeżywalność wybranych szczepów potencjalnie probiotycznych podczas trawienia *in vitro*. Dodatkowo oceniano wpływ zaprojektowanych produktów na skład i funkcjonalność mikrobioty jelitowej. W pracy opracowano produkty na bazie puree jabłkowego oraz ketchupu z dodatkiem czerwonego buraka, obydwie mieszaniny fermentowano z udziałem wyselekcjonowanych szczepów posiadających cechy probiotyczne.

Skład dwóch bezmlecznych matryc był następujący: (1) puree jabłkowe, nasiona chia i otręby owsiane oraz (2) ketchup z burakiem, w którego skład wchodził koncentrat pomidorowy, buraki i przyprawy. Do fermentacji wykorzystano szczepy *Lacticaseibacillus rhamnosus* K3 lub *Lactobacillus johnsonii* K4. Matryca żywnościowa miała istotny wpływ na żywotność probiotyków; w puree jabłkowym początkowa liczba bakterii probiotycznych przekraczała 9 log jtk/g i pozostała stabilna podczas przechowywania. W fermentowanym ketchupie z burakiem również stwierdzono wysoką liczbę mikroorganizmów w czasie przechowywania. Analiza sensoryczna produktów wykazała, że fermentacja poprawiała smak, teksturę i zapach, w obydwu badanych produktach.

Produkty fermentowane charakteryzowały się zwiększoną ilością kwasów organicznych, cukrów oraz aktywnością antyoksydacyjną, co podkreśla znaczenie fermentacji w poprawie wartości odżywczej.

W badaniach przeżywalności probiotyków podczas trawienia *in vitro* zaobserwowano stabilność przeżywalności w fazach jelitowych, mimo zmniejszenia ich liczby po fazie trawienia żołądkowego.

W badaniach fermentacji jelitowej *in vitro* wykazano, że niefermentowany i fermentowany ketchup z burakiem sprzyjał wzrostowi korzystnych bakterii jelitowych

przy jednoczesnym ograniczeniu liczby potencjalnych patogenów. Zaobserwowano zwiększoną produkcję krótkołańcuchowych kwasów tłuszczowych oraz metabolitów fenolowych, co wskazuje na złożone interakcje pomiędzy składnikami żywności a mikrobiotą jelitową. Badania te dowodzą, że bezmleczne produkty roślinne mają potencjał do działania probiotycznego i wspierania zdrowia jelit.

Słowa kluczowe: żywność bezmleczna, trawienie, fermentacja, mikrobiota jelitowa, probiotyk

The list of publications making up the doctoral thesis

P1 Küçükgöz K, Trząskowska M. Nondairy Probiotic Products: Functional Foods That Require More Attention. Nutrients. 2022; 14(4):753. https://doi.org/10.3390/nu14040753. **Impact Factor: 5.9, Ministry Point: 140**

P2 Küçükgöz K, Kruk M, Kołożyn-Krajewska D, Trząskowska M. Investigating the Probiotic Potential of Vegan Puree Mixture: Viability during Simulated Digestion and Bioactive Compound Bioaccessibility. Nutrients. 2024; 16(4):561. https://doi.org/10.3390/nu16040561. **Impact Factor: 4.8, Ministry Points: 140**

P3 Küçükgöz, K., Venema, K., & Trząskowska, M. (2024a). Beetroot Ketchup as a Stable Carrier of Potential Probiotic *Lacticaseibacillus rhamnosus* K3 and *Lactobacillus johnsonii* K4: A Study on Sensory Attributes, Storage Viability, and *In Vitro* Gastrointestinal Survival. *Food and Bioproducts Processing*. https://doi.org/10.1016/j.fbp.2024.10.004

Impact Factor: 3.5, Ministry Points: 140

P4 Küçükgöz, K., Venema, K., & Trząskowska, M. (2024b). Gut Microbiota Modulatory Capacity of Fermented Ketchup in a Validated *In Vitro* Model of The Colon. *Food Research International*, *192*, 114801. https://doi.org/10.1016/j.foodres.2024.114801

Impact Factor: 7.0, Ministry Points: 140

P5 Küçükgöz, K., Venema, K., Chamorro, F., Cassani, L., Donn, P., Prieto, M. A., & Trząskowska, M. (2025). Unlocking the Potential of Fermented Beetroot Ketchup: Enhancing Polyphenol Recovery and Gut Microbiota Interactions. *Food Chemistry*, *463*, 141141. https://doi.org/10.1016/j.foodchem.2024.141141

Impact Factor: 8.5, Ministry Points: 200

Total Impact Factor: 29.7, Total Ministry Points: 900

1. Introduction

Probiotics are living microorganisms that can improve health when administered in adequate amounts and can show the potential benefits (Hill et al., 2014). Traditionally, dairy-based products have been considered as the primary carriers for these beneficial microbes. However, dietary restrictions, such as lactose intolerance, dairy allergies, and the increase of veganism, have improved the development of non-dairy probiotic products. These alternatives, which include products based on fruits, vegetables, grains, and legumes, offer health benefits and meet consumer expectations (Panghal et al., 2018). Non-dairy matrices are often rich in vitamins, minerals, and prebiotic fibers that can support probiotic growth (Ranadheera et al., 2017). Additionally, the interaction between probiotics and the food matrix can improve the bioavailability of nutrients and bioactive compounds, increasing their absorption and use in the host. Selecting food matrices is therefore crucial to optimize the delivery and functionality of probiotic strains in non-dairy products. Because the food matrix has a critical role in determining the stability, viability, and efficacy of probiotics (Mandalari et al., 2016; Shori, 2016). These matrices can also influence the fermentation process by providing essential nutrients and protective compounds that enhance the survival of probiotics through the gastrointestinal tract (Küçükgöz & Trząskowska, 2022).

Fermentation is a traditional preservation technique that changes the nutritional profile and probiotic potential of food products. During fermentation, beneficial microorganisms, including potential probiotic strains, metabolize carbohydrates into organic acids, gases, and alcohol. This metabolic activity results in several changes, such as the production of lactic acid and acetic acid, which lower the pH of the product and create an environment not suitable for growth of pathogenic bacteria (Rezac et al., 2018). Additionally, fermentation can increase the production of vitamins, peptides, and phenolic compounds, which have antioxidant, anti-inflammatory, and antimicrobial properties (Shahbazi et al., 2021). The process also breaks down complex carbohydrates, proteins, and fats into simpler forms, making the nutrients more accessible and easier to digest (Leeuwendaal et al., 2022).

The sensory qualities of non-dairy probiotic products, including flavour, texture, odour, and appearance, are critical factors influencing consumer acceptance and market success. Fermentation can significantly change these organoleptic properties. During

fermentation, the metabolic activities of microorganisms transform the food matrix, resulting in flavour texture and odour enhancement (Arratia-Quijada et al., 2024). The production of organic acids, esters, and other volatile compounds during fermentation creates complex flavour profiles, with modifications in the texture of non-dairy products. Additionally, the metabolic by-products of fermentation, such as alcohols and aldehydes, contribute to changes in sensory properties, effecting the overall sensory experience (Zhang et al., 2023).

Evaluating the performance of non-dairy probiotic products under gastrointestinal conditions is crucial to understand their efficacy and behaviour. *In vitro* digestion models can mimic the human digestive process and assess the nutritional changes and survival rates of probiotic strains. These models are divided into two main types: static and dynamic digestion systems (Minekus et al., 2014). Static digestion models working with exposing the food product to a series of simulated digestive fluids (saliva, gastric juice, pancreatic juice, and bile) in a sequential matter. These models provide valuable information on the release of nutrients and the survival of probiotics but not for the dynamic changes simulated in the human digestive system (Brodkorb et al., 2019). Dynamic digestion models, such as the TNO Gastro-Intestinal Model (TIM-1 and TIM-2), offer a more specific simulation of the human digestive process. These systems replicate the physiological conditions and dynamic changes in pH, enzyme concentrations, and transit times in the gastrointestinal tract (Minekus, 2015; Venema, 2015). The TIM-1 system simulates the upper gastrointestinal tract, including the stomach and small intestine. It consists of four compartments that mimic the stomach, duodenum, jejunum, and ileum. TIM-1 system includes dynamic pH control, peristaltic movements, enzyme and bile addition, and multiple sampling parts for collecting digested products at different stages of digestion. The TIM-2 system simulates the large intestine, focusing on colon fermentation. It consists of four connected compartments that mimic the ascending, transverse, descending, and sigmoid colon. Key properties of the TIM-2 system include maintaining anaerobic conditions, inoculating with human fecal microbiota and analyzing microbial composition and metabolic activity (Venema, 2015).

By using these advanced digestion models, researchers can assess the stability, viability, and functional properties of probiotics in non-dairy products, ensuring they can simulate gastrointestinal conditions and deliver health benefits to the host (Minekus et al., 2014).

The consumption of non-dairy probiotic products can have showed effects on gut microbiota composition and metabolic activity. Probiotics introduced through these products can modulate gut microbiota by inhibiting pathogenic bacteria and promoting the growth of beneficial species (Latif et al., 2023). This modulation enhances the production of short-chain fatty acids (SCFAs) such as butyrate, propionate, and acetate, which play crucial roles in maintaining gut health, reducing inflammation (Louis & Flint, 2017). Changes in gut microbiota composition and increased SCFA production are associated with numerous health benefits, including improved digestion, enhanced immune function, reduced risk of chronic diseases, and overall better metabolic health (Portincasa et al., 2022).

The publication **P1** also gives a detailed overview of the potential health benefits of probiotics and the growing interest in non-dairy probiotic products. It shows the importance of examining different strains and raw materials for these products, as well as using *in vitro* assessment methods to evaluate their effectiveness. The review covers different aspects, including choosing the potential probiotic strains, properties of raw materials, the sensory qualities of non-dairy products, and the use of artificial gastrointestinal tract models for evaluation. The publication also shows the need for more research and development in this area to meet consumer demand and address environmental concerns related to dairy production (Küçükgöz & Trząskowska, 2022).

In conclusion, non-dairy probiotic products represent a promising and innovative approach to delivering the health benefits of probiotics. Selection of food matrices, optimization of fermentation processes, and testing of probiotic viability and sensory attributes, these products can provide significant nutritional and health benefits, supporting diverse dietary preferences and contributing to overall health and wellness.

The above chapter is based on the following publication included in the doctoral dissertation:

P1 Küçükgöz, K., & Trząskowska, M. (2022). Nondairy Probiotic Products: Functional Foods That Require More Attention. *Nutrients*, *14*(4), 753. https://doi.org/10.3390/nu14040753

2. Purpose and scope of the research

The dissertation aimed to assess the possibility of using non-dairy food matrices as carriers of probiotic bacteria and to evaluate the nutritional value of the obtained products based on an *in vitro* model of the human gastrointestinal tract.

The specific objectives of the research were (1) to develop an innovative non-dairy potentially probiotic product and (2) to assess the survival of selected beneficial bacteria and the product's nutritional value during *in vitro* digestion in the human gastrointestinal tract.

Hypothesis

H1. Non-dairy food matrices are suitable carriers of probiotic strains.

H2. Deeper insight into probiotic bacteria survival in the human gastrointestinal tract is possible by *in vitro* digestion simulation.

H3. Fermentation of non-dairy matrices improves product nutritional value, i.e., bioaccessibility and recovery of the bioactive compounds present in these foodstuffs.

H4. Non-dairy fermented products with potential probiotics can positively influence microbiota composition in the gut microbiota.

H5. Based on *in vitro* digestion simulation of the human gastrointestinal tract, it is possible to evaluate the modulatory capacity of food products on gut microbiota.

The scope of the research

1. Literature review and searching for data regarding non-dairy food products and probiotic potential for application in this group of foodstuffs.
2. Developing innovative non-dairy potentially probiotic products, i.e. selection of raw materials, proportions of ingredients and fermentation parameters (temperature and time). Followed by an evaluation of the developed products' physicochemical, microbiological and sensory characteristics.

3. Investigating the probiotic potential of apple puree mixture: viability during simulated digestion and bioactive compound bioaccessibility.
4. Evaluate the viability, stability, and sensory quality of beetroot ketchup fermented during storage and simulated digestion.
5. Assessment of the potential of fermented beetroot ketchup: enhancing polyphenol recovery and gut microbiota interactions.
6. Analysis of gut microbiota modulatory capacity of fermented ketchup in a validated *in vitro* model of the colon.

3. Materials and sample preparation

The research stages of the doctoral thesis were divided into three main stages (Table 1).

Table 1: Research scheme.

	Research Task	Tests
Stage I Developing innovative non-dairy potentially probiotic products	→ ingredients and product formulations → physicochemical characteristic → fermentation process → viability assessment during storage → sensory analysis of developed products	→ pH measurements → sensory analysis → microbial counting
Stage II Assessment of the survival of selected beneficial bacteria and the nutritional value during in vitro digestion	→ *in vitro* digestion models (static and dynamic) → survival of probiotic strains → nutritional value assessment	→ ABTS, Folin-Ciocalteu Test → microbial counting → HPLC analysis
Stage III Assessment of the impact of developed non-dairy potentially probiotic products on gut microbiota composition and functionality	→ gut microbiota simulation with *in vitro* dynamic colon systems → gut microbiota composition changes → functionality assessment in gut microbiota	→ SCFAs production → HPLC analysis → sequencing → GC-MS analysis

Probiotic Strains

Lacticaseibacillus rhamnosus K3 and *Lactobacillus johnsonii* K4 strains were selected for testing because of their favourable acidity and minimal sensory alteration after fermentation. Both strains were sourced from the collection of the Department of Food

Gastronomy and Food Hygiene, Warsaw University of Life Sciences, Poland (Zielińska et al., 2015, 2019).

Ingredients and Product Formulations

Apple Puree Mixture

Non-dairy ingredients included "Golden Delicious" apples, chia seeds, oat bran, and oat flakes, all procured from local markets in Poland. Apples were washed, peeled, and cut into small pieces, then cooked with 200 mL of water per 1000 g of apple in a pressure cooker for five minutes. The softened apples were then homogenized using a Bosch ErgoMixx 1000W mixer to ensure a uniform consistency. Ingredient proportions for each sample are listed in publication **P2**.

Ketchup with beetroots

Ketchup with beetroots was formulated with tomato concentrate, beetroots, garlic powder, black pepper, sugar, and apple vinegar. Fresh beetroots were washed, boiled, and homogenized before mixing with the other ingredients according to the specified proportions in publication **P3**.

Fermentation and storage procedures

All ingredients were mixed and pasteurized at 72°C for 15 minutes, then cooled to room temperature. Probiotic strains were inoculated by adding a 1 mL (9 log10 CFU/g) solution of either *L. rhamnosus* K3 or *L. johnsonii* K4 in saline to 100 g of each sample. The apple puree mixtures were incubated for 15 hours at 30°C, while the ketchup samples were incubated for 5 hours at 37°C. Post-fermentation, samples were cooled to 4°C and stored in a refrigerator for 24 hours before *analysis.*

4. Research methods

pH analyses

The pH value of the samples was determined by a calibrated pH meter (ORION STAR A211, Thermo SCIENTIFIC). pH of fermented beetroot ketchup was measured post-

incubation and weekly over three weeks; the apple puree mixture was assessed post-incubation and after 21 days (**P2, P3**).

The viability of probiotics

Viability of *L. rhamnosus* K3 and *L. johnsonii* K4 was evaluated in ketchup (weekly over three weeks) and apple puree mixture (post-incubation and after three weeks) using serial dilution on MRS agar. It is possible to see the methodology in publications **P2** and **P3**.

Sensory analysis

Sensory evaluation followed the Quantitative Descriptive Profile (QDP) method as per ISO 13299:2016, with ten trained assessors using a 100 mm scale (**P2, P3**).

Static *in vitro* gastrointestinal digestion (GIS)

A static digestion model simulated human digestion by sequentially exposing samples to artificial saliva, gastric juice, pancreatic juice, and bile, following the method of (Minekus et al., 2014). This method provided insights into nutrient stability and probiotic survival. It is possible to see methodology in **P2.**

***In vitro* dynamic digestion with TIM-1 systems**

The TIM-1 in vitro gastrointestinal model was used to simulate human digestion, consisting of chambers representing the stomach, duodenum, jejunum, and ileum. Prior to experiments, the system was sterilized overnight with 70% ethanol. The five-hour digestion process involved adjusting the pH of a mixture of samples, enzymes, and electrolyte solutions to 4.5 before introducing it into the gastric compartment. Pancreatic function was simulated by adding 10% pancreatin, and bile was mimicked with a 4% bile solution at specific rates. Dialysis fluids in the jejunum and ileum compartments enabled the passive diffusion of polyphenols through hollow fiber membranes. Flow rates were standardized at 10 mL/min, and experiments were conducted with two replicates for reliability. During the digestive process, polyphenols were released in the jejunum and ileum compartments and passed through semipermeable hollow capillary membranes with a pore size of 0.05 μm (Spectrum Milikros 205 modules M80S-300-01P) through passive diffusion, entering the jejunum and ileum dialysis fluids. Flow rates through the hollow fibers are standardized at 10 ml/min. Each experimental condition incorporates two replicates to ensure reliability in the experiments. The methodology detailed for survival of selected strains in **P3** and for polyphenols in **P5** publication**.**

Bioactive properties' determination

Antioxidant activity was measured via the ABTS assay, with results expressed in ascorbic acid equivalents. Polyphenol content was assessed by the Folin-Ciocalteu method and reported as gallic acid equivalents. Further details on these methodologies are provided in the **P2** and **P5** publications.

Organic acids and sugar detection

Organic acids and sugars were quantified via HPLC, with samples pretreated by dilution, centrifugation, and filtration. The compounds were detected using a (Shimadzu, USA), system with an Aminex HPX-87H column. Further details are available in **P2**.

***In vitro* batch digestion**

Fermented and non-fermented ketchup samples underwent simulated oral, gastric, and intestinal digestion to prepare for colon fermentation (**P4, P5**).

***In Vitro* colon model TIM-2 and SCFA analysis**

The TIM-2 model simulated colon conditions over 72 hours with SCFAs measured by GC-MS **(P4).**

Microbiota composition

Microbiota was assessed via 16S rRNA sequencing and analyzed with QIIME-2 software **(P4)**.

Statistical analysis

Statistical analyses were performed using R software (version 3.6.2) and Statistica 13.3 (StatSoft, Kraków, Poland). Detailed description is available in publications **P2, P3, P4,** and **P5**.

5. Results and discussion

5.1 Development of non-dairy potentially probiotic products

The main goal of the first stage of the research was to assess if the food matrices that can carry potential probiotic strains and help their growth in the food matrices also protect and stabilize pH changes throughout storage. The research results are presented in publications **P2** and **P3** to show the changes in the developed "Apple puree mixture" and "Fermented Beetroot Ketchup".

Research shows that the survival of probiotic strains is significantly influenced by the interaction between the chosen raw materials and the potential probiotic strains. Typically, the final product must contain 6 to 7 $\log_{10}$ CFU/mL of the strains to be considered probiotic. It is also essential that these strains stay viable during storage until consumption, maintaining at least 6 $\log_{10}$ CFU/mL (Maia et al., 2023).

To understand the effect of fermentation on apple pure mixtures, total count of selected strains and pH levels were followed and showed in Table 2. The apple puree mixture samples initially contained approximately 9 log CFU/g of both *L. rhamnosus* K3 and *L. johnsonii* K4 after fermentation. The highest count of *L. rhamnosus* K3 was observed in apple samples with oat bran after 21 days of storage, with no significant change in the bacterial counts from after fermentation to the end of storage in samples prepared with either oat bran or oat flakes ($p>0.05$). This trend was similarly observed in samples inoculated with *L. johnsonii* K4. Both oat bran and oat flakes demonstrated no significant difference in the viability of the bacterial strains, with *L. rhamnosus* K3 and *L. johnsonii* K4 counts increasing during the 21-day storage period.

The pH values of fermented apple puree mixtures after fermentation ranged from 4.09 to 4.2, with control samples showing similar pH ranges between 4.25 and 4.30 after fermentation and 4.23 to 4.30 during storage ($p>0.05$). In parallel studies, Valerio et al. (2020) examined the viability of *Lactobacillus paracasei* IMPC2.1 in probiotic apple snacks, finding no significant differences in viability during 30 days of storage at 4°C ($p>0.05$) (Valerio et al., 2020). These findings suggest that apple and apple-based products can effectively support the growth of selected probiotic strains.

Table 2: The count of selected strains in the apple puree mixtures and pH values after fermentation and after 21 days of storage

Samples	Total count (log CFU/g)		pH	
	After fermentation	After storage	After fermentation	After storage
BRC	nd	nd	4.30±0.01	4.30±0.02
FLC	nd	nd	4.25±0.02	4.23±0.01
FL3	9.2	9.0	4.14 ±0.04	4.07±0.09
FL4	9.1	9.1	4.20±0.02	4.14±0.02
BR3	9.1	9.2	4.09±0.03	4.09±0.02
BR4	9.1	9.2	4.11±0.05	4.06±0.02

Explanatory notes: BRC—control sample with oat bran, FLC control sample with oat flakes, BR3 and FL3 apple puree mixtures with *Lacticaseibacillus rhamnosus* fermentation, BR4 and FL4 apple puree mixtures with *Lactobacillus johnsonii* fermentation. Values (mean ± standard error of the mean) in each column, $p > 0,05$ for total counts and pH measurements.

A similar analysis was also conducted for "Fermented Beetroot Ketchup", and microbial counts and pH levels changed during storage and shown in publication **P3**. KT3 and KT4 showed a decrease from total counts to storage period. Despite this reduction, both samples maintained counts above 8 $\log_{10}$ CFU/ml throughout storage. The control sample KTC consistently showed no detectable microbial growth, confirming successful pasteurization and indicating no growth other than the added strains. Another research found that a mixture of *L. johnsonii* and *L. plantarum* in fermented vegetables maintained viability above 8.5 $\log_{10}$CFU/g for one month at 4°C (Manowan et al., 2020). Similarly, Czyżowska et al. (2020) showed that lactic acid bacteria in fermented beetroots and beetroot juices remained stable after eight months of storage (Czyżowska et al., 2020). This supports the suitability of our developed beetroot ketchup as a carrier for *L. johnsonii* K4 or *L. rhamnosus* K3, offering potential probiotic benefits. The pH of the ketchup samples was monitored over three weeks. KTC remained stable at around 4.6, while KT3 and KT4 showed significant pH decreases from 3.84 to 3.79 and 3.96 to 3.69, respectively ($p<0.05$). These findings align with research on fermented vegetable matrices; it was also observed significant pH decreases during storage (Vatansever et al., 2017). Similarly, Bah et al. (2019) reported pH decreases in tomato juices with added *Lactobacillus plantarum* and *Leuconostoc mesenteroides* during storage at 4°C, while non-fermented control samples remained stable (Bah et al., 2019). The decrease in pH could be attributed to microbial enzyme activity, which hydrolyses substrates and produces metabolites during

storage (Nematollahi et al., 2016). These observations highlight the impact of fermentation and storage on the acidity and potential shelf stability of ketchup formulations. The selected non-dairy matrices not only provided a conducive environment for the initial growth of potential probiotics but also maintained their viability throughout fermentation and storage. This stability indicates that non-dairy matrices, such as apple puree and fermented beetroot ketchup, can effectively support the potential probiotic strains, offering a promising alternative to traditional dairy-based carriers for delivering probiotics. **These results validate the hypothesis: non-dairy food matrices are suitable carriers of probiotic strains (H1), highlighting their potential as functional food products with extended probiotic viability.**

The above results were described based on the following publications included in the doctoral dissertation:

P2 Küçükgöz, K., Kruk, M., Kołożyn-Krajewska, D., & Trząskowska, M. (2024). Investigating the probiotic potential of vegan puree mixture: Viability during simulated digestion and bioactive compound bioaccessibility. *Nutrients, 16*(4), 561. https://doi.org/10.3390/nu16040561.

P3 Küçükgöz, K., Venema, K., & Trząskowska, M. (2024a). Beetroot Ketchup as a Stable Carrier of Potential Probiotic Lacticaseibacillus rhamnosus K3 and Lactobacillus johnsonii K4: A Study on Sensory Attributes, Storage Viability, and In Vitro Gastrointestinal Survival. *Food and Bioproducts Processing*. https://doi.org/10.1016/j.fbp.2024.10.004

5.2 Sensory analysis of the developed non-dairy potentially probiotic products

It is known that fermentation can impact organoleptic properties, including flavour, texture, odour, and overall quality. The process involves the metabolic activities of microorganisms, which convert sugars and other compounds in the food matrix into various metabolites(*ISO 13299:2016(En), Sensory Analysis — Methodology — General Guidance for Establishing a Sensory Profile*, n.d.). That's why it is critical to assess sensory changes after fermentation to prove the acceptability of the product. In this study, we also focused on fermentation impacts on the sensory quality of apple puree mixtures and fermented tomato ketchup with beetroot, using potential probiotic strains *L. johnsonii* K4 and *L. rhamnosus* K3 with using Quantitative Descriptive Profiling (QDP), explained

also in **P2** and **P3** publication. This technique that provides a detailed sensory profile by evaluating attributes such as flavour, aroma, texture, and appearance. This method involves trained panellists who use standardized vocabularies to describe and quantify the intensity of sensory attributes. QDP is helpful in understanding consumer preferences and ensuring product consistency (*ISO 13299:2016(En), Sensory Analysis — Methodology — General Guidance for Establishing a Sensory Profile*, n.d.).

For the apple puree mixture, the sensory evaluation showed the findings on the impact of fermentation on sensory properties. The addition of oat flakes and oat bran with strains such as *L. rhamnosus* K3 and *L. johnsonii* K4 resulted in an apple puree mixture with acceptable sensory properties with the changes of odour, flavour and texture attributes. In particular, the sample containing oat flakes and fermented with *L. johnsonii* (FL4) had the most intense odours of apple, cinnamon and fermentation.

Similarly, sensory evaluation of fermented ketchup samples highlighted the effect of fermentation on sensory attributes. Significant differences in fermentation odour and flavour profiles were observed between ketchup samples fermented with different strains, as described in **P3**. Notably, the sample containing *L. johnsonii* K4 (KT4) showed the highest overall quality scores, indicating the superiority of this fermentation process in enhancing sensory attributes.

The results of this study show the importance of fermentation in improving the sensory quality and overall acceptability of food products. Fermentation-induced changes in sensory attributes, such as flavour complexity and texture improvement, contribute to the appeal of fermented foods (Zhang et al., 2023). These results highlight the important role of lactic acid bacteria in modifying the sensory attributes of food products.

The overall quality of fermented plant-based products, as assessed by QDP, typically shows improvements in sensory attributes that are critical to consumer acceptance. Fermented products are often perceived as desirable due to the dynamic changes induced by microbial activity (Xiang et al., 2019).

These results also validate the hypothesis: non-dairy food matrices are suitable carriers of probiotic strains (H1), highlighting their potential as functional food products with good sensory quality.

The above results were described based on the following publications included in the doctoral dissertation:

P2 Küçükgöz, K., Kruk, M., Kołożyn-Krajewska, D., & Trząskowska, M. (2024). Investigating the probiotic potential of vegan puree mixture: Viability during simulated digestion and bioactive compound bioaccessibility. *Nutrients, 16*(4), 561. https://doi.org/10.3390/nu16040561

P3 Küçükgöz, K., Venema, K., & Trząskowska, M. (2024a). Beetroot Ketchup as a Stable Carrier of Potential Probiotic Lacticaseibacillus rhamnosus K3 and Lactobacillus johnsonii K4: A Study on Sensory Attributes, Storage Viability, and In Vitro Gastrointestinal Survival. *Food and Bioproducts Processing*. https://doi.org/10.1016/j.fbp.2024.10.004

5.3 Survival analysis of the probiotic strains

Probiotics must resist the acidic stomach environment and bile salts in the small intestine and compete with other microbes to have potential health benefits. That's why it is important to observe potential probiotic microorganisms throughout the gastrointestinal system. For this purpose, developed non-dairy potential probiotic products were assessed for survival analysis with *in vitro* digestion systems. A static *in vitro* digestion system was applied for the developed apple pure mixture samples, and it gave the possibility to observe survival after the gastric phase and after the intestinal phase of digestion, also described in **P2.** Dynamic *in vitro* digestion system TIM-1 was applied for the survival analysis of developed fermented ketchup with beetroots; it also gave the opportunity to observe the dynamic process of upper gastrointestinal system effects that are also explained in **P3**. This research also provides valuable information about potential probiotic strains' viability and potential health benefits in different non-dairy food matrices during simulated digestion.

For assessing apple pure mixtures fermented with *L. rhamnosus* K3 and *L. johnsonii* K4 during simulated gastric and intestinal digestion, the initial probiotic counts were approximately 9 log CFU/g for both strains. After simulated gastric digestion, there was a significant decrease in total counts. However, no significant change was noted during the simulated intestinal phase compared to the gastric phase. All the sample's survival

changes are described in Table 3 and also detailed in **P2.** Both *L. rhamnosus* K3 and *L. johnsonii* K4 had better survival rates when combined with oat bran compared to flakes, likely due to oat bran's high protein and fibre content. Oat bran also showed the most positive effect on the growth of *Bifidobacterium* in the gut microbiome. Other studies have explored the survival of probiotics in apple-based matrices. Other research found that *Lactobacillus plantarum* incorporated into apple cubes survived a 2-hour simulated gastrointestinal digestion without a significant reduction in counts (Emser et al., 2017). Similarly, dehydrated apple slices as a carrier for *L. paracasei* demonstrated high viability, with only a ~2 log reduction after simulated digestion (Valerio et al., 2020). Encapsulation of *L. salivarius* spp. *salivarius* in dried apple improved its survival during simulated digestion compared to the non-encapsulated form, highlighting the importance of delivery form and matrix composition for probiotic survival (Ester et al., 2019).

Table 3: The count of potential probiotic bacteria in the apple puree mixtures after fermentation and during digestion (log CFU/g).

Samples	After Fermentation	After Gastric Phase	After Intestinal Phase
BR3	9.10^{aA}	6.05^{aB}	6.20^{aB}
BR4	9.10^{aA}	6.30^{aB}	6.03^{aB}
FL3	9.20^{aA}	5.94^{bB}	5.80^{bB}
FL4	9.10^{aA}	5.38^{cB}	5.70^{bC}

Explanatory notes: Significant differences between samples are represented by means in the same column followed by different lowercase letters of the alphabet and significant differences between samples are represented by means in the same row followed by different uppercase letters of the alphabet. Tukey HSD test shows that statistical differences in lowercase apply to all samples in the same column; ($p<0.05$).

Table 4: Cumulative CFUs as determined by microbiological cell count in the ketchup samples KT3 and KT4, and average cumulative survival during complete TIM-1 runs.

Sample	Time of Interval					
	0-60	**60-120**	**120-180**	**180-240**	**240-300**	**Residue**
KT3	6.78×10^{7}	1.03×10^{9}	1.33×10^{9}	1.57×10^{9}	1.60×10^{9}	1.68×10^{9}
KT4	3.78×10^{9}	1.13×10^{10}	1.31×10^{10}	1.46×10^{10}	1.69×10^{10}	2.41×10^{10}

Explanatory notes: KT3 ketchup mixtures with *L. rhamnosus* K3 fermentation, KT4 ketchup mixtures with *L. johnsonii* K4 fermentation.

The survival of selected strains for ketchup samples is explained as cumulative survival. Cumulative survival in dynamic *in vitro* gastrointestinal systems refers to the overall survival rate of probiotic bacteria as they pass through simulated sequential digestive stages. This approach aims to mimic the complex and variable conditions of the gastrointestinal tract more accurately than the static model. This process is also described in **P3** in detail**.** It is also possible to see the cumulative CFU amounts for the samples with different time points of digestion. For both samples, cumulative CFU was always more than 10^7 (Table 4), which makes our strains potentially probiotic because it is recommended that it is recommended that for foods with probiotics to have a therapeutic effect, between 10^6 and 10^8 CFU of viable probiotic cells should remain after the intestinal digestion stage (Wendel, 2022). However, when it comes to cumulative survival percentage compared with the number of bacteria during intake, it shows that *L. johnsonii*, K4 survived around 30% while *L. rhamnosus* K3 did not show high percentages compared with the bacteria density at the beginning. These results showed that different bacteria strains in the same food matrices can have different survival abilities (Figure 1).

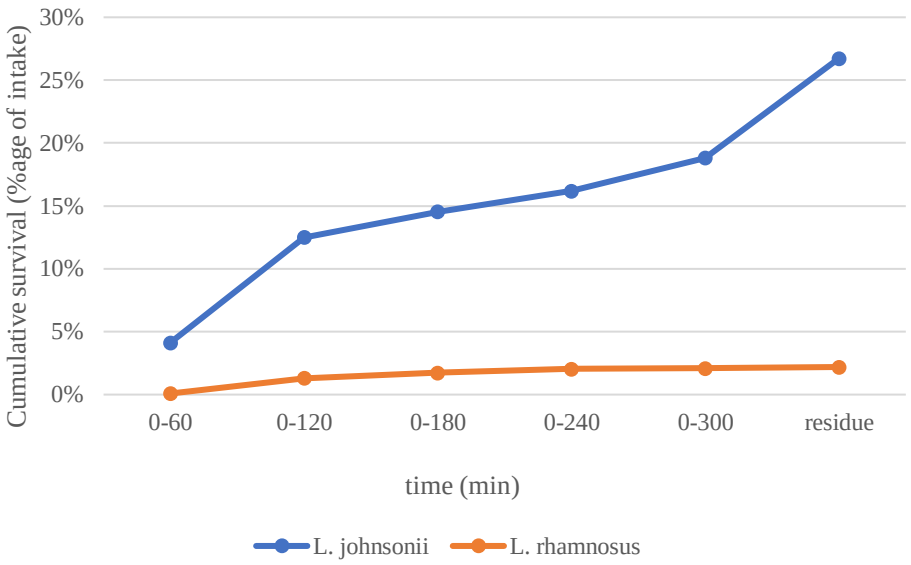

Figure 1: Survival of *L. johnsonii* K4 and *L. rhamnosus* K3 (expressed as cumulative delivery from the ileal compartment) in the samples collected from the *in vitro* model at different time points (min).

Similar food matrices are also examined for their ability to carry probiotic bacteria explored the viability of probiotic *Lactobacillus strains* in red beetroot matrices, showing

that all strains had viable cell counts exceeding 3.5×10^{10} CFU after simulated digestion, indicating the protective effect of the food matrix (de Oliveira et al., 2023). Additionally, Valero-Cases et al. (2017) demonstrated that *Lactobacillus plantarum* in tomato juice survived the digestion process and improved intestinal barrier function in in vitro cell cultures. This underscores the potential of plant-based matrices to enhance probiotic survival and functionality (Valero-Cases et al., 2017).

The other studies worked on the survival of potential probiotic strains survival on the TIM-1 system also showed that different formulations of *Lactobacillus* probiotic strains using the TIM-1 system reported a cumulative survival rate of up to 12% of the initial intake, emphasizing the potential of this dynamic model to predict the behaviour of probiotics during human digestion (Venema et al., 2019).

These research results confirmed Hypothesis 2 (H2): deeper insight into probiotic bacteria survival in the human gastrointestinal tract is possible by *in vitro* digestion simulation. These findings underscore that non-dairy food matrices can act as suitable carriers, supporting probiotics' survival and potential health benefits through digestive processes, and further validate the usefulness of *in vitro* models for studying probiotic stability and nutrient delivery in plant-based matrices.

The above results were described based on the following publications included in the doctoral dissertation:

P2 Küçükgöz, K., Kruk, M., Kołożyn-Krajewska, D., & Trząskowska, M. (2024). Investigating the probiotic potential of vegan puree mixture: Viability during simulated digestion and bioactive compound bioaccessibility. *Nutrients, 16*(4), 561. https://doi.org/10.3390/nu16040561

P3 Küçükgöz, K., Venema, K., & Trząskowska, M. (2024a). Beetroot Ketchup as a Stable Carrier of Potential Probiotic Lacticaseibacillus rhamnosus K3 and Lactobacillus johnsonii K4: A Study on Sensory Attributes, Storage Viability, and In Vitro Gastrointestinal Survival. *Food and Bioproducts Processing*. https://doi.org/10.1016/j.fbp.2024.10.004

5.4 Nutritional value of the developed non-dairy potential probiotic products

Organic Acids

One of the aims of this research was to examine the nutritional value changes of the developed non-dairy potentially probiotic products nutritional changes after fermentation and during gastrointestinal digestion. In the **P2** publication, organic acids production and changes in the sugar content of apple puree mixture were described. It is known that lactic acid fermentation plays an important role in changing and producing organic acids composition. The levels of sugars and organic acids present in fermented foods also help to understand microbial activity during fermentation and can impact the final product's sensory and health attributes (Küçükgöz et al., 2024). Before fermentation, control samples contained citric, malic, and acetic acids. After fermentation, all fermented samples (BR3, BR4, FL3, FL4) exhibited malic, lactic, and acetic. The significant increase in malic and acetic acids in fermented samples with oat bran (BR3, BR4) compared to oat flakes samples (FL3, FL4) highlights the influence of the raw material selection on fermentation outcomes (Figure 3). Lactic acid, produced by LAB during carbohydrate metabolism, increased as expected during fermentation, a trend also observed in other LAB-fermented products (Li et al., 2019). In another study on apple juice after lactic acid fermentation, the organic acid profile changed significantly to malic acid, and acetic acid levels increased significantly, consistent with the energy metabolism of LAB turning sugars into lactic and acetic acids (Bintsis, 2018; Ji et al., 2023). Citric acid was only detected in non-fermented samples, as LAB can utilize it for carbon sourcing; similar results were also observed in fermented pomegranate juice with *L. acidophilus* and *L. plantarum* (Mousavi et al., 2013). In our research also, the stability of organic acids was assessed during *in vitro* digestion. Organic acid content increased significantly in all samples, showing stability under gastrointestinal conditions. Propionic acids in digested fermented oat flake samples (FL3i, FL4i) were similar to behaviours observed in studies on different vegetables and fruits (Igual et al., 2023; Renaud et al., 2020). This stability and transformation are crucial for the health benefits of these acids, as they must survive digestion to exert beneficial effects in the gut.

Sugar profiles are also changed during digestion, concentrations of disaccharides, glucose and fructose increased significantly in all samples due to enzymatic hydrolysis of complex carbohydrates like starch and polysaccharides (Leong et al., 2019).

Bioactive Components

This part explains the polyphenol content and antioxidant activity of the developed products. Polyphenol content and antioxidant activity in food products are critical factors for their potential health benefits, such as helping neutralize free radicals, reducing oxidative stress and preventing chronic diseases. Scientific studies have demonstrated that polyphenols improve cardiovascular health, possess anti-cancer properties, and offer neuroprotection by modulating biological pathways and reducing inflammation (Rudrapal et al., 2022). Besides, it is shown that fermentation can influence the polyphenol content and antioxidant activity of plant-based products. Total polyphenol content, organic acids and antioxidant capacity were evaluated in this study for an apple puree mixture in the P2 and for a fermented beetroot ketchup in the P5 sample with a polyphenol profile.

The levels of sugars and organic acids in fermented foods are key indicators of microbial activity during fermentation. Before fermentation, control samples contained citric, malic, and acetic acids, as shown in Figure 2. After fermentation, the composition changed significantly, with fermented samples showing increased malic, lactic, and acetic acids, and occasionally propionic acid. Samples with oat bran exhibited higher malic and acetic acid levels than those with oat flakes. In vitro digestion further increased organic acid content, indicating their stability under gastrointestinal conditions, with the appearance of propionic acids reflecting metabolic transformations. These findings align with studies on LAB-fermented products and their digestion outcomes in various plant-based matrices.

Fermentation in the apple puree mixture did not significantly decrease the total polyphenol content and antioxidant activity compared to non-fermented control samples ($p>0.05$). Additionally, the total polyphenol content is higher in the fermented samples after the digestion process was completed. Moreover, the bioaccessibility index was higher for the fermented samples' total polyphenol content and antioxidant activity. Detailed measurements are provided in Table 5. Throughout the simulated digestion, fermenting apple puree mixtures with *L. rhamnosus* K3 and *L. johnsonii* K4 significantly increased the bioaccessibility of total polyphenol content. Similar results were also observed for the mixed vegetable juices fermented with *Lactobacillus plantarum* during the in vitro digestion, which showed a positive impact on the recovery and

bioaccessibility of bioactive compounds like phenolics and flavonoids, proving beneficial effects of fermentation on polyphenol absorption (Valero-Cases et al., 2017).

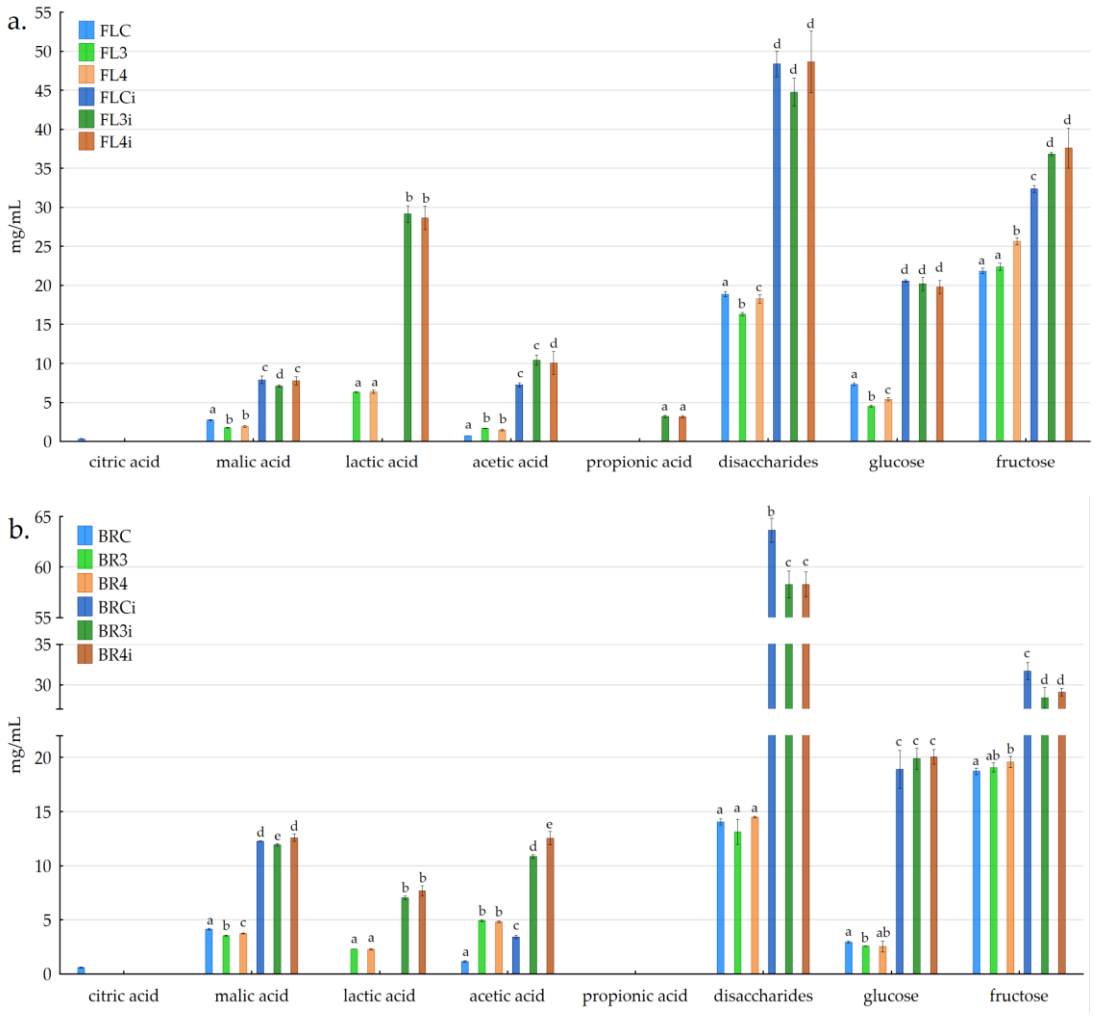

Figure 2: Organic acids and sugars before and after digestion in the analysed samples.

BRC: control sample with oat bran, FLC: control sample with oat flakes, BR3 and FL3: apple puree mixtures with *L. rhamnosus* K3 fermentation, BR4 and FL4: apple puree mixtures with *L. johnsonii* K4 fermentation, FLCi, FL3i, FL4I, BRCi, BR3i, and BR4i: samples after digestion; (A) samples with oat flakes; (B) samples with bran; a, b, c, d, and e mean the statistical difference between the samples in the post hoc Tukey's test ($p < 0.05$); error bars mean standard deviation; n = 3.

The bioactive components were examined in ketchup with beetroots using dynamic *in vitro* digestion systems allowed to check for recovery amounts means showing all the release during digestion, including jejunal and ileal filtrates, ileal efflux and residue during the process with bioaccessibility amounts, including jejunal and ileal filtrates proving these amounts are absorbed by the small intestine. Non-filtered materials are also described as available for gut microbiota.

Table 5: Data of antioxidant capacity, total phenolic content, and bioaccessibility of apple puree mixture samples in the initial (non-digested) phase and after digestion.

Samples	Initial Samples TPC (GAE mg/100 g)	Digested Samples TPC (GAE mg/100 g)	Bioaccessibility of TPC	Initial Samples Phase ABTS (VCEAC mg/100 g)	Digested Samples ABTS (VCEAC mg/100 g)	Bioaccessibility of ABTS
BRC	40.9 ± 2.05 ^{eA}	12 ± 2.9 ^{aC}	29.30%	122.0 ± 5.83 ^{cA}	34.7 ± 2.71 ^{bC}	28.40%
FLC	33.6 ± 2.74 ^{bcA}	11.42 ± 8.9 ^{abC}	34.00%	110.2 ± 5.8 ^{aA}	36.2 ± 2.88 ^{bC}	32.70%
BR3	39.8 ± 4.78 ^{deA}	17.7 ± 1.2 ^{cC}	44.50%	102.3 ± 5.47 ^{aA}	44.48 ± 4.9 ^{aC}	43.40%
BR4	36.1 ± 2.01 ^{cdA}	16.6 ± 0.1 ^{bcC}	46.00%	106.0 ± 3.52 ^{aA}	37.3 ± 1.3 ^{bC}	35.18%
FL3	31.0 ± 3.27 ^{abA}	12.6 ± 1.9 ^{cB}	40.60%	78.4 ± 5.01 ^{bA}	38.05 ± 2.17 ^{bC}	48.50%
FL4	28.8 ± 1.48 ^{aA}	14.5 ± 9.8 ^{cB}	50.30%	84.1 ± 6.77 ^{bA}	36.5 ± 1.03 ^{bC}	43.40%

Explanatory notes: BRC: control sample with oat bran, FLC: control sample with oat flakes, BR3 and FL3: apple puree mixtures with *L. rhamnosus K3* fermentation, BR4 and FL4: apple puree mixtures with *L. johnsonii* fermentation. Significant differences between samples separately for total polyphenol content and antioxidant activity are represented by means in the same row followed by different lowercase letters, and significant differences between samples are represented by means in the same column followed by different uppercase letters. Tukey HSD test shows that statistical differences in lowercase are applicable to all samples in the same row ($p < 0.05$).

The cumulative bioaccessibility percentage of polyphenols is divided into groups: phenolic acids, flavonoids, and betalains during 5 hours of upper gastrointestinal digestion. The cumulative bioaccessibility varied between non-fermented ketchup (KTC) and fermented ketchup (KT4) with *L .johnsonii* K4. The bioaccessibility of compounds varied between samples. KTC generally showed higher percentages for most compounds compared to KT4. However, specific compounds, including betagarin, caffeic acid, dihydroisorhamnetin, gallic acid, p-coumaric acid, and rutin, showed higher bioaccessibility in the fermented ketchup. These findings suggest that fermentation impacts the bioaccessibility of certain compounds, potentially due to changes in chemical composition and structure induced by the fermentation process. Further studies are needed to understand why fermented and non-fermented ketchup products exhibit different bioaccessibility profiles, as described in **P5**.

Figure 3 shows the recovery of polyphenols percentages in the upper gastrointestinal tract during digestion. Despite KTC having higher cumulative absorption percentages, KT4 exhibited higher recovery percentages for most phenolic acids, flavonoids, and betalains, frequently exceeding 100%. This indicates that fermentation with lactic acid bacteria can

convert polyphenols and release more bioactive components during digestion. The higher recovery of polyphenols in KT4, alongside lower bioaccessibility levels compared to KTC, suggests these compounds might be more available for the gut microbiota. Supplementary material 2 in the **P5** confirms that for KT4, compounds such as betagarin, catechin, chlorogenic acid, nariningen, betanidin, betanin, neobetanidin, vulgaxanthin III, vulgaxanthin IV, total antioxidant capacity, and total polyphenol content are more available for gut microbiota compared to the non-fermented sample. Notably, ferulic acid and gallic acid were undetected in KTC samples for gut microbiota availability but were present in KT4 samples.

Moreover, figure 3 shows metabolic transformations and recovery of bioactive compounds in non-fermented and fermented beetroot ketchup with *L. johnsonii* K4. Fermentation may involve enzymes produced by *L. johnsonii* K4 that hydrolyse macromolecules like polysaccharides and polyphenols into smaller metabolites (Yang et al., 2023). This process changes the content of specific phenolic compounds when comparing non-fermented samples with fermented ones, with phenolic compounds converting into specific bioactive metabolites depending on the bacterial metabolic pathways. This conversion is influenced by substrate composition, affecting the metabolic pathways and biochemical transformations during fermentation (Leonard et al., 2021).

Our study identified that KTC had a higher concentration of chlorogenic acid, with recovery percentages of 73% for KTC and 62% for KT4. Conversely, caffeic acid showed recovery percentages of 229% for KTC and 277% for KT4, exceeding 100%. Chlorogenic acid, an ester formed by caffeic acid and quinic acid, undergoes breakdown and absorption necessary for its metabolic fate and potential effects on human metabolism (Colombo et al., 2023). Additionally, fermentation can transform kaempferol into glycosylated forms or quercetin derivatives through microbial enzymes. Kaempferol present in KTC but undetected in KT4 indicates its transformation, while quercetin showed higher recovery in KT4. Nariningen displayed low recovery percentages (3% for KTC and 8% for KT4), while quercetin recoveries were high (183% for KTC and 226% for KT4), indicating significant enzymatic transformations during microbial fermentation (Tartik et al., 2023). Gallic acid was higher in KT4 with a higher recovery percentage during digestion. Other studies have shown that lactic acid bacteria fermentation

decreases quercetin content while increasing gallic acid, likely due to the direct effect of the C-ring in the chemical structure (Tang et al., 2023).

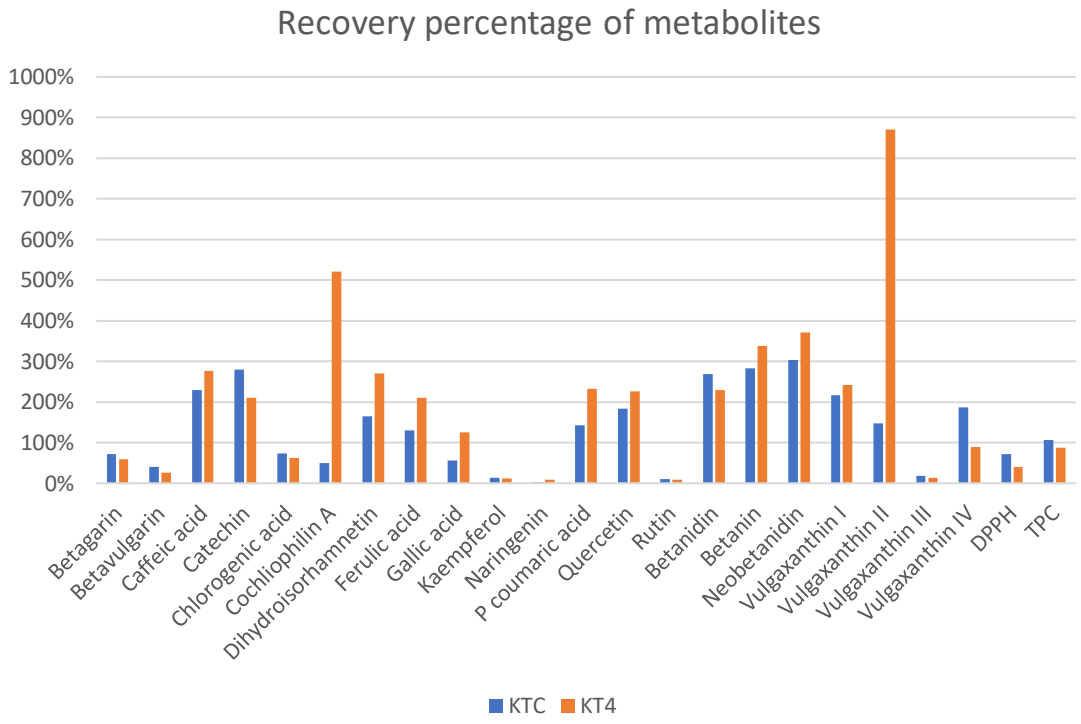

Figure 3: The total recovery percentage of metabolites during TIM-1 *in vitro* gastrointestinal digestion.

Despite the reduction in polyphenol amounts after fermentation, overall recovery during digestion can be higher in fermented samples. Fermentation's protective effects on polyphenols, stabilizing them and protecting them from enzymatic breakdown, contribute to higher overall recovery (Yang et al., 2023). Fermentation with *L. johnsonii* K4 increased total polyphenol content and antioxidant activity (Supplementary material 2 in **P5**), enhancing the nutritional value of the sample and confirming the hypothesis that fermentation improves the sample's nutritional value. Similar findings are reported for lactic acid fermentation in tomato juices (Ricci et al., 2020). Additionally, fermentation of beetroot juice by probiotics such as *L. brevis* and *L. paracasei* increased the contents of betanin, isobetanin, neobetanin, betanidine, and isobetanidine compounds not found in unfermented beetroot juices, known for their free radical-neutralizing ability (Klewicka et al., 2015). The addition of tomato ketchup to beetroots improves polyphenol diversity and adds betalains, potentially offering health benefits like reducing oxidative stress,

having anti-inflammatory effects, and contributing to cardiovascular health by potentially lowering blood pressure and improving endothelial function. Betalains have been also linked to improved exercise performance and liver health (Nirmal et al., 2024).

Further research is required to understand lactic acid fermentation's effects on polyphenols. Combining in vitro, *in vivo*, and clinical studies can confirm these findings and explore the health benefits of fermented foods rich in polyphenols, potentially leading to the development of new health-improving foods.

These findings confirm Hypothesis 3 (H3): Fermentation improves product nutritional value, i.e. bioaccessibility and recovery of the bioactive compounds in foodstuffs. The research shows that lactic acid fermentation significantly enhances the nutritional profile of these products, including organic acids, bioactive compounds, and antioxidant activity, throughout gastrointestinal digestion. Fermentation increased the stability and bioavailability of essential nutrients and bioactive compounds in apple purees and beetroot ketchup, even after digestion. For example, lactic acid, malic, and acetic acid remained stable. In contrast, compounds like quercetin, gallic acid, and betanin showed increased recovery in fermented samples, supporting their potential antioxidant, anti-inflammatory, and cardiovascular benefits. These results support the use of *in vitro* models to predict probiotic and nutritional performance, as they effectively assess probiotic survival and nutrient bioaccessibility, demonstrating the enhanced functionality and potential health benefits of non-dairy fermented foods.

The above results were described based on the following publications included in the doctoral dissertation:

P2 Küçükgöz, K., Kruk, M., Kołożyn-Krajewska, D., & Trząskowska, M. (2024). Investigating the probiotic potential of vegan puree mixture: Viability during simulated digestion and bioactive compound bioaccessibility. *Nutrients, 16*(4), 561. https://doi.org/10.3390/nu16040561

P5 Küçükgöz, K., Venema, K., Chamorro, F., Cassani, L., Donn, P., Prieto, M. A., & Trząskowska, M. (2025). Unlocking the potential of fermented beetroot ketchup: Enhancing polyphenol recovery and gut microbiota interactions. *Food Chemistry, 463*, 141141. https://doi.org/10.1016/j.foodchem.2024.141141

5.5 Gut microbiota composition and functionality effects of beetroot ketchup

In publications P4 and P5, the effects of developed ketchup samples with beetroot on gut microbiota composition and functionality were presented. The results include taxa changes, SCFA production, and analysis of bioactive components. The analysis of microbiota composition showed significant differences between control and ketchup-supplemented samples.21 taxa showed significant differences (q-value < 0.2) between these groups, with no significant differences between the fermented ketchup samples with *L. johnsonii* K4 and non-fermented ketchup samples. That's why results are grouped into ketchup-added and control categories.

Several taxa known for their roles in gut health, particularly in butyrate production, were significantly more abundant in ketchup-added samples than controls (Figure 4). For instance, *Blautia*, *Faecalibacterium*, and various *Ruminococcaceae* groups significantly increased (q=0.093). The higher abundance of these butyrate producers, crucial for maintaining gut health, suggests that ketchup consumption positively influences the gut microbial community. The potential benefits of increased butyrate producers like *Faecalibacterium* and *Ruminococcaceae* in the gut microbiota are significant. Butyrate enhances the barrier function of the colonic gut wall and increases mucus production (Genua et al., 2021; V. Singh et al., 2023). On the other hand, Eubacterium *ruminantium* group, which was absent in control samples, was significantly higher in ketchup-added samples (q=0.194). *Anaerostipes* (q=0.150) also showed a notable increase in ketchup-supplemented samples. The observed shifts, including the decrease in *Intestinimonas* and the increase in butyrate-producing *Anaerostipes* and *Collinsella* in ketchup-supplemented samples, prove the beneficial impact of ketchup on gut microbial composition (Bailén et al., 2020; Qin et al., 2019). Conversely, potential pathogens like *Escherichia/Shigella* and *Desulfovibrio* were significantly reduced in ketchup samples, indicating a possible inhibitory effect of ketchup on these harmful bacteria. The reduction of *Desulfovibrio*, associated with gastrointestinal disorders, bacteria's hydrogen sulfide production is linked to inflammatory bowel diseases (S. B. Singh & Lin, 2015). The complete absence of *Escherichia-Shigella* in ketchup samples further indicates a protective effect against potential pathogens.

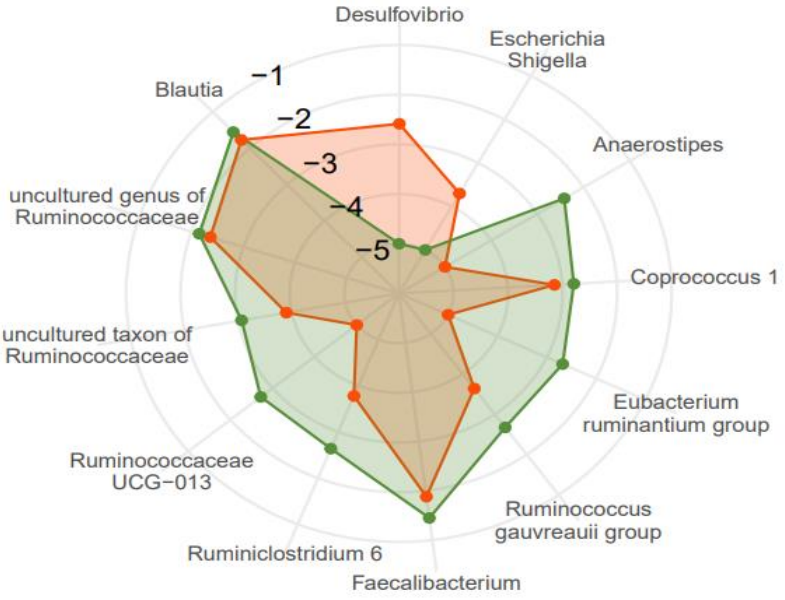

Figure 4: Relative abundance of selected bacteria species (several butyrate producers and the two potential pathogenic genera) after supplementation with standard ileal efflux medium (control; red points) or 60 g predigested ketchup (green points), Log10 relative abundance (plotted as −5 when taxon was absent).

Further dynamic analysis of specific taxa over time (Figure 5) highlighted that *Lactobacillus* significantly increased in fermented ketchup samples with *L. johnsonii* K4, and in ketchup-supplemented samples, suggesting that ketchup may promote the growth of beneficial lactic acid bacteria. Moreover, *Prevotella-9* and *Roseburia* were more abundant in ketchup-supplemented samples compared to controls, although the latter showed more inconsistent trends.

Furthermore, it is found that fermented tomato products can promote beneficial bacteria such as *Bifidobacterium* and *Akkermansia*, which further support gut health (Wei et al., 2024). Similarly, beet-derived pectin and oligosaccharides are noted for their prebiotic effects, particularly in increasing *Faecalibacterium prausnitzii* and *Roseburia intestinalis* populations, both of which are beneficial for gut health (Gullón et al., 2013). Tomato consumption has been shown to positively affect gut microbiota composition, as seen in studies using piglets, leading to a more favourable microbiota phenotype (Goggans et al., 2022). **These results also confirmed hypothesis 5 (H5), Non-dairy fermented products with potential probiotics can positively influence microbiota composition in the gut microbiota.**

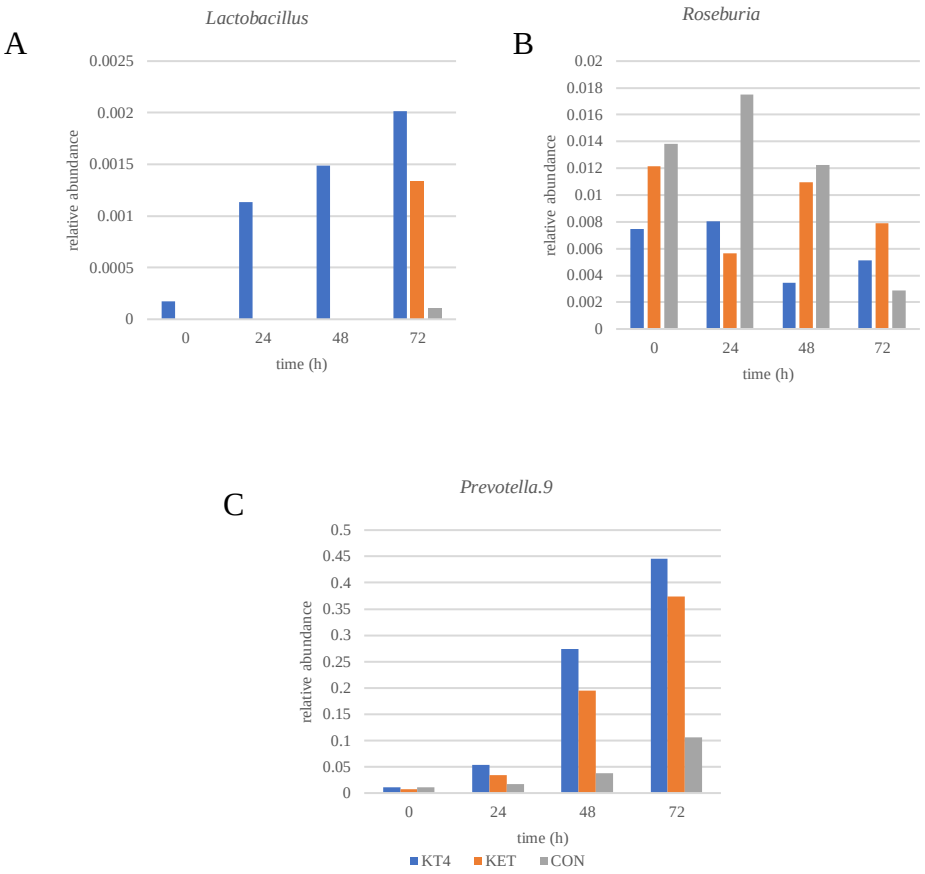

Figure 5: Changes in the relative abundance overtime of **(A)** *Lactobacillus*, **(B)** *Roseburia* and **(C)** *Prevotella-9* in gut microbiota intervention with the addition of KT4; fermented ketchup; KET; Non-fermented ketchup and CON; (standard ileal efflux medium (SIEM).

The other part of the results for effects of developed ketchup addition explained SCFA production changes on gut microbiota using the TIM-2 *in vitro* colon systems. The findings presented in **P4** also showed that developed ketchup supplementation, whether fermented or non-fermented, led to a higher abundance of acetate, propionate and butyrate compared to control. The cumulative SCFA production during colon fermentation, detailed in Figure 6, revealed distinct profiles for each intervention, emphasizing the substantial influence of substrate composition on the metabolic outcomes in the TIM-2 system.

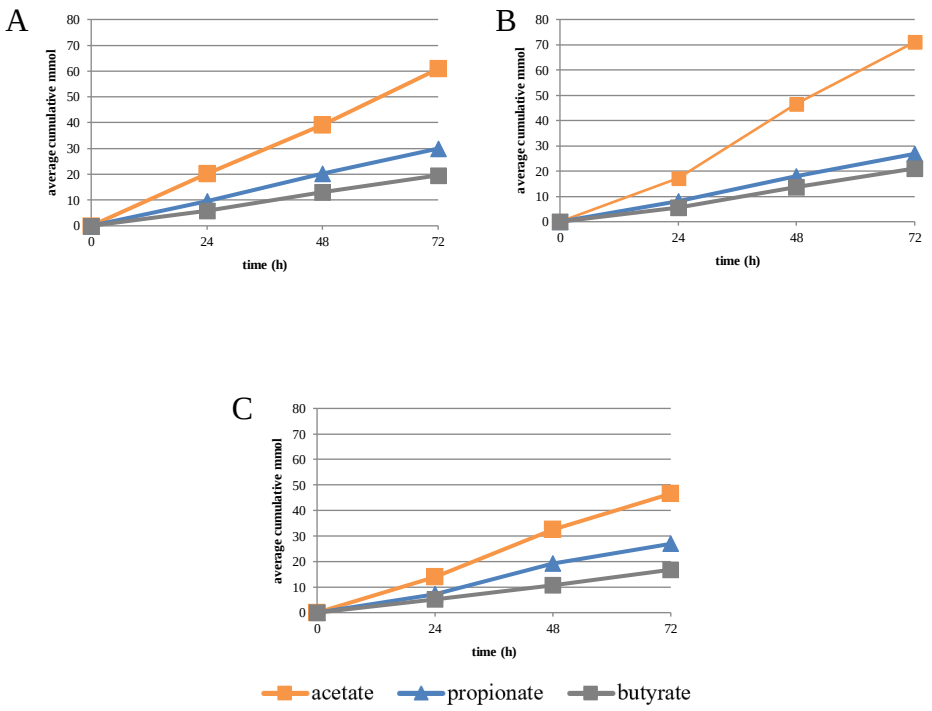

Figure 6: Cumulative production (mmol) of the SCFAs; acetate, propionate, and butyrate at 24 h, 48 h, and 72 h after supplementation with (**A**) KT4; fermented ketchup; (**B**) KET; Non-fermented ketchup, (**C**) CON; (standard ileal efflux medium (SIEM).

Non-fermented ketchup (KET) resulted in a balanced mixture of acetate, propionate, and butyrate. In contrast, the control (SIEM) was the least effective in producing butyrate. Specifically, the total acetate, propionate, and butyrate production was higher in the ketchup-supplemented samples.

Besides SCFAs production in simulated colon systems, correlations between SCFAs and specific microbial taxa at the genus level were assessed and illustrated in Figure 7.

Acetate production was significantly correlated with most taxa except for the *Christensenellaceae R-7* group, the Clostridiales family, and the *Eubacterium ruminantium* group. Notably, *Blautia* and *Ruminoclostridium 6* were positively correlated with both acetate and butyrate production, whereas *Bacteroides* showed a negative correlation with these SCFAs. Propionate production, however, did not exhibit significant correlations with any specific taxa, suggesting a more complex interaction in its synthesis.

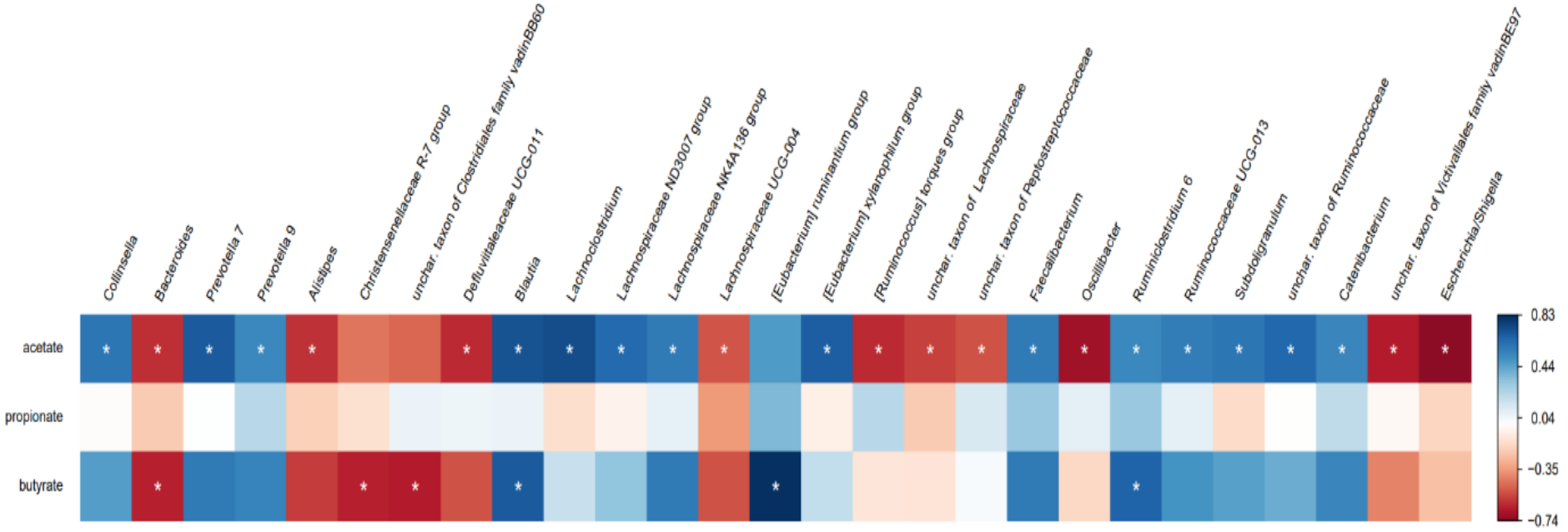

Figure 7: Correlation between metabolite production and ASVs at the genus level. White asterisks (*) $q \leq 0.2$; blue: positive correlation; red: negative correlation.

Research has proven the health benefits of short-chain fatty acids (SCFAs) for humans. Acetate is an important energy source for the liver. It helps with the creation of fat in fat tissue, which can help control appetite, increase fat burning, and improve metabolism, especially in overweight people (González Hernández et al., 2019). Propionate is essential for glucose metabolism and modulating the immune system, which helps maintain metabolic balance and control inflammation (Pang et al., 2023). Butyrate, produced when dietary fibre is fermented, helps maintain the intestinal barrier, has anti-inflammatory properties, and is the main energy source for cells in the colon. Also, butyrate can help prevent cancer by causing transformed cells to mature (Bekebrede et al., 2021; Liu et al., 2018).

Overall, our research detailed in **P4** presents the critical role of substrate composition in SCFA production and the microbial interactions in this process. The observed correlations between specific microbial taxa and SCFA concentrations highlight the diverse contributions of the gut microbiota to SCFA synthesis, with potential implications for metabolic health and disease prevention(Ramos Meyers et al., 2022).

In publication **P5**, microbial metabolites produced during *in vitro* colon fermentation and biotransformation of polyphenols were also presented. The *in vitro* colon fermentation of ketchup with beetroots led to changes in metabolite profiles, showing the complex interplay.

The *in vitro* colon fermentation of non-fermented beetroot ketchup and the ketchup fermented with *L. johnsonii* K4 and control sample SIEM presented different alterations in metabolite profiles, showing the complex interactions between food components, microbial activity, and metabolite transformations.

In publication P5, the detailed quantification of various polyphenol metabolites identified throughout the colon fermentation process at different time points (0h, 24h, 48h, and 72h) is possible. Polpyphenols such as betagarin, betavulgarin, kaempferol, naringenin, betanin, and neobetanidin were consistently detected in both ketchup variants across all fermentation stages, suggesting the resilience and stability of these compounds in the gut environment. Notably, the concentration of these metabolites generally increased after

colon fermentation, proving a potential improvement in their bioactivity modulated by the colon microbiota. For example, betagarin levels showed a substantial increase from 4.37 µg/L at 0h to 8.92 µg/L at 72h in KT4 samples, and similar trends were observed in KTC samples.

Conversely, compounds such as catechin, chlorogenic acid, cochliophilin A, gallic acid, betanidin, vulgaxanthin I, and vulgaxanthin IV exhibited degradation over time in all samples. This degradation trend points to the susceptibility of these compounds to microbial enzymatic activities. For instance, catechin levels in KT4 decreased from 1497.7 µg/L at 0h to 749.48 µg/L at 72h, reflecting microbial degradation dynamics. The degradation of compounds like catechin and chlorogenic acid are also similar, with previous findings showing microbial enzymatic activity leading to their transformation into other metabolites. For instance, chlorogenic acid degradation to caffeic acid through microbial activity has been also found and explained (Tomas-Barberan et al., 2014). This transformation is also reliable in our study, where higher levels of caffeic acid were detected in fermented ketchup samples compared to non-fermented ketchup.

Figure 8 illustrates the complex correlations between specific operational taxonomic units (OTUs) at the genus level and the phenolic metabolites. Significant positive correlations were observed between betagarin and genera such as *Methanobrevibacter*, *Prevotella 9*, and *Lachnospiraceae NK4A136* group, indicating potential microorganisms involved in its biotransformation. In contrast, negative correlations with genera such as *Bacteroides* and *Parabacteroides* proposed that metabolic pathways or competitive interactions within the gut microbiota be considered as potential factors.

The results underscore the dynamic nature of polyphenol biotransformation during colon fermentation, driven by the diverse enzymatic capabilities of the gut microbiota. The observed increase in polyphenols suggests that the gut microbiota can enhance the bioavailability and possibly the bioactivity of these compounds. This enhancement could be due to the breakdown of complex polyphenol structures into more readily absorbable forms.

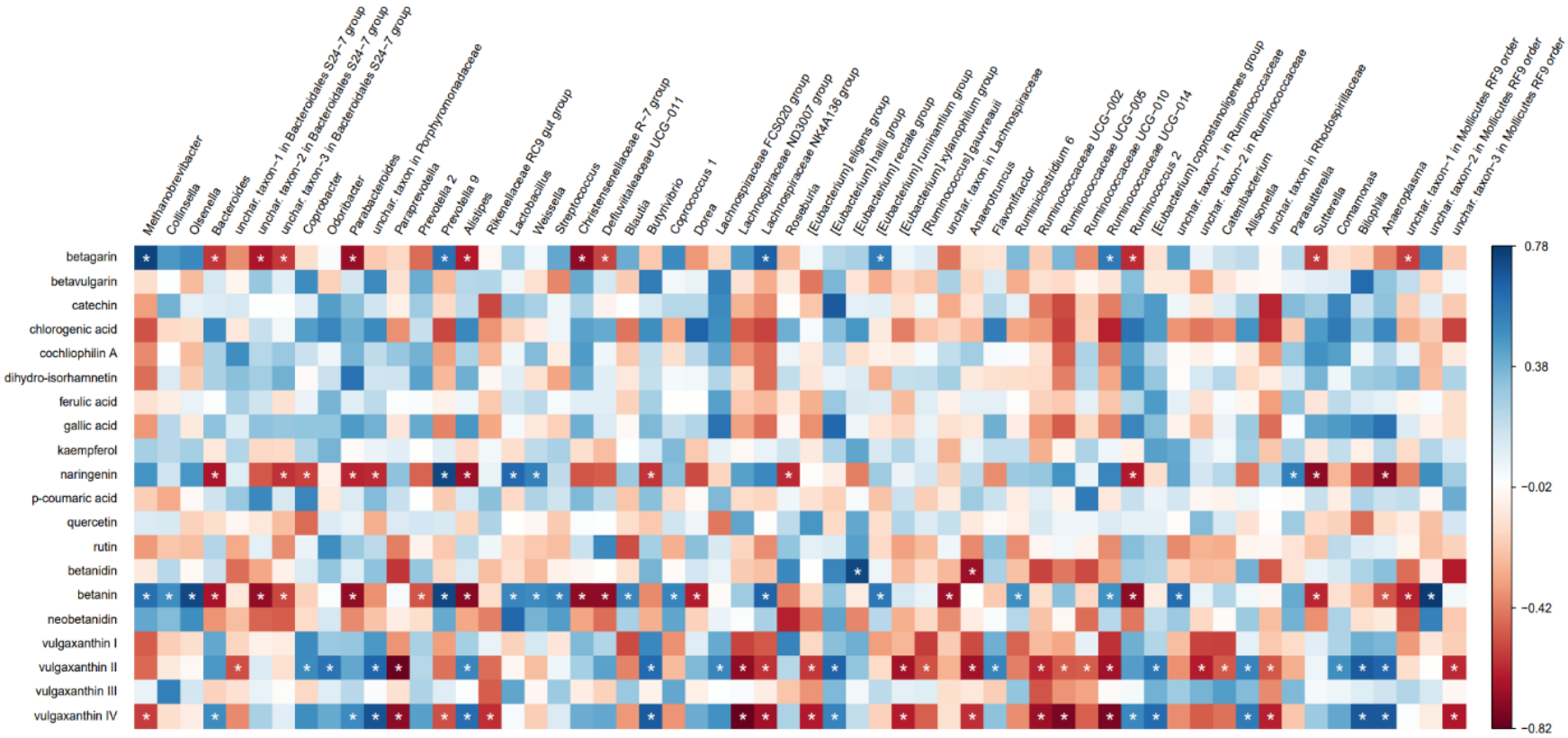

Figure 8: Correlation between bioactive components and specific operational taxonomic units (OTUs) at genus level. White asterisks (*) $q \leq 0.2$; blue: positive correlation; red: negative correlation.

The results from publications P4 and P5 confirm Hypothesis H5; based on *in vitro* digestion simulation of the human gastrointestinal tract, it is possible to evaluate the modulatory capacity of food products on gut microbiota. Supplementing with ketchup containing beetroot led to significant changes in gut microbiota, with an increased abundance of beneficial butyrate-producing bacteria and a reduction in harmful pathogens. Additionally, higher production of short-chain fatty acids (SCFAs), particularly butyrate, was observed, supporting gut health. The fermentation process also enhanced the bioavailability of polyphenols, with some metabolites increasing during colon fermentation. These findings underscore the beneficial impact of fermented products on both microbiota composition and nutritional value.

The above results were described based on the following publications included in the doctoral dissertation:

P4 Küçükgöz, K., Venema, K., & Trząskowska, M. (2024). Gut microbiota modulatory capacity of fermented ketchup in a validated in vitro model of the colon. *Food Research International*, *192*, 114801. https://doi.org/10.1016/j.foodres.2024.114801

P5 Küçükgöz, K., Venema, K., Chamorro, F., Cassani, L., Donn, P., Prieto, M. A., & Trząskowska, M. (2025). Unlocking the potential of fermented beetroot ketchup: Enhancing polyphenol recovery and gut microbiota interactions. *Food Chemistry*, *463*, 141141. https://doi.org/10.1016/j.foodchem.2024.141141

6. Conclusions

In conclusion, the results demonstrated the possibility of using non-dairy food matrices as carriers of probiotic bacteria. Developed food products, apple puree mixture and ketchup with beetroots support the growth and stability of *L. rhamnosus* K3 and *L. johnsonii* K4, maintaining viable counts above 6 $\log_{10}$ CFU/mL throughout storage. These products exhibited pH stability during storage, confirming their suitability for probiotic delivery. Moreover, survival analysis of *L. rhamnosus* K3 and *L. johnsonii* K4 in apple puree mixtures and beetroot ketchup shows that both strains endure during static and dynamic *in vitro* digestion simulation. The results from the TIM-1 dynamic model also demonstrated cumulative survival, indicating the potential of the strain to survive digestion.

The assessment of the nutritional value of developed non-dairy foods revealed that fermentation increased a few dietary characteristics. For example, enhancing polyphenol recovery and altering the organic acid profile (malic, lactic, and acetic acids) were observed. These compounds remained stable during digestion, confirming fermentation's role in increasing bioaccessibility and nutritional value.

Regarding the gut microbiota, fermented beetroot ketchup positively modulated its composition by promoting beneficial microbes and SCFA production. The increase in butyrate-producing bacteria and suppression of harmful taxa highlight the potential of fermented non-dairy products to enhance gut health. Adding developed ketchup samples to gut microbiota resulted in significant compositional changes, including an increase in butyrate-producing bacteria and a reduction of potential pathogens. SCFAs analysis showed higher acetate, propionate, and butyrate levels than controls.

These results also confirm the properties of *L. rhamnosus* K3 and *L. johnsonii* K4 as potential probiotics. The limitation of the research is reliance on *in vitro* models, which cannot fully replicate human physiological conditions. However, studies based on *in vitro* dynamic digestion simulations can provide essential data in some ethically difficult cases, e.g. when studying interactions with pathogens.

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8. Copies of the articles making up the doctoral thesis and statements of co-authors

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Review

Nondairy Probiotic Products: Functional Foods That Require More Attention

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Abstract: The potential health benefits of probiotics have been illustrated by many studies. However, most functional foods containing probiotics are from dairy sources. This review provides an overview of potential strains and raw materials for nondairy probiotic products together with the role of its in vitro assessment. Probiotic-containing products from raw nondairy materials are known both in terms of quality and nutritional values. The sensory properties of raw plant-based materials are generally improved as a result of fermentation with probiotics. Increased market shares for plant-based probiotic products may also help to curb environmental challenges. The sustainability of this food results from reductions in land use, greenhouse gas emissions, and water use during production. Consuming nondairy probiotic food can be a personal step to contribute to climate change mitigation. Since some people cannot or do not want to eat dairy products, this creates a market gap in the supply of nutritious food. Therefore, the promotion and broader development of these foods are needed. Expanding our knowledge on how to best produce these functional foods and increasing our understanding of their in vivo behaviours are crucial. The latter may be efficiently achieved by utilizing available in vitro digestion systems that reliably recapitulate the in vivo situation without introducing any ethical concerns.

Keywords: fermentation; functional food; nondairy food; plant foods; probiotic; in vitro digestion

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1. Introduction

Growing consumer interest in health and wellness also affects nutritional habits and food choices. Consumers' nutritional understanding has changed from only meeting their energy needs to also providing a healthy and balanced nutrition profile. Functional foods including probiotic-containing products belong to this diet category [1]. Consumers are also becoming more concerned about the sustainability of the food chain; thus, this encourages manufacturers to give importance to the development of such functional foods. The key to the successful marketing and acceptance of new foods depends on the concept of added value based on food quality and food functions [2]. The global probiotic food market is growing very quickly due to increasing consumer awareness about the impact of food on health. Today, probiotic products account for 60% to 70% of the total functional food market [3,4].

Probiotics are a common ingredient in functional foods, as they confer health benefits when consumed in adequate amounts [5]. There are various health benefits associated with probiotic strains, including intestinal and nonintestinal effects. Intestinal benefits include the prevention of diarrhoea, the reduction in symptoms associated with inflammatory bowel disease, the prevention of gastrointestinal cancers, the alleviation of lactose intolerance, and a reduction in *Helicobacter pylori* infections [6]. Moreover, probiotics may play a role in the prevention and treatment of intestinal inflammatory disorders [7] such as Crohn's disease and pouchitis, and paediatric atopic disorders. The impact of using probiotics on bacterial infections and immunological conditions such as adult asthma, cancer, diabetes, and arthritis is unconfirmed in humans [7,8].

The intestinal microbiota is as a potential factor in pathophysiology and associated metabolic disorders. Studies investigating the effect of probiotic intake on serum lipids, cholesterol levels, and more recently on blood pressure and glucose regulation indicate that probiotics may also benefit these factors [9].

Additionally, supplementing pregnant mothers with probiotics impacts mother and infant metabolism and later health [10,11]. The above-mentioned advantages of probiotics justify the indepth research of nondairy probiotic products, encompassing strain selection and characteristics, functional food development, and health properties.

The purpose of this review is to draw attention to and provide an overview of potential strains and raw materials for the production of nondairy probiotic products, along with the role of in vitro evaluation of such functional foods to accelerate the research and development of this functional food category.

2. Literature Search Methodology

For this review, a literature search was conducted in the Web of Science, PubMed, Google Scholar, and ScienceDirect search engines with keywords "fermentation", "functional food", "nondairy food", "plant foods", "probiotic", and "in vitro digestion". All selected terms were used in one search.

The timeline for our literature survey was set from 2011 to 2021 (in January 2022). The article titles and abstracts were reviewed, and duplicates were removed. Only studies on probiotics and nondairy food products were considered for inclusion. The literature concerning animals was excluded. Eligible sources of evidence included research articles, review articles, short communications, and book chapters. Full articles with appropriate references were obtained, the full text was read, and they were evaluated for final inclusion. Additional studies with respect to our search terms were only used for limited and specific purposes.

3. Potential Strains and Raw Materials for Nondairy Probiotics

3.1. Probiotic Strains and Viability Properties

The benefits of probiotic products are related to the selection of probiotic strains and their survival. The functionality of probiotics is generally strain-dependent. Strains should be resistant to gastric acid and bile, and be safe for human consumption [12,13]. Furthermore, a food must contain an adequate number of viable bacteria to have probiotic properties [14]. The stages of probiotic food production affect probiotic microorganisms' viability and stability. Microorganisms should also survive during processing, storage, handling, transport, and shelf life [4].

Due to these criteria and safety regulations, *Lactobacillus*, *Streptococcus*, *Propionibacterium, Enterococcus, Pediococcus,* and *Saccharomyces* can be used as probiotic microorganism sources for nondairy probiotic products [15].

Fermented nondairy food products can also be a source of probiotic bacteria. For example, bacterial strains isolated from cucumber and cabbage prepared by traditional methods have probiotic properties. Ten different *Lactobacillus* strains were isolated: *L. johnsonii* K4, *L. rhamnosus* K3, *L. brevis* (O22, O24), *L. plantarum* (O19, 020), and *L. casei* (O12, 013, 016, O18). Isolated strains were examined in gastrointestinal conditions to test safety for human consumption by in vitro experiment. Most of the isolated *Lactobacillus* strains could survive in gastrointestinal conditions and are safe for human consumption [12,13].

However, different raw materials play a specific role in bacterial growth, functionality, viability, and survival with their food matrix. Therefore, well-suited strains should be selected for each type of product [16–20]. Many studies have been conducted to incorporate microbes into different food matrices, some of which are discussed below.

Research has been undertaken to determine the suitability of tomato juice as a raw material for the production of probiotic juice by four lactic acid bacteria. Tomato juice was inoculated with probiotics such as *L. acidophilus*, *L. casei*, *L. delbrueckii*, and *L. plantarum*. The bacteria isolate fermented tomato juice from pH 4.1 to 3.5 in 72 h. They reached a viable cell population of more than 8 log CFU/mL (of <5 log CFU/mL) after 48 h of fermentation at 30 °C [18]. In other

research, *L. sanfranciscensis* was added to tomato juice and stored for 4 weeks at 4 °C. After storage, the number of surviving bacteria was determined and there was a decrease in probiotic viability. However, decreasing amount from 8 to 7.5 log CFU/mL was still acceptable and showed that tomato juice is a possible carrier of probiotic *L. sanfranciscensis* [20].

Oats are important sources of beta-glucan, recognized as the most important functional component in cereal fibre. In addition, beta-glucan is known as a prebiotic as it stimulates the growth of some beneficial microorganisms in the colon [21,22]. Furthermore, beta-glucan supports the viability of probiotic strains during cold storage [21–23]. In a study, the effects of beta-glucan obtained from oatmeal and modified beta-glucan samples obtained with xylanase treatment on the probiotic *Bifidobacterium bifidum* were investigated. While the two components had a significant effect on the growth of *Bifidobacterium bifidum*, the effect of modified beta-glucan was greater [22–24].

Beetroots have rich nutrient content and bioactive compounds [25,26]. Fermented beetroot with *Lactobacillus* bacteria had good biological viability and antimutagenic activity for up to 30 days at refrigerated storage [26]. Research about enriched ready-to-eat beetroot products with *L. plantarum* showed 8–9 log probiotic cells in 100 g. In addition, probiotic viability was greater than 7 log CFU/g after 21 days of storage at 4 °C; these results showed that the beetroot food matrix is favourable for probiotic survival [27].

Research that focused on producing potentially probiotic orange juice showed that different microorganisms have different viability. *L. rhamnosus* and nettle (*Urtica dioica* L.) additions were used for production, while *L. rhamnosus* was able to remain above 6 log CFU/mL at 4 °C storage for 28 days, but nettle could not improve the viability of the product [28].

Another study reported on orange juice with *Bacillus coagulans* GBI-30 6086 in animal models compared with yoghurt samples of the same probiotic. The probiotic orange juice food matrix adversely affected the functionality of probiotics in rats. The rats that had been fed the probiotic yoghurt group also showed higher gut bacterial diversity than that of orange juice [29].

Some features of the raw materials can cause the loss of viability of probiotic microorganisms such as natural antimicrobial compounds, acidity, diacetyl, and hydrogen peroxide [30].

Table 1 provides data on probiotic bacterial viability in different types of food.

3.2. Properties and Environmental Concerns of Raw Nondairy Materials for Probiotic Products

All over the world, most probiotic products are dairy-based. The increased health awareness of consumers and some health-related issues has led to the exploration of nondairy-based products. For example, plant-based alternative yoghurts are being developed and marketed in increasing numbers [40]. Statistical analysis shows that there are more than 380 types of probiotic products in the world, but 80% of these products are from dairy sources. Nondairy probiotic products with fruit and vegetable origins are very rare [5]. The lack of nondairy probiotic products means that various human groups do not benefit from functional foods containing probiotics. However, industry and people's interest in nondairy probiotic products is increasing for a variety of reasons [41]. The strongest drivers of nondairy products are vegetarianism, milk cholesterol content, lactose intolerance, and consumer interest in shelf diversity and sensory appeal. From the industry viewpoint, many manufacturers are seeking ways to create and increase value, which has further increased the product profile. However, a more compelling reason and the stronger driver is the emerging evidence of health benefits that can be acquired from a symbiotic relationship between plant components and probiotics, and gut commensals [42,43]. Nondairy products also contain more antioxidant phytochemicals such as phenolic acids, carotenoids, and flavonoids that have positive effects on oxidative stress in the body, prevent cell damage, and help to change the lipid metabolism and reduce obesity risk factors [43].

Table 1. Viability of probiotic bacteria in the different types of foods.

Genus	Species	Product Type	Viability (log CFU per mL or g)	References
Lactobacillus	*L. rhamnosus* ATCC7469	Fruit-Based Product Dried apple slices	1.0–3.0 log in slices dried by freezing and a combination of air drying and vacuum drying after 120 days storage at 25 °C, but higher viability of 9.3–7.8 log was found at 4 °C for 180 days.	[31]
	L. plantarum B2, *L. fermentum* PBCC11.	Fruit-Based Product Fresh-cut cantaloupe	*L. plantarum* (8.1 log) and *L. fermentum* (7.8 log) after 11 days of storage at 4 °C	[32]
	L. helveticus 76 (Lh76)	Fruit-Based Product Kiwifruit juice	Above 9.0 log CFU/mL after fermentation	[33]
	L.delbrueckii subsp. *bulgaricus*	Legume Based ProductSoy Protein	First day after fermentation 54×10^6 CFU/mL, after period of 15 days 43×10^7 CFU/mL	[34]
	L. paracasei LBC-81	Cereal-Based Product Maize-based substrate	Viable cell count, 10^6 CFU/mL	[35]
	L. reuteri NCIMB11951	Grain-based Product Fermented beverage made from oats, barley or malt	Viability between 7.8 and 8.1 log of the three species in fermented beverage after 10 h of fermentation at 37 °C.	[36]
	L. johnsonii	Vegetable-Based Product Traditional fermented cabbage and cucumber	Above 9 log CFU/g	[12,13]
	B. bifidum	Fruit-Based Product Blueberry and Black Berry Juices	Increased CFU/mL and 7.3 $\log_{10}$ CFU/mL to 8.2 $\log_{10}$ CFU/mL after 48 h fermentation,	[37]
Bifidobacterium strains	*B. lactis* Bb-12	Fruit-Based Product Cashew apple juice	After 1 day fermentation 2.16×10^{10} CFU/L h	[38]
	B. longum Bifidobacterium longum Bb-46	Fruit-Based Product Apricot Fruit Juice	After 24 h of fermentation were higher than 10^8 CFU/mL,	[38]
Saccharomyces	*Saccharomyces cerevisiae* CCMA 0731,	Cereal-Based Product Maize-based substrate	Viable cell counts 10^6 CFU/mL	[35]
Streptococcus	*Streptococcus thermophiles*	Grain-based product Oat Flour	Viable cell counts 10^6 CFU/mL	[39]

Considering product categories, cereal- and legume-based products increase their nutritional quality by a fermentation process using lactic acid bacteria and probiotic microorganisms [44]. Cereals have a rich content of dietary fibre, carbohydrates, and vitamins. Their nondigestible carbohydrate content also helps the growth of probiotic microorganisms in the human colon such as *Lactobacilli* and *Bifidobacteria*. Microbial processes on cereals such as fermentation also affect the improvement of protein digestibility and the reduction in allergens with microbial proteases [45,46]. Moreover, water kefir increased beneficial short-chain fatty acid production at the microbial level, reduced detrimental proteolytic fermentation compounds, and increased *Bifidobacterium* genus abundance [47]. Vegetables and fruits are also used in the production of nondairy probiotic products. These products have excellent nutritional values due to the presence of many phytochemicals, antioxidants, zero cholesterol, vitamins, minerals, and dietary fibre [1]. Fruit and vegetable juices can improve the viability of probiotics because additional nutrients can be obtained from the raw material by cellular synthesis, which is similar to the processes used during the fermentation of fruit and vegetable juice. This may make them ideal substrates for probiotic growth. Cutting or grating vegetables and fruits also helps to release their cellular content of vitamins, minerals, sugars, and other nutrients, and creates a good environment for probiotic microbial growth [31,48].

It is possible to find nondairy probiotic products in the market with different combinations of food matrices [18,26,49]. Nondairy probiotic beverages, frozen desserts, spoonable products, and probiotic vegan milk replacements are already on the market. Nondairy probiotics and prebiotics also have a great marketing future, as recent research shows the application of strains that are well-suited to alternative matrices [42,50,51].

In recent years, plant-based dairy alternatives have received more attention due to consumer demands and environmental concerns [52]. The production of dairy products is related to environmental externalities, including greenhouse gas emissions, soil degradation from overgrazing, soil erosion, deforestation, loss of biodiversity, the contamination of surfaces and groundwater arising from waste management, and soil salinization [53]. The report of the Lancet Commission on Food, Planet, and Health explained that contemporary research concluded that vegetarian and vegan diets are associated with reductions in land use, greenhouse gas emissions, and water use [54].

There is, therefore, another motivation to develop and popularize nondairy and vegan products that are involved in reducing climate change to encourage personal actions to reduce individual carbon footprints by switching to a plant-based diet. This kind of diet also helps to prevent diet-related chronic diseases and decrease expenses [45,55,56].

This growing interest in plant-based diets not only impacts sustainable consumption behaviour, but is also being noticed by the food industry [57]. Companies in the food sector need to create innovative products on market research while developing marketing skills in addition to scientific and R&D capacity [2]. Reasonable prices and lactose-free content increase the demand for these products.

Short-term marketing strategies should focus not only on vegan consumers but also on consumers who want to reduce their consumption of animal products and are looking for new strains of nonanimal origin.

3.3. Sensory Properties

Acceptable sensory properties are most important in probiotic food production, and are directly related to product quality, consumer acceptability, and processing characteristics [18,46]. Sensory changes can occur while producing probiotic products after the probiotic bacteria had been added to raw materials. Probiotic microorganisms produce different metabolic compounds such as lactic acid during storage and fermentation. Probiotic microorganisms also ferment the raw materials' carbohydrate content, and increase alcohol content and production gases. This also affects the consumer acceptance of the product [58]. The development of nondairy probiotic products with vegetables and fruits can be undertaken in three different ways, namely, the fermentation, nonfermentation, and minimal processing of raw materials. Probiotic cultures and fermentation can also affect sensory aspects. For instance, lactic acid fermentation of fruits and vegetables enhances sensory and nutritional quality, and retains nutrients and coloured pigments [31,59]. Probiotic blackcurrant juice prepared with *L. plantarum* strains and blackcurrant juice have more acceptable sensory properties to consumers, such as flavour, appearance, aroma, and texture [58]. Another study regarding the fermentation of grape juice found that the sensory properties of a probiotic product prepared with *Lactobacillus rhamnosus* strains were highly regarded by the consumers [60].

Raw cereal grains do not have enough active organoleptic compounds with their taste and texture. This situation also affects the preferences of consumers. Fermentation can lead to reducing flavouring additives to cereals. In particular, lactic acid bacteria's enzymatic activity on cereals contributes to the taste changes, such as the sweet and sour taste generated from nonvolatile and volatile compounds [60].

Plant-based milk is one of the most common materials used to produce probiotic beverages. It has a similar appearance to animal milk but offers different sensory properties, kinetic stability, and nutrient composition. In general, plant-based milk substitutes can be defined as homogenised extracts of vegetable matrices such as cereals, vegetables, and nuts. The nutritional profile of plant-based milk alternatives is usually unbalanced, and their

flavour profiles limit their acceptance. Probiotic fermentation was shown in several studies to improve sensory acceptability compared with unfermented alternatives [61–63].

Many researchers proved and studied that probiotic cultures did not affect the overall acceptability of the products, but these products are yet to come to the market [61,63].

4. In Vitro Assessment of Probiotic Product by Artificial Gastrointestinal Tract

The health-promoting effects of probiotics often depend on their survival during transit through the gastrointestinal tract. To show health benefits, probiotic microorganisms should be resistant to digestion conditions and colonise in adequate amounts in the host [64,65]. Their survival rate depends on some factors such as galenic form, food matrix, and dosage. To prove and understand the beneficial effects and the survival of probiotic microorganisms in the host, the passage of these microorganisms must be observed throughout the gastrointestinal transit [66]. However, it is difficult to investigate this phenomenon with in vivo study. Research shows that in vitro models of the upper and lower gastrointestinal tract can provide significant insight into the behaviour of probiotic strains during digestion in humans. They are particularly relevant for screening purposes, such as for studying the effects of biopharmaceutical factors (such as dosage form, food matrix, and dose regimen) on the viability of probiotic strains throughout the human digestive tract [67].

In addition, in vivo studies can be complex and expensive to investigate microorganisms. On the other hand, in vivo probiotic research generally focuses on the recovery of beneficial microorganisms from faeces, which makes it difficult to observe probiotics' behaviour on the gastrointestinal transit. All these reasons show the importance of in vitro research in probiotic studies. Artificial digestion models are also quicker, less difficult to undertake, and have fewer ethical concerns. Research tools of artificial digestion tracts help in understanding chemical and structural changes of food components in the digestion tract parts and the gut microbiome [67].

Two different in vitro digestion models are developed, namely, static and dynamic digestion models, and they are used for research purposes. Generally, protocols for static digestion systems describe food in bioreactors where enzymatic, physical, and chemical conditions of each digestive part are recreated. However, these digestive models have limitations because digestion is a dynamic procedure. In these systems, there is no possibility to replace food between the different digestive parts, and environmental conditions such as enzymes, bile concentrations, and pH are stable [68].

Dynamic digestion models have better simulation advantages, such as physical conditions with constant biological and chemical changes. Generally, dynamic digestion models mimic all sections of the gastrointestinal tract for complete simulation. The main difference between dynamic digestion systems is configuration. Currently, the TNO artificial gastrointestinal model with specific variations (TIM-agc, tinyTIM, TIM-1, TIM2) is used [62]. The mainly used generic platform is TIM-1, which includes the stomach, duodenum, jejunum, and ileum. These four compartments are connected with peristaltic valve pumps. This configuration has several variants for animals and humans for different kinds of meals. TinyTIM does not include separate intestinal steps and is a more basic version of TIM. TIM-agc is a more qualified version of TIM systems and it helps to compare the compounds of digestion under controlled conditions. As it is possible to observe the movement of foods and drugs, the design of this version enables a more accurate assessment of the behaviour of the stomach [57].

Another currently used dynamic digestive system is the Simulator of Human Intestinal Microbial Ecosystem (SHIME®) model, which is a computer-controlled gastrointestinal simulation device. It is possible to examine the microbial ecology and physiology of the gastrointestinal system. The model allows for simulating various age groups and some diseases. The simulator consists of five different reactors that help to see parts of the gastrointestinal system, the stomach, the ileum, and three parts of the colon (ascendant, transversal, and descendent). First, reactors allow for simulating steps of food intake

and digestion with fill and drawing reactor. The peristaltic pumps, SHIME® nutritional medium, pancreatic enzymes, and bile liquid set off physiological conditions in the large intestine [69,70]. The model also maintains microbiota stability for a determined time and helps to observe the adaptation of microbiota. The different subjects can also be examined at the same time and the subject's microbiome can be stored to set up unique features. The Mucus SHIME® is the specific variation of the SHIME® model. The model is used for the investigation of the adhesion ability of bacteria and changes in the microbiome in the mucosal parts of the gastrointestinal system [71].

For instance, the following studies were conducted on in vitro digestion models. *Lactobacillus crispatus* strain, added to cheese as a probiotic culture and isolated from a healthy human vaginal environment, was tested for its digestion system process using SHIME®. Results showed that the survival of *L. cripatus* BC4 was not affected by gastric digestion, but was significantly affected by bile salts and pancreatic juice. During colon simulation, *L. cripatus* BC4 was able to grow under sterile colon conditions and survive in the presence of a complex microbiota [72]. Another study also investigated soybean polysaccharides' bioavailability and the metabolites on the gut microbiota by using SHIME. Results showed that soybean polysaccharides were only partially decreased in the oral, gastric, and small intestine parts of SHIME [73].

Increasing our understanding of probiotic behaviours in the product and during gastrointestinal passage is crucial in the development of nondairy probiotic food. This may be efficiently achieved by utilising available in vitro digestion systems that reliably recapitulate the in vivo situation without introducing any ethical concerns.

5. Conclusions

The potential health benefits of probiotics have been illustrated by many studies. Most of the functional foods containing those beneficial microorganisms are from dairy sources. However, the high fat, cholesterol, lactose, or allergen content of dairy products may induce health problems and cause the exclusion of valuable functional foods from the diet. One of the solutions to this problem may be products containing probiotics produced from nondairy raw materials. The value and benefit of the probiotics themselves, combined with raw plant materials, give rise to unique advantages, for example, additional content of fibre or phytochemicals with quality sensory properties.

There is a market gap in the supply of the discussed nutritious food, especially for people who are unable or unwilling to eat dairy products. To address this issue, there is a need to intensify the indepth research and development of nondairy probiotic foods. In particular, advances in product evaluation through in vitro digestion models lead to faster and more accurate data on the health value of the product. In vitro artificial digestion systems are reliable, and this research methodology has no ethical concerns.

In addition, paying attention to nondairy and vegan foods benefits the environment by reducing land use, greenhouse gas emissions, and water consumption compared to the production of raw dairy materials.

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Article

Investigating the Probiotic Potential of Vegan Puree Mixture: Viability during Simulated Digestion and Bioactive Compound Bioaccessibility

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Abstract: This study aimed to develop a fermented puree mixture containing plant-based ingredients and potential probiotic strains *Lacticaseibacillus rhamnosus K3* and *Lactobacillus johnsonii K4.* The survival of potential probiotic strains, changes in sugar and organic acid concentrations, bioaccessibility of polyphenols, and antioxidant capacity after simulated digestion were examined with sensory quality. The mixture of apple puree, chia seeds, and oat bran or oat flakes was fermented. The sensory quality of the puree mixture was assessed by the quantitative descriptive profile (QDP) method. In vitro digestion was simulated using a static gastrointestinal model. Antioxidant capacity and total polyphenol content were analyzed before and after the digestion phases. All samples changed sensory profiles after fermentation. The overall quality was above six out of ten for every product. Fermentation also changed the organic acid composition, with significant increases in lactic, succinic, and acetic acids. After the digestion process, the survival rate remained above 5.8 $\log_{10}$ CFU/g. As a result of fermentation with potential probiotics, the bioaccessibility of the total phenolics and antioxidant activity increased. These results showed that the addition of potential probiotic strains increases nutritional value and could help with healthy nourishment habits. This knowledge can guide the development of consumer-satisfying products in the food industry, expanding the probiotic food market with innovative alternatives.

Keywords: bioactive compounds; digestion; fermentation; lactobacillus; probiotic; vegan

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1. Introduction

Food products have been extensively researched to determine whether they enhance consumers' health and prevent diseases [1]. Probiotics, live microorganisms offering health benefits, are also used for preventing gastrointestinal diseases, antimicrobial activity, and regulating lactose metabolism [2,3]. Scientific evidence supports their safety and efficacy. Probiotic-added products are increasingly available in the market, including drinks and snacks generally containing *Lactobacillaceae*, *Bifidobacterium*, *Saccharomyces*, and *Bacillus* strains [4]. Notably, non-dairy matrices, such as cereals and fruits, have gained prominence due to both health and sustainability concerns and the growing appeal of vegan options in the probiotic-containing foods sector [4,5]. It is of great importance that the type of food matrix carrying probiotic microorganisms may affect the survival of probiotics in the gastrointestinal system, their susceptibility to gastrointestinal conditions (acidity, bile, and various enzymes), as well as their functionality in the body [6,7]. Our selected strains for this research were sourced from a study that studied 38 strains from traditional fermented foods as part of a probiotic selection study. These strains were isolated from cucumber and cabbage pickles in households in the central region of Poland and have shown the capability of survival at low pH levels, good tolerance to bile salts, phenol addition, and moderate hydrophobicity. *L. johnsonii K4* even stands out among these strains due to its

ability to multiply under phenol exposure and a hydrophobicity exceeding 60%. This makes *L. johnsonii K4* a promising probiotic strain for further investigation and potential inclusion in functional foods [8].

Fruit, grain, and oil seed mixtures are tasty, nutritious puree mixture choices that are widely consumed around the world. Puree mixtures containing oats, apples, and chia seeds, with the addition of potential probiotic strains, improve the nutritional value of these selected ingredients. The antioxidants in whole oat grains refer to health benefits. Specifically, oat bran includes high amounts of antioxidants in comparison to other parts of grain such as potent avenanthramides [9,10]. In addition to the beta-glucan content, this cereal is a potential source of prebiotics, which stimulate the growth of beneficial microorganisms in the colon [11,12]. Regarding fruits, apples have been studied as a suitable carrier for probiotic microorganisms due to their nutritional properties and food matrix function. Apples, with their prebiotic content, are a good carrier of probiotic strains with amounts of pectin [13]. According to research, functional coatings with probiotic and prebiotic compounds have been applied with success on sliced apples [14–16]. Chia seeds have been used as a dietary supplement and in the production of bars, cereals, and cakes [17], while chia seed flour has been used to formulate gluten-free bread with high nutritional value [18]. Fermentation technology is used all around the world for developing functional foods, as it can improve sensory and nutritional qualities and process primary food substrates to remove undesirable compounds [19]. Additionally, fermentation methods have been targeted and developed to enable the synthesis of biologically active metabolites using the right microbial target and suitable substrate for diverse functional purposes. Lactic acid bacteria (LAB)-based fermentation contributes to inadvertently improving the nutritional value and digestibility of various foods, decreasing lactose intolerance, and controlling possible infections [20,21]. Soluble sugars and organic acids are also crucial factors that contribute to determining the chemical stability, pH, nutritional value, acceptability, and storage stability of a product [22]. Lactic acid fermentation is a process that converts and utilizes organic acids found in plant materials, such as LAB, and can decompose the organic acids present in food products and transform them into lactic acid. This process gives a sour taste to the fermented products [23].

Furthermore, fiber and phenolic compounds have been shown to play an important role in colon microbiota interactions [24]. Polyphenols may not always be at their peak biological activity after digestion. Biologically active compounds, which are generally transformed into metabolites during digestion and transported to target organs via blood, have different biological properties from their primary forms [25]. Various methods have been proposed to increase the bioaccessibility of polyphenols and their stability in digestion. One of them is fermentation with LAB, which can convert polyphenols into compounds with higher bioavailability and bioactivity [26]. In addition, LAB can prevent the chemical breakdown of some polyphenols [27].

It is essential to examine how digestion affects the survival of potential probiotic strains, bioactive compounds, and antioxidant activity. Moreover, in vitro simulation of gastrointestinal digestion can be used to monitor changes in different parts of the digestive tract [28].

Therefore, this research aims to investigate the survival of potential probiotic bacteria within selected food matrices and assess the bioaccessibility of polyphenols and antioxidant capacity during simulated digestion. Furthermore, the research aims to evaluate the sensory quality of a developed puree mixture incorporating plant-based ingredients and a potential probiotic strain.

2. Materials and Methods

2.1. Selection of the Probiotic Strains

Potential probiotic strains were obtained from the collection of the "Department of Food Gastronomy and Food Hygiene, Warsaw University of Life Sciences in Poland". A few strains of *L. brevis*, *L. casei*, *L. rhamnosus*, and *L. johnsonii* were tested, and, due to optimal

acidity and acceptable sensory changes on the products after fermentation, *L. rhamnosus* K3 and *L. johnsonii* K4 were used for further research [8,29]. Before application, bacteria were activated from a frozen culture stored at −80 °C. They were incubated at 30 °C for 24 h in 10 mL of MRS broth (Merck-110.660, Darmstadt, Germany). After completing the incubation period, the tubes were centrifuged at 10,000 rpm for 5 min (laboratory centrifuge MPW-251; MPW MED Instruments, Warsaw, Poland) to separate bacterial cells from the medium. The supernatant was replaced with 8.5 g/kg of saline, and the centrifugation procedure was performed three times to remove residual growth medium.

2.2. Development of Potential Probiotic Puree Mixture

The food matrix for fermentation was selected from non-dairy raw materials with high nutritional properties. Apples ("Golden Delicious"), chia seeds, oat bran, and oat flakes were purchased from local markets in Poland. Fresh apples were peeled, washed, and cut into small pieces. The apple pieces were then cooked for five minutes in a pressure cooker with the addition of 200 mL of water for every 1000 g of peeled apples. After cooking, the softened apple pieces were mashed using a high-powered mixer (Bosch ErgoMixx 1000W) to achieve a homogeneous mixture. The ingredients were then mixed in the ratios specified in Table 1. The mixture was pasteurized at 72 °C for 15 min, followed by cooling to room temperature for inoculation. For each sample, a 1 mL (9 $\log_{10}$ CFU/g) bacterial solution of *L. rhamnosus* K3 or *L. johnsonii* K4 in saline solution was added to 100 g of the puree mixture and inoculated for 15 h at 30 °C. After inoculation, the samples were immediately cooled to 4 °C and stored in the refrigerator for 24 h before further analysis.

Table 1. Proportion of the ingredients in the developed puree mixture.

Sample	Ingredients					
	Apple	**Oat Flakes**	**Oat Bran**	**Chia Seeds**	**Cinnamon**	**Probiotic**
BRC	100 g	-	10 g	2 g	0.3 g	-
FLC	100 g	10 g	-	2 g	0.3 g	-
BR3	100 g	-	10 g	2 g	0.3 g	*L. rhamnosus K3*
BR4	100 g	-	10 g	2 g	0.3 g	*L. johnsonii K4*
FL3	100 g	10 g	-	2 g	0.3 g	*L. rhamnosus K3*
FL4	100 g	10 g	-	2 g	0.3 g	*L. johnsonii K4*

Explanatory notes: BRC: control sample with oat bran, FLC: control sample with oat flakes, BR3 and FL3: puree mixtures with *L. rhamnosus K3* fermentation, BR4 and FL4: puree mixtures with *L. johnsonii K4* fermentation.

2.3. In Vitro Gastrointestinal Digestion (GIS)

In vitro digestion studies play a pivotal role in evaluating the bioaccessibility of nutrients and bioactive compounds within diverse food matrices. These studies emulate the human digestive process, simulating oral, gastric, and small intestinal phases to understand changes in food components during digestion. The methodology was completed according to Minekus et al. (2014) [30]. Oral phase was started by combining 3.5 mL simulated salivary fluid (pH 7) with 5 g of the food sample and then adding 0.5 mL amylase solution (75 U/mL) from *Aspergillus oryzae* (Sigma-Aldrich, Lot SLCD1111, Poznan, Poland), 25 g $CaCl_2$ (0.3 M) (Chempur, Piekary Śląskie, Poland), and 975 mL water.

The resulting mixture, to represent the oral phase, underwent grinding with 2.5 mL $CaCl_2$ (0.3 M (Chempur, Piekary Śląskie, Poland)) and 975 mL distilled water. Lastly, pH was adjusted to 7 with NaOH (1 M) (Chempur, Piekary Śląskie, Poland) and shaken for 2 min at 37 °C and 100 rpm. Gastric phase was started by adjusting the mixture's pH to 2.5, then 7.5 mL simulated gastric juice was added, including 1.6 mL pepsin from porcine gastric mucosa (2000 U/mL) (Sigma Aldrich, Lot SLBH3879V, Poznan, Poland), 5 µL $CaCl_2$ (0.3 M) (Chempur, Piekary Śląskie, Poland), and 690 µL distilled water. The mixture was shaken for 2 h at 37 ± 2 °C and 100 rpm. Small intestinal phase began with combining the gastric chyme with 5.5 mL simulated intestinal fluid (pH 7) and then 2.5 mL pancreatin from porcine pancreas (800 U/mL) (Sigma Aldrich, Lot SLBX1822, Poznan,

Poland), 1.25 mL porcine bile extract (10 mM) (Sigma Aldrich, Lot SLCJ7934), 20 µL $CaCl_2$ (0.3 M), and 585 µL distilled water were added. Finally, pH was maintained at 7 with NaOH (1 M) and incubated for 2 h at 37 ± 2 °C and 100 rpm.

2.4. Microbiological Viability Analysis

For the analysis of *L. rhamnosus K3* and *L. johnsonii K4* CFUs, 1 g of the puree mixtures with probiotics was added to 9 mL of sterile peptone water and homogenized with a stomacher at medium speed for 2 min. Seven-fold serial dilutions were prepared from the homogenized samples with peptone (Sigma-Aldrich, Poznań, Poland) water. Appropriate dilutions were inoculated onto MRS Agar (pH 6.8 ± 7.2, Merck-110,660, Darmstadt, Germany), and Petri plates were incubated at 37 °C for 48 h. After the digestion in the gastric and intestinal phases, an aliquot of the digested sample was decimally diluted with peptone water and plated onto MRS agar plates at 37 °C for 48 h. Colony counts were calculated as CFU/g of mixtures, and the obtained means of the data were transformed to $\log_{10}$ CFU/g.

2.5. Bioactive Properties' Determination

2.5.1. 12,2′-Azino-Bis(3-ethylbenzothiazoline-6-sulfate) (ABTS•+) Radical Cation Depolarization Assay

ABTS stock solution was dissolved in sodium acetate–acetic acid buffer (20 mM, pH 4.5) to make a 7 mM ABTS stock solution. A total of 20 mL of distilled water was added to 0.0256 g of potassium persulfate ($K_2S_2O_8$) (weighed to an accuracy of 0.0001 g). To 0.0384 g of ABTS radical reagent (2′2-zinobis-3-ethylbenzothiazoline-6-sulfonic acid) (weighed to the nearest 0.0001 g), 5 mL of distilled water was added, followed by 5 mL of the previously prepared aqueous potassium persulfate solution. The solution was prepared at least 12 h before the planned determination. The solution was stored at room temperature and protected from light. Before the assay, the absorption of radical solution in PBS was adjusted to absorbance 0.7. For carrying out the assay, 50 µL of the prepared sample dilution was added to a polystyrene plate (96 well, 300 µL), and 150 µL of ABTS radical solution was added in PBS. Measurements were performed exactly after the sample had been incubated with ABTS for 6 min at room temperature. The absorbance at a wavelength ÿ = 734 nm was measured using a microplate reader. The result is expressed as mg of ascorbic acid per a given volume or mass of the material tested. The calculation of the result was based on the 10-point standard curve created by ascorbic acid (Poch, Gliwice, Poland).

2.5.2. Total Polyphenolic Content Determination

For the determination of total polyphenolic content, the colorimetric method with the use of the Folin-Ciocalteu reagent was used. The reaction was carried out in an alkaline environment by using an anhydrous sodium carbonate solution in which the phenolate anion reduces the molybdenum. The intensity of the color produced is proportional to the total amount of phenolic compounds. Total polyphenols are expressed as gallic acid equivalent (mg GAE) based on the weight or volume of the sample. For carrying out the assay, 20 µL of the previously prepared sample dilution was added to a polystyrene plate (96 wells, 300 µL), 100 µL of Folin-Ciocalteu reagent was added, and it was left for 5 min at room temperature in a dark place. An amount of 80 µL of sodium carbonate solution was poured into the wells, mixed at 150 rpm for 5 min, and left for 2 h in the dark. The samples were mixed for one minute at 150 rpm on a reading machine before measurement. The absorbance was measured at a wavelength ÿ = 750 nm using a microplate reader. The calculation of the result was based on the 10-point standard curve created from gallic acid (Merck, Poznań, Poland).

2.6. Organic Acids and Sugar Detection

Before the analysis, samples were diluted in deionized water at a 1/10 ratio and then centrifuged for 15 min at 10,000 rpm using an Eppendorf Centrifuge 5804 R (Hamburg,

Germany). Then, 1 mL of the samples was firstly filtered with 0.45 µm syringe PES filter into the vials. Organic acids and sugars were analyzed with an HPLC system (Shimadzu, USA Manufacturing Inc, USA, consisting of two LC-20AD pumps, a CBM-20A controller, a CTD-20AC oven, a SIL-20AC autosampler, RID-10A detector, and UV/Vis SPD-20AV detector). For the separation of related compounds, we used Aminex HPX-87H column 300 × 7.8 mm (Bio-Rad, USA) at 40 °C with a flow rate of 0.5 mL/min and a mobile phase of 10 mM H_2SO_4. Quantification was based on the detection of each analyte at 210 nm wavelength using UV/Vis, RI, and external standard curves ranging from 0.12 to 40 µg per injection.

2.7. Bioaccessibility Index

In this study, the bioaccessibility index of phenolic compounds was calculated according to [31,32], based on the equation below:

$$BI\ (\%) = A/B \times 100$$

where A is the quantification of total phenolic content or antioxidant capacity (ABTS) after in vitro digestion; B is the quantification of total phenolic content or antioxidant capacity (ABTS) puree mixture.

2.8. Sensory Evaluation

The quantitative descriptive profile (QDP) method was utilized to objectively determine the sensory quality of the produced puree mixture. This procedure is in accordance with ISO Standard 13299:2016 [33], which provides general guidance for establishing a sensory profile. A linear graphical scale (100 mm) was used and converted to numerical values (0 to 10 conventional units). A list of descriptors was chosen and defined during the panel discussion and then verified in the preliminary session. Most of the tested attributes were measured on a scale from no intensity to high intensity, with an overall quality rating ranging from very low to very high. The trained panel consisted of 10 assessors, each with 4 to 12 years of experience in sensory evaluation, a good understanding of sensory methodology, and familiarity with the sensory quality being evaluated. To achieve this objective assessment, a set of 11 sensory descriptors was carefully selected and defined, as outlined in the supplementary materials (Table S1). To prepare the samples for evaluation, 50 mL transparent containers with lids were used, and each sample was assigned a unique 3-digit code and served randomly to the experts at room temperature. To ensure neutrality between samples, still water was provided as a neutralizer after each sample. Overall, these measures ensured a scientifically rigorous and standardized approach to the evaluation of the sensory quality of the produced puree mixture. An overall quality rating was determined on a scale of 0 to 10, where a rating below 6 was considered "poor", 6 to 7 was considered "fair", and 8 to 10 was considered "good" [34].

2.9. Statistical Analysis

The statistical analyses were conducted using Statistica 13.3 (StatSoft, Kraków, Poland). Standard deviation (SD) and arithmetic mean were calculated. The data were analyzed by multivariate analysis of variance (ANOVA) and Tukey HSD post hoc test. In order to analyze the sensory analysis results, principal component analysis (PCA) was conducted using a correlation matrix. The difference was considered statistically significant when $p < 0.05$ in relation to the count of bacteria, the results of chemical analyses, pH, and the results of sensory evaluation. Error bars in numbers and values after "±" in tables represent SD. All laboratory analyses were performed in triplicate.

3. Results and Discussion

3.1. Evaluation of the Survival of L. rhamnosus K3 and L. johnsonii K4 in Puree Mixtures during Simulated Digestion

It is recommended that for food to have a therapeutic effect, between 10^6 and 10^8 CFU/g or mL of viable probiotic cells should remain after the intestinal digestion stage [35]. The survival of probiotic bacteria during oral and gastric digestion is a crucial factor in their effectiveness in delivering health benefits. In order to assess the survival of potential probiotics added to puree mixtures, a study was conducted to evaluate the survival of *L. rhamnosus K3* and *L. johnsonii K4* in puree mixtures during simulated gastric and intestinal digestion. The initial number of probiotic bacteria in the puree mixtures was found to be 9 $\log_{10}$ CFU/g for both *L. rhamnosus K3* and *L. johnsonii K4.* After undergoing simulated gastric digestion, a significant decrease in the total counts of both strains was observed. However, during the simulated intestinal phase, there was no significant change in the counts of *L. rhamnosus K3* and *L. johnsonii K4* compared to the gastric phase, except for sample BR3, which showed growth during the intestinal phase, although this growth was not statistically significant. The numbers of *L. rhamnosus K3* in the puree mixtures samples were detected as ranging from 9.1 to 9.2 $\log_{10}$ CFU/g, while the numbers of *L. johnsonii K4* in the puree mixtures samples were detected as ranging from 9.13 to 9.20 $\log_{10}$ CFU/g. These findings suggest that the probiotics added to the puree mixtures were able to survive the simulated gastric and intestinal digestion and maintained their viability during the process (Table 2).

Table 2. The count of potential probiotic bacteria in the puree mixtures after fermentation and during the digestion process ($\log_{10}$ CFU/g).

Samples	After Fermentation	After Gastric Phase	After Intestinal Phase
BR3	9.10 ^{aA}	6.05 ^{aB}	6.20 ^{aB}
BR4	9.10 ^{aA}	6.30 ^{aB}	6.03 ^{aB}
FL3	9.20 ^{aA}	5.94 ^{bB}	5.80 ^{bB}
FL4	9.10 ^{aA}	5.38 ^{cB}	5.70 ^{bC}

Explanatory notes: Significant differences between samples are represented by means in the same column followed by different lowercase letters, and significant differences between samples are represented by means in the same row followed by different uppercase letters. Tukey HSD test shows that statistical differences in lowercase are applicable to all samples in the same column ($p < 0.05$).

It was found that the *L. rhamnosus K3* strain with samples of oat bran survived at 6.05 log CFU/g under gastric digestion and 5.94 log CFU/g with samples of flakes, with an increase to 6.20 $\log_{10}$ CFU/g for the bran samples and a slight decrease for the flake samples to 5.80 $\log_{10}$ CFU/g during the intestinal phase. Similarly, for *L. johnsonii K4*, a higher total count of 6.30 $\log_{10}$ CFU/g was observed after gastric digestion for samples with bran, and a higher recovery after the intestinal phase was also observed with bran samples after the intestinal phase (6.03 $\log_{10}$ CFU/g). Overall, these findings showed that both *L. rhamnosus K3* and *L. johnsonii K4* had better survival rates during the digestion process when combined with oat bran compared to flakes. This result can be related to oat bran's high protein and fiber content. In another research study on the different oat compounds' effects on the gut microbiome, oat bran showed the most positive effect on the growth of Bifidobacterium [36].

The results of the study help us to understand how important it is for microbes to survive during digestion. One study conducted by Emser et al. (2017) [15] investigated the survival of *Lactobacillus plantarum* incorporated in apple cubes during simulated in vitro digestion. The results showed that *L. plantarum* survived the quick simulation of gastrointestinal digestion for 2 h and was not reduced. The study suggested that apple-based products can provide a protective matrix for probiotic bacteria during digestion, which can improve their survival. Other research examined the viability and survival of *L. paracasei* in

dehydrated apple slices as a carrier. The researchers observed the successful adherence of the bacterial strain to the apple matrix and its ability to tolerate the harsh conditions of the gastrointestinal (GI) tract. The bacterial load in the dehydrated apple slices met the recommended threshold of more than 7 $\log_{10}$ CFU/g for a probiotic product. After simulated digestion, the survival rate of *L. paracasei* remained high, with only a 2 $\log_{10}$ CFU/g reduction in bacterial cells. These findings suggest that dehydrated apple slices can effectively deliver viable probiotic cells to the gut [37]. Another study focused on *L. salivarius* spp. *salivarius* as the bacterial strain and investigated its survival rate after digestion using encapsulation. The study compared the viability of encapsulated and non-encapsulated forms of *L. salivarius* spp. *salivarius* in dried apple during simulated gastrointestinal digestion. The researchers found that the encapsulated form exhibited higher resistance to the GI simulation than the non-encapsulated form. Encapsulation improved the survival rate and total microorganism content of *L. salivarius* spp. *salivarius* compared to the non-encapsulated form. However, both encapsulated and non-encapsulated forms showed a decrease in survival with storage time during the GI stages, highlighting the importance of freshness and timely consumption for optimal probiotic effects [38].

All these studies contribute to our understanding of how apple-based matrices can serve as carriers for probiotics and enhance their survival during digestion. Among factors that are relevant to their studies are the strain of probiotics, the form of delivery (e.g., puree mixtures, dehydrated apple slices, encapsulation), and the simulated digestion conditions. The acidic pH of apples offers a potentially favorable environment for probiotic strain growth. Moreover, apples have a rich source of prebiotics, including soluble fibers like pectin, which can act as substrates for beneficial bacteria within the gastrointestinal tract. Prebiotic fibers in apples promote a balanced gut microbiota by improving probiotic populations [37]. Based on the findings, oat bran in particular can protect potential probiotic bacteria in apple-based matrices, allowing them to survive gastrointestinal conditions.

3.2. Evaluation of the Bioactive Components of Fermented and Non-Fermented Puree Mixtures during Simulated Digestion

The bioaccessibility of dietary polyphenols, despite their presence in plant-based foods, is often limited by several factors, including their large molecular size, molecular weight, polarity, shape, and susceptibility to degradation within the small intestine [39,40]. Furthermore, certain microorganisms possess the capacity to metabolize complex phenolic compounds into bioactive derivatives, thereby enhancing their bioavailability [41]. Apple products that contain probiotic bacteria also contain polyphenols, which may improve gut health and immunity. It is crucial to understand whether these polyphenols and antioxidants are bioaccessible within the gastrointestinal tract to show their health benefits. The amount of TPC in puree mixtures with oat flakes and oat bran decreased during the digestion process. Before digestion, in the initial phase, non-fermented samples with oat bran and fermented samples with oat bran were significantly higher than the other samples. Notably, for the fermented mixtures, TPC decreased less. The fermentation of the puree mixtures probably favored the positive results achieved in this study. The TPC was significantly increased for BR3, BRC, and FLC samples, and for the FL4 and FL3 samples, TPC amounts did not change significantly in the last stage of digestion, the intestinal phase. In this stage, non-fermented samples showed a significantly lower amount of TPC compared with fermented samples ($p < 0.05$). It is particularly interesting to use LAB fermentation as a way of increasing the bioaccessibility of polyphenols. The promising results for improved bioactivity of phenolic extracts pretreated with LAB have been demonstrated (Table 3).

Fermented samples have a higher bioaccessibility of total polyphenol content at the end of the intestinal digestion; for example, non-fermented samples with oat bran's bioaccessibility was 29.30%, while a sample fermented with *L. rhamnosus K3* was 44.5% and with *L. johnsonii K4* was 46%. The same scenario also applied to samples with oat flakes: non-fermented samples' bioaccessibility was 34%, while samples fermented with

L. rhamnosus K3 were 40.6% and with *L. johnsonii K4* were 50.3% (Table 3). Additionally, the stability of phenolic compounds may be linked to the production of lactic acid during fermentation [41]. For instance, the fermentation of various fruit and vegetable juices using specific LAB strains, including *L. plantarum* ASCC 292, *L. brevis* 145, *Weissella cibaria* 64, *Leuconostoc mesenteroides* 12b, *L. brevis* POM4, and *Weissella confusa* LK4, has been reported to enhance their antioxidant activities [42].

Table 3. Data of antioxidant capacity, total phenolic content, and bioaccessibility of puree mixture samples in initial phase (non-digested) and after digestion process.

Samples	Initial Samples TPC (GAE mg/100 g)	Digested Samples TPC (GAE mg/100 g)	Bioaccessibility of TPC	Initial Samples Phase ABTS (VCEAC mg/100 g)	Digested Samples ABTS (VCEAC mg/100 g)	Bioaccessibility of ABTS
BRC	40.9 ± 2.05^{eA}	12 ± 2.9^{aC}	29.30%	122.0 ± 5.83^{cA}	34.7 ± 2.71^{bC}	28.40%
FLC	33.6 ± 2.74^{bcA}	11.42 ± 8.9^{abC}	34.00%	110.2 ± 5.8^{aA}	36.2 ± 2.88^{bC}	32.70%
BR3	39.8 ± 4.78^{deA}	17.7 ± 1.2^{cC}	44.50%	102.3 ± 5.47^{aA}	44.48 ± 4.9^{aC}	43.40%
BR4	36.1 ± 2.01^{cdA}	16.6 ± 0.1^{bcC}	46.00%	106.0 ± 3.52^{aA}	37.3 ± 1.3^{bC}	35.18%
FL3	31.0 ± 3.27^{abA}	12.6 ± 1.9^{cB}	40.60%	78.4 ± 5.01^{bA}	38.05 ± 2.17^{bC}	48.50%
FL4	28.8 ± 1.48^{aA}	14.5 ± 9.8^{cB}	50.30%	84.1 ± 6.77^{bA}	36.5 ± 1.03^{bC}	43.40%

Explanatory notes: BRC: control sample with oat bran, FLC: control sample with oat flakes, BR3 and FL3: puree mixtures with *L. rhamnosus K3* fermentation, BR4 and FL4: puree mixtures with *L. johnsonii* fermentation. Significant differences between samples separately for total polyphenol content and antioxidant activity are represented by means in the same row followed by different lowercase letters, and significant differences between samples are represented by means in the same column followed by different uppercase letters. Tukey HSD test shows that statistical differences in lowercase are applicable to all samples in the same row ($p < 0.05$).

3.3. Evaluation of the Organic Acids of Fermented and Non-Fermented Puree Mixtures during Simulated Digestion

The levels of sugars and organic acids present in fermented food are important indicators of the activity of microorganisms during fermentation. Specific organic acids, including malic, succinic, lactic, and acetic, are found after fermentation and in in vitro digestion (Figure 1). Before fermentation, the control samples (BRC, FLC) exhibited the presence of citric acid, malic acid, and acetic acid. After lactic acid fermentation, significant changes in the organic acid composition were observed. All fermented samples (BR3, BR4, FL3, and FL4) showed the presence of malic acid, lactic acid, acetic acid, and, in some instances, propionic acid. Malic acid and acetic acid were significantly higher in fermented samples with oat bran (BR3 and BR4) than in fermented samples with oat flakes (FL4 and FL3). Lactic acid, which is produced by lactic acid bacteria during carbohydrate metabolism, increases as expected during fermentation. Similar observations have been made in other lactic acid bacteria-fermented products, highlighting the importance of residual sugars in maintaining probiotic activity in fermented foods [42]. In another research study on apple juice after lactic acid fermentation, the organic acid profile changed to become mostly malic acid [43]. The concentration of acetic acid also increased significantly during fermentation. This could be explained again with LAB energy metabolism turning sugars into lactic acid and also other metabolites such as acetic acid [44]. The citric acid concentration was only detected in non-fermented samples and could not be detected after fermentation. It was also similar for fermented pomegranate juice with *L. acidophilus* and *L. plantarum*, which led to a significant decrease in citric acid levels. This phenomenon can be explained as LAB can utilize citric acid for carbon sourcing [45]. Upon in vitro digestion, further alterations in organic acid composition were observed. All detected organic acid content increased significantly after digestion, suggesting its stability under gastrointestinal conditions. Additionally, propionic acids were detected in the digested samples (FL3i and FL4i), indicating the generation or liberation of these acids during digestion. A similar behavior was also observed in the studies working on different vegetables and fruits [46,47].

After in vitro digestion, there was a significant increase in the concentrations of disaccharides, glucose, and fructose in all samples. The increase in sugars after digestion can be attributed to the breakdown of complex carbohydrates, such as starch and polysaccharides, present in the food matrix. When these complex carbohydrates are subjected to the diges-

tive process, they undergo enzymatic hydrolysis, leading to the release of simpler sugars such as glucose and fructose [48].

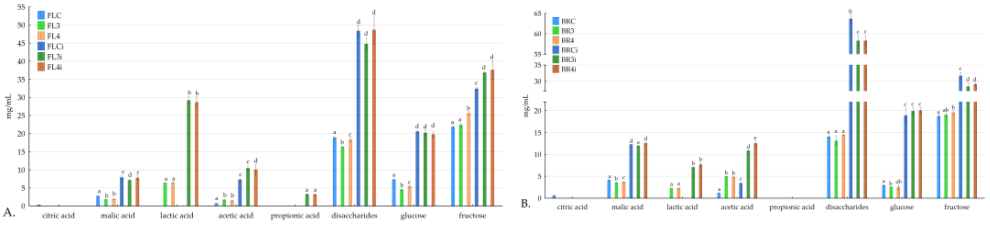

Figure 1. Organic acids and sugars before and after digestion in the analyzed samples; BRC: control sample with oat bran, FLC: control sample with oat flakes, BR3 and FL3: puree mixtures with *L. rhamnosus K3* fermentation, BR4 and FL4: puree mixtures with *L. johnsonii K4* fermentation, FLCi, FL3i, FL4I, BRCi, BR3i, and BR4i: samples after digestion; (**A**) samples with oat flakes; (**B**) samples with bran; a, b, c, d, and e mean the statistical difference between the samples in the post hoc Tukey's test ($p < 0.05$); error bars mean standard deviation; $n = 3$.

3.4. Sensory Evaluation

In this study, a potential probiotic-enriched puree mixture was prepared as an alternative functional food for human consumption. It is therefore crucial that fermented probiotic puree mixtures have acceptable sensory properties. Table 4 shows the sensory evaluation results of the puree mixtures enriched with potential probiotics. The fortification of oat flakes and oat bran, including samples with *L. rhamnosus K3* and *L. johnsonii K4*, provided acceptable sensory properties of puree mixtures after probiotic addition. The research also focuses on the addition of a potential probiotic strain called *L. paracasei* ssp. *paracasei* to apple juice formulations and its impact on sensory characteristics. In this study, the probiotic strain was added to apple juice in order to determine whether it affected appearance, aroma, texture, or purchase intent. When evaluated for these sensory attributes, there was no significant difference ($p > 0.05$) in acceptance between the various formulations, indicating that the inclusion of the probiotic culture did not affect acceptance [49]. Fermentation positively influenced the sensory characteristics of the potential probiotic-added puree mixtures, enhancing acidity and fermented odors and flavors. Samples with oat flakes fermented with *L. johnsonii K4* (FL4) showed the most intense apple, cinnamon, and fermented attributes, making them appealing options for human consumption. Overall, fermentation was a beneficial process in improving the sensory properties of the puree mixtures. The results of the descriptive sensory profile analysis of our products were consistent with this finding. PCA showed common characteristics between the flakes' control product and fermented samples. A direct effect of fermentation on the enhancement of the acid and fermented odor and flavor characteristics is shown in Figure 2A. As a result of fermentation, the samples changed their sensory profile. The greatest change after fermentation occurred in the range of attributes of cinnamon flavor, which corresponded to the high intensity of cinnamon flavor in the control sample and the low intensity in the fermented samples. This is also confirmed by the correlation obtained between the analyzed determinants in terms of PCA. In the PCA of the flakes, the distinguishing feature of the cinnamon flavor is opposite to the distinguishing features of the odor, the fractionated flavor, and the acid flavor. The results presented in Figure 1B illustrate the common characteristics between the bran control product and fermented samples. The common features were sweetness, apple odor and flavor, and structure characteristics. The fermentation directly affected the acid and fermented flavor characteristics. As a result of fermentation, the samples changed their sensory profile. Also, in this case, as in the bran samples, the greatest change after fermentation occurred in the range of notes of the cinnamon flavor, which corresponded to the high intensity of the cinnamon flavor in the control sample and the lower intensity in the fermented samples. This is also confirmed by the correlation obtained between the

analyzed determinants in terms of PCA. In the PCA of the flakes, the distinguishing feature of the cinnamon flavor is opposite to the distinguishing feature of the acid flavor.

Table 4. Intensity of defined attributes for developed puree mixture (0–10 c.u.) (n = 26).

Attribute	BRC	FLC	BR3	BR4	FL3	FL4
Apple o.	3.57 ^{bc}	4.73 ^{a}	5.85 ^{c}	5.92 ^{a}	6.75 ^{ab}	6.87 ^{ab}
Cinnamon o.	2.60 ^{ac}	3.19 ^{a}	4.56 ^{b}	5.69 ^{a}	6.14 ^{b}	6.54 ^{c}
Fermented o.	2.01 ^{b}	2.12 ^{b}	2.93 ^{a}	3.44 ^{a}	3.49 ^{a}	4.08 ^{ab}
Sweet o.	2.89	3.34	2.75	2.90	2.79	2.51
Other o.	0.47 ^{a}	1.21 ^{ab}	1.36 ^{ab}	1.81 ^{b}	1.93 ^{a}	2.06 ^{a}
Thickness	5.75	5.88	5.45	6.43	6.38	5.37
Stickiness	4.93 ^{ab}	5.24 ^{b}	5.85 ^{ac}	6.04 ^{ab}	6.08 ^{ab}	6.23 ^{c}
Apple f.	4.67 ^{ac}	5.33 ^{ab}	5.76 ^{abc}	6.40 ^{b}	6.52 ^{ab}	6.84 ^{c}
Cinnamon f.	2.83 ^{c}	3.24 ^{d}	3.85 ^{a}	5.06 ^{a}	5.23 ^{bc}	7.01 ^{ab}
Fermented f.	1.88 ^{a}	2.53 ^{ab}	3.35 ^{a}	3.74 ^{a}	3.75 ^{a}	3.81 ^{a}
Sweet f.	2.22 ^{a}	2.96 ^{b}	3.28 ^{a}	3.43 ^{a}	3.52 ^{ab}	4.79 ^{ab}
Acid f.	1.43 ^{a}	1.85 ^{a}	2.19 ^{a}	2.33 ^{a}	2.35 ^{a}	4.03 ^{b}
Bitter f.	1.21	1.28	1.33	0.86	1.00	1.41
Other f.	1.00	1.06	0.94	2.55	1.22	0.66
Overall Quality	6.16	6.96	6.89	6.63	6.63	6.47

Explanatory notes: c.u.: conventional units, BC: control sample with oat bran, FC: control sample with oat flakes, BR3 and FL3: puree mixtures with *Lacticaseibacillus rhamnosus K3* fermentation, BR4 and FL4: puree mixtures with *L. johnsonii K4* fermentation, o.: odor, f.: favor. Significant differences between samples are represented by means in the same row followed by different lowercase letters ($p < 0.05$).

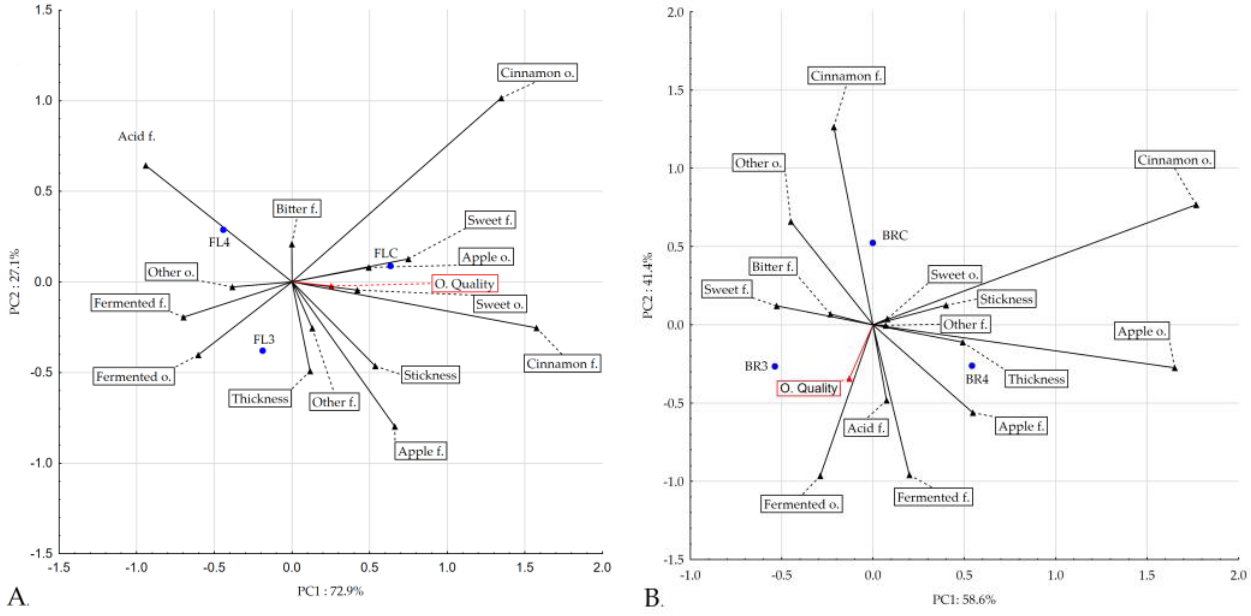

Figure 2. PCA of the puree mixture samples with oat flakes (**A**) and the PCA of the puree mixture samples with oat brans (**B**).

4. Conclusions

The presented study successfully developed a puree mixture with probiotics added and a suitable food matrix that promotes the growth of *L. rhamnosus K3* and *L. johnsonii K4*. Sensory properties were not compromised, with all samples scoring above six out of ten for overall quality. This suggests the mixture's potential commercialization in the probiotic food market. During simulated digestion, oat bran samples exhibited higher survival rates under gastric digestion compared to flakes. BR3 and BR4 also showed increased survival during the intestinal phase, suggesting oat bran's better carrier properties in

terms of probiotic survival during digestion. The total polyphenol content in fermented samples was more bioaccessible at the end of intestinal digestion than in non-fermented samples. BR3 and BR4 also had significantly increased bioaccessibility compared to non-fermented bran samples. Similar trends were observed for oat flakes, with fermented samples exhibiting higher bioaccessibility percentages than non-fermented samples. Lactic acid fermentation also changed the organic acid composition of the food matrix. The study observed significant changes in malic, succinic, lactic, and acetic acids, and it may also bring potential health benefits. In conclusion, this study provides insights for developing probiotic-enriched puree mixtures with a suitable food matrix. Probiotic viability, sensory properties, and improved bioaccessibility of phenolic compounds were demonstrated. This knowledge can guide the development of consumer-satisfying products in the food industry, expanding the probiotic food market with innovative alternatives.

Supplementary Materials: The following are available online at https://www.mdpi.com/article/10.3390/nu16040561/s1, Table S1: Sensory descriptors and their definitions used in the sensory analysis of breakfast mixture; Table S2: BRC—control sample with oat bran, FLC control sample with oat flakes, BR3 and FL3 puree mixtures with L. rhamnosus K3 fermentation, BR4 and FL4 puree mixtures with L. johnsonii K4, fermentation, FLCi,FL3i,FL4I,BRCi,BR3i,BR4i are samples after digestion. Organic acids and sugars before and after digestion in the analyzed samples; a. samples with oat flakes; b. samples with bran.

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Table S1: Sensory descriptors and their definitions used in the sensory analysis of breakfast mixture.

Descriptors	Definition	Anchors
	Odour descriptors	
Apple	Odour characteristic of apple and apple-based products	"none" to "very intense."
Cinnamon	Odour characteristic of cinnamon	"none" to "very intense."
Fermented	Odour characteristic of fermented apple	"none" to "very intense."
Sweet	The basic differentiator does not require characterization	"none" to "very intense."
Other	Odour other than those described above	"none" to "very intense."
	Texture attributes	
Thickness	A bulky characteristic of a product as sensed in mouth or by feeling and seeing e.g. During pouring of the product.	"very liquid" to "very thick."
Stickiness	Adhesion to processing equipment, cohesion of powders, sticking to packaging, and sticking to fingers and parts of the mouth	"imperceptible" to "very stick."
	Flavour descriptors	
Apple	Flavour characteristic of apple and apple-based products	"none" to "very intense."
Cinnamon	Flavour characteristic of cinnamon	"none" to "very intense."
Fermented	Flavour characteristic of products subjected to fermentation.	"none" to "very intense."
Sweet	The basic differentiator does not require characteristics (flavour characteristic of white sugar)	"none" to "very intense."
Acid	Flavour characteristic of products subjected to acids.	"none" to "very intense."
Bitter	The basic distinguishing feature does not require characteristics (bitter flavour characteristic of grapefruit)	"none" to "very intense."
Other	Enter the intensity and name or associate	"none" to "very intense."
Overall quality	Impression based on all testes	"very low" to "very high."

Table S2. BRC—control sample with oat bran, FLC control sample with oat flakes, BR3 and FL3 puree mixtures with *L. rhamnosus K3* fermentation, BR4 and FL4 puree mixtures with *L. johnsonii K4, fermentation,* FLCi,FL3i,FL4I,BRCi,BR3i,BR4i are samples after digestion. Organic acids and sugars before and after digestion in the analyzed samples; a. samples with oat flakes; b. samples with bran.

Sample	Citric Acid (mg/mL)	Malic Acid (mg/mL)	Lactic Acid (mg/mL)	Acetic Acid (mg/mL)	Propionic Acid (mg/mL)	Disaccharides (mg/mL)	Glucose (mg/mL)	Fructose (mg/mL)
BR3	0.0 ± 0.0	3.53 ± 0.07	2.3 ± 0.0	4.933 ± 0.057	0.0 ± 0.0	13.167 ± 0.577	2.6 ± 0.0	19.07 ± 0.192
BR3i	0.0 ± 0.0	11.9 ± 0.0	7.033 ± 0.057	11.067 ± 0.057	0.0 ± 0.0	58.0 ± 0.67	19.9 ± 0.47	28.43 ± 0.53
BR4	0.0 ± 0.0	3.733 ± 0.057	2.3 ± 0.0	4.8 ± 0.057	0.0 ± 0.0	14.467 ± 0.167	2.533 ± 0.167	19.567 ± 0.167
BR4i	0 ± 0	12.57 ± 0.19	7.67 ± 0.25	12.87 ± 0.34	11.9 ± 0.25	57.83 ± 0.37	20.13 ± 0.37	28.83 ± 0.37
BRC	0.6 ± 0.0	4.133 ± 0.057	0.0 ± 0.0	1.166 ± 0.057	0.0 ± 0.0	14.033 ± 0.167	2.933 ± 0.057	18.733 ± 0.167
BRCi	0.0 ± 0.0	12.3 ± 0.0	0.0 ± 0.0	3.433 ± 0.057	0.0 ± 0.0	63.633 ± 0.577	19.6 ± 0.47	31.7 ± 0.472
FL3	0.0 ± 0.0	1.767 ± 0.057	6.3 ± 0.0	1.7 ± 0.0	0.0 ± 0.0	16.3 ± 0.167	4.533 ± 0.057	22.4 ± 0.2
FL3i	0.0 ± 0.0	7.1 ± 0.0	29.167 ± 0.167	10.467 ± 0.057	3.233 ± 0.057	45.1 ± 0.577	20.167 ± 0.167	36.833 ± 0.1
FL4	0.0 ± 0.0	1.933 ± 0.057	6.3 ± 0.0	1.467 ± 0.057	0.0 ± 0.0	18.267 ± 0.167	5.367 ± 0.167	25.633 ± 0.167
FL4i	0 ± 0	7.8 ± 0.16	28.3 ± 0.76	10.07 ± 0.53	3.17 ± 0.09	48.6 ± 1.33	19.8 ± 0.41	37.6 ± 0.53
FLC	0.333 ± 0.057	2.733 ± 0.057	0.0 ± 0.0	0.7 ± 0.0	0.0 ± 0.0	18.9 ± 0.167	7.333 ± 0.057	21.833 ± 0.167
FCLi	0.0 ± 0.0	7.9 ± 0.0	0.0 ± 0.0	7.267 ± 0.057	0.0 ± 0.0	48.4 ± 0.833	20.567 ± 0.167	32.0 ± 0.353

Warsaw, 07.11.2024

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Beetroot ketchup as a stable carrier of potential probiotic *Lacticaseibacillus rhamnosus* K3 and *Lactobacillus johnsonii* K4: A study on sensory attributes, storage viability, and *in vitro* gastrointestinal survival

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ABSTRACT

This research aimed to assess the viability of *Lacticaseibacillus rhamnosus* K3 and *Lactobacillus johnsonii* K4 in beetroot ketchup during storage and simulated digestion to examine fermentation effects on sensory quality. The findings revealed that both strains maintained viability above 8 $\log_{10}$ CFU/ml during storage, confirming their potential as probiotics. pH levels changed significantly over three-week storage period indicating fermentation's impact on shelf stability. The control sample maintained consistent pH level of 4.6, while pH of ketchup fermented with *L. rhamnosus* K3 decreased from 3.84 to 3.79, and ketchup fermented with *L. johnsonii* K4 decreased from 3.96 to 3.69. Sensory evaluations showed statistically significant differences in odor, texture, flavor, and overall quality between samples. Fermentation with *L. johnsonii* K4 improved sensory attributes, achieving the highest overall quality score with mean value of 7.31 out of 10, compared to 6.28 for the control and 6.23 for the *L. rhamnosus* K3 fermented ketchup. Survival rate of *L. johnsonii* K4 was 27 %, compared to 2.8 % for *L. rhamnosus* K3 in dynamically simulated gastrointestinal system TIM-1. Both fermented ketchups contained over 10^9 CFU of viable cells. These results demonstrate that plant-based food products can effectively serve as carriers for potential probiotic strains, preserving their viability during storage and digestion, while enhancing sensory quality of food products.

1. Introduction

Probiotics, defined as "live microorganisms that, when administered in adequate amounts, confer a health benefit on the host," have gained importance in the development of functional foods and health promotion (Cordaillat-Simmons et al., 2020). They are primarily found in fermented foods and dietary supplements with strains of the *Bifidobacterium* genus and lactic acid bacteria such as *Enterococcus, Lactobacillus*, *Lactococcus*, *Leuconostoc*, *Oenococcus, Pediococcus*, and *Streptococcus* prevailing (Soemarie et al., 2021). Food products containing probiotics are generally derived from dairy sources, which means that not everyone can benefit from them. It is especially important for people who suffer from allergies, or lactose intolerance, have vegan eating habits, or are concerned about their health and sustainability (Küçükgöz and Trząskowska, 2022). As a result of these concerns, the market has seen an increase in the number of non-dairy probiotic-containing foods. The food matrix specialties of vegetables, including their low pH, sugar and fiber content, along with their overall nutritional values, make them ideal candidates for carrying probiotic strains (Maia et al., 2023). However, bioactive compounds in plant-based food matrices, and the amount of vegetables included in the product, can affect the survival of probiotic strains. In this context, interesting and little researched are beetroot and tomato, which are rich in bioactive compounds that can affect probiotic viability. Beetroot contains betalains like betacyanins and betaxanthins, phenolic compounds such as flavonoids and phenolic acids, and nitrates, all of which have antioxidant properties that can create a favorable environment for probiotics (Czyżowska et al., 2020; Sentkowska and Pyrzyńska, 2023). Tomatoes are packed with carotenoids, including lycopene and beta-carotene, which protect probiotics from oxidative damage (Boulaajine and Hajjaj, 2024). They also contain phenolic compounds such as caffeic acid, p-coumaric acid, and ferulic acid and glycoalkaloids

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and can enhance the growth environment for probiotics by reducing oxidative stress and inflammation. Vitamins and essential minerals in tomatoes further stabilize and support probiotics in tomato-based food matrices (Coelho et al., 2023). These bioactive compounds collectively can create a conducive environment for probiotics growth, enhancing their viability and efficacy in non-dairy, vegetable-based fermented products.

Therefore, food matrix selection will have a direct impact on probiotic strains (Dinkçi et al., 2019). Probiotic strains have already been successfully tested in different kinds of vegetables and have shown good sensory properties with ability to carrying these strains (Lillo-Pérez et al., 2021). As part of the potential probiotic selection for this study, 38 strains from traditional fermented foods were screened and strains were isolated from pickled cucumbers and sauerkraut found in homes in central Poland and was characterised by its ability to survive at low pH levels, tolerance of bile salts, excess of phenols. It is also important to draw attention for the remarkable abilities of *L. johnsonii* K4 growth during the exposure of phenols and hydrophobicity by over 60 %. This makes *L. johnsonii* K4 a promising probiotic species that could benefit from further study and potential integration into functional foods. (Zielińska et al., 2015). Beetroot and tomato, both rich sources of essential nutrients, antioxidants, and bioactive compounds, offer a unique opportunity for the development of fermented products that combine taste and nutrition (Ali et al., 2020; Punia Bangar et al., 2022). Nevertheless, these food matrices should also ensure that the probiotic strains remain viable during storage and be resistant to digestion. The recommended minimum viability level of 10^6 colony-forming units (CFU) per millilitre or gram of the consumed product is crucial for potential health effect (Shori, 2016). pH is one of the most important criteria to determine the potential activity of microorganisms and food quality, as the low pH levels are one of the main restrictive parameters for storage viability of selected strains, while at the same time necessary for preventing growth of other, food-spoilage, microorganisms (Nematollahi et al., 2016; Tripathi & Giri, 2014). It is also possible for acidity and pH to have opposite effects on the sensory characteristics and overall quality of a product.

Survival of probiotics during food processing and digestion is a critical factor in determining the efficacy of probiotic-containing products. Several challenges, including acidic conditions, enzymes, and bile salts in the gastrointestinal tract, can impact the viability and functionality of probiotics. Digestion is a complex process of breaking down food into its nutrients for absorption by the body and plays an important role in determining the bioavailability of bioactive compounds present in foods (Naissinger da Silva et al., 2021). To gain a comprehensive understanding of the survival of probiotics during gastrointestinal passage, it is a reliable choice to use simulated digestion models, because *in vitro* digestion models allow us to examine the digestion process in every step. The TNO Intestinal Model (TIM) system is a computer controlled dynamic *in vitro* model that closely simulates the conditions of the human gastrointestinal tract, including the stomach, small intestine, and colon. This model allows for the precise control and monitoring of digestion parameters, the survival of probiotics, and the overall nutritional quality of food products (Barroso et al., 2015). The use of the TIM-1 system to simulate gastrointestinal conditions for evaluating the survival of probiotic strains in these novel food matrices is another innovative aspect of this study.

This study aims to evaluate beetroot ketchup as a novel non-dairy carrier for probiotics, focusing on sensory attributes, storage viability, and gastrointestinal survival, which distinguishes it from previous research on dairy-based matrices.

2. Materials and methods

2.1. Selection of potential probiotic strains

The strains of *L. rhamnosus* K3(ID: KM186164) and *L. johnsonii* K4 (ID: KM186165) were collected from the laboratory of the "Department of Food Gastronomy and Food Hygiene, Warsaw University of Life Sciences in Poland" (Zielińska et al., 2015, 2019). Bacteria were activated from a frozen culture stored at −80°C and incubated at 37°C in 10 ml of MRS broth (pH 6.8 ± 7.2, Merck, Darmstadt, Germany) for 24 hours, with non-inoculated media as controls to ensure no contamination. After completing the incubation period, the tubes were centrifuged at 10, 000 rpm for 5 minutes (MPW-251; MPW MED Instruments, Warsaw, Poland) to separate bacterial cells from the medium. The supernatant was replaced with 8.5 g/kg of saline and the centrifugation procedure was performed three times to remove residual growth medium.

2.2. Preparation of beetroot ketchup

The raw materials are selected from common ketchup recipes and for improving the food matrices and decrease acidy of tomatoes and vinegar, beetroots were added. During product development, various proportions of ingredients were tested to obtain optimal textural and sensory parameters, which allowed the establishment of target amounts (Table 1). Tomato concentrate, beetroots, garlic powder, black pepper, white sugar and apple vinegar were purchased from local market. Beetroots were washed, boiled in a pressure cooker for 30 minutes. After cooking, the softened beetroots were mashed using a high-powered mixer (Bosch ErgoMixxThe mixture was pasteurized at 72°C for 15 minutes, followed by cooling to room temperature for inoculation. For each sample, a 1 ml bacterial solution (9 $\log_{10}$ CFU/ml) of *L. rhamnosus* K3 or *L. johnsonii* K4 in 0.85 % saline solution was added to 100 g of the ketchup mixture and fermented for 5 hours at 37°C. Next, the samples were immediately cooled to 4°C and stored in the refrigerator for 24 hours before further analysis.

2.3. pH analyses

The pH value of the samples was determined by a calibrated pH meter (ORION STAR A211, Thermo SCIENTIFIC). Measurements were made the day after fermentation and weekly for 3 weeks of storage. During the measurement, the product temperature was equal to the ambient temperature of 21 ± 1°C.

2.4. The viability of probiotics during storage

The viability of *L. rhamnosus* K3 and *L. johnsonii* K4 in ketchup samples was checked every week during the storage of 3 weeks at 4°C. A serial dilution method was conducted to check bacterial viability with MRS agar (pH 6.8 ± 7.2, Merck-Darmstadt, Germany) used for the plating at 37°C for 48 hours. The counting results are shown as log CFU/ml.

2.5. In vitro digestion

The TIM-1 *in vitro* gastrointestinal model (TNO Nutrition and Food Research Institute, Zeist, the Netherlands) has been extensively explained (Venema et al., 2019) and has been used on numerous occasions to study probiotic survival (Minekus, 2015; Venema et al., 2020). Before starting the experiments, a cleaning process involved immersing glass and plastic components in a solution containing 40 g of sodium hydroxide (NaOH) and 200 g of a phosphate-free cleansing agent (RBS® by Carl Roth) per liter of distilled water. These parts were then manually brushed to enhance the removal of contaminants and subsequently rinsed with tap water. Following this, the entire system was sequentially submerged in a solution of sodium hypochlorite (2.5 % w/v) for approximately 30 minutes and afterward rinsed with tap water, a 0.5 M hydrochloric acid (HCl) solution, distilled water, and finally cleaned with a 70 % ethanol solution. This model includes different compartments to simulate the stomach, duodenum, jejunum, and ileum, with a connection with peristaltic pumps. The digestion process is

Table 1
Proportion of the ingredients in the developed ketchup.

Sample	Ingredients						
	Tomato concentrate	**Beetroots**	**Garlic powder**	**Black Pepper**	**Sugar**	**Apple Vinegar**	**Probiotic**
KTC	100 g	30 g	1 g	0.3 g	5 g	1 g	-
KT3	100 g	30 g	1 g	0,3 g	5 g	1 g	*L. rhamnosus* K3
KT4	100 g	30 g	1 g	0,3 g	5 g	1 g	*L. johnsonii* K4

Explanatory notes: KTC—control sample, KT3 ketchup with *L. rhamnosus* K3 fermentation, KT4 ketchup with *L. johnsonii* K4 fermentation.

computer-controlled by different sensors and stays at 37°C during the entire process. Digestion lasted for 5 hours, during which approximately 90 % of the intake passed through the model. At the end of the experiment, the residue in the model was collected as well, to measure remaining viable counts in the system for details see (Venema et al., 2019). The process of mixing via peristalsis was controlled using computer-regulated peristaltic valve pumps, which simultaneously managed the movement of meals through distinct compartments. The pH levels within these compartments were under computer surveillance, with adjustments made by introducing HCl (1 M) for the stomach compartment and $NaHCO_3$ (1 M) for the small intestinal compartments. Electrolytes, bile, enzymes and pancreatic juice were adjusted according to a healthy adult. Pancreatic solution was simulated by secreting 10 % pancreatin ((Pancrex V) in a small intestinal electrolyte solution (containing NaCl 5 g l−1, KCl 0·6 g l−1, $CaCl_2$ 0·22 g l−1) at 0.25 ml per minute. We simulated biliary output by secreting 4 % bile solution (porcine bile extract, Sigma) at 0.5 ml per minute. The compartments were filled with start residues as described before (Minekus et al., 1995), except for the gastric residue, which was mixed with the 'meal'. Digested and dissolved low-molecular weight compounds were continuously dialyzed from the jejunum and ileum compartments through hollow fiber membrane systems, mimicking nutrient absorption in the body, and maintaining physiological bile and electrolyte concentrations. The dialysis fluid in the jejunum comprised 5.43 g per liter of NaCl, 0.65 g per liter of KCl, 0.37 g per liter of $CaCl_2$, and included 1.55 % bile extract. Similarly, the ileum dialysis fluid had the same composition, with the exception of bile salts, which were absent. The flow rates through the hollow fibers were set at 10 ml/min. In each TIM-1 run, 30 grams of citrate buffer ($C_6H_5Na_3$-O_7·2 H_2O, pH 4.5, Sigma) and approximately 5 g of a starting residue solution with 30 g of samples (non-fermented or fermented ketchup) were introduced. The starting residue solution consisted of 5 g from a prepared gastric electrolyte solution containing the enzymes Lipase (37.5 mg; Rhizopus F-AP15 from Amano Pharmaceuticals), Pepsin (42.0 mg, Sigma P7012), and acetate buffer pH 5.0 (prepared by adding 21.6 g of acetic acid to 87.1 g/liter sodium salt trihydrate, Sigma). For each experimental condition, two replicates were included in the experiments.

2.6. Determination of bacterial survival

The viable counts of *L. rhamnosus* K3 and *L. johnsonii* K4 were assessed at the start of the experiment (designated as t=0) and in the ileum efflux of TIM-1 over a five-hour period, with hourly sampling (at t=1, 2, 3, 4 and 5 h). To determine the viable counts, all collected samples were subjected to serial ten-fold dilution in sterile PBS. Subsequently, 10 µl from each dilution (ranging from 10^0 to 10^7-fold) were evenly spread onto the surface of Rogosa agar plates, which were then placed in anaerobic conditions (Anaerobic System Anaerogen from Oxoid, Basingstoke, UK), and incubated for 48 hours at 37°C. Viable counts were determined by examining agar plates that resulted in colony counts ranging from 3 to 30 Colony Forming Units (CFU). The cumulative survival percentage was calculated by dividing the viable bacteria in every-hour efflux samples by the number of bacteria in the intake.

2.7. Sensory analysis

The Quantitative Descriptive Profile (QDP) method, following ISO Standard 13299:2016 (ISO 13299:2016*(En), Sensory Analysis — Methodology — General Guidance for Establishing a Sensory Profile*, n.d.), was utilized to objectively evaluate the sensory quality of the produced ketchup samples. In the preliminary session, descriptors were selected and defined during the panel discussion. Sensory descriptors, including Tomato Odour, Beetroot Odour, The Odour of Spices, Fermented Odour, Sweet Odour, Other Odour, Density, Viscosity, Fermented Flavour, The Flavour of Salt, Beetroot Flavour, Acid Flavour, Sweet Flavour, Flavour of Spices, Flavour of Bitter, Other Flavour, and Overall Quality, were employed for this purpose. Table S1 in the supplementary materials contains the definitions of these descriptors. The trained panel included eight assessors, each with two to ten years of experience in sensory evaluation. They very command understanding of the sensory methodology and were familiar with the sensory quality being evaluated. To maintain scientific accuracy and standardization, 50 ml transparent containers with lids were used for sample preparation, each assigned a unique 3-digit code, and served randomly to experts at room temperature. Neutralization between samples was ensured by providing still water. Experts evaluated the sensory quality of samples using a 100 mm scale, converted to numerical values from 0 to 10 and named as conventional units [c.u.]. Attributes were rated from "none" to "very strong". Evaluation was conducted twice with 16 individual results calculated to determine the average result. Samples were coded with three-digit codes and served randomly to avoid bias. These measures collectively established a systematic and rigorous approach to assess the sensory attributes of the produced ketchup, aiming for precision and objectivity in the evaluation process.

2.8. Statistical analysis

The statistical analyses for this study involved a combination of R software package (version 3.6.2) and Statistica 13.3 (StatSoft, Kraków, Poland). The data were analyzed by multivariate analysis of variance (ANOVA) and Tukey HSD post hoc test and for sensory analysis results, a principal component analysis (PCA) was conducted in R, utilizing a correlation matrix to explore patterns and relationships within the sensory data. The difference was considered statistically significant when $p < 0.05$ in relation to bacterial counts, pH, and the outcomes of sensory evaluation.

3. Results and discussion

3.1. Viability of L. rhamnosus K3 and L. johnsonii K4 during storage

Studies show that the viability of probiotic strains is highly dependent on the relationship of the selected raw materials with probiotic strains. In general, the final product should contain 10^6 to 10^7 CFU/ml counts of strains to have a probiotic effect. Additionally, it is crucial that the selected strains remain viable throughout the storage process until consumption and have a minimum of 10^6 CFU/ml (Maia et al., 2023; Marinova et al., 2019). For the samples, KT3 and KT4 the microbial counts varied over the three-week period. The count of selected probiotic strains dropped significantly ($p<0.05$) after one week of storage

but there was no significant change during the following 2 weeks of storage ($p>0.05$). Specifically, directly after fermentation, KT3 exhibited a log CFU/ml of 9.12, which slightly decreased to 8.73 after one week of storage and further to 8.49 after three weeks. Similarly, KT4 showed values of 9.16 log CFU/ml after fermentation and 8.55 log CFU/ml, 8.56 log CFU/ml, 8.47 log CFU/ml at one, two, and three weeks, respectively (Table 2). Thus, both KT3 and KT4 showed a decreasing trend in microbial counts over the storage period, suggesting potential changes in the microbial population or metabolic activity. However, all samples stayed at more than 8 log CFU/ml throughout the storage process. The control sample, KTC, consistently showed non-detectable levels of microbial growth throughout the entire fermentation period, indicating the absence of viable bacteria and showed that the pasteurization process was successful, which suggests that in the fermented ketchup, there is no growth other than the added strains. Other research on the storage stability of a mixture of *L. johnsonii* and *L. plantarum* strains in fermented vegetables showed that the storage period did not affect the viability of strains for one month at 4°C and the counts of bacteria stayed above 8.5 log CFU/g (Manowan et al., 2020)[OBJ]. Other research on fermented beetroots and beetroot juices showed that lactic acid bacteria stayed viable after 6 months of storage (Klewicka & Czyzowska, 2011). Table 3 also illustrates the storage viability of similar food matrices with probiotic strains. Hence, our developed ketchup with beetroots can be considered suitable food carriers for *L. johnsonii* K4 or *L. rhamnosus* K3 and have potential probiotic effects due to the amounts of viable cells of the selected strains.

3.2. pH Changes After Fermentation and During Storage

The pH changes in ketchup samples were monitored over the three-week storage period. The control sample, KTC, maintained a consistent pH level of 4.6 at the start and throughout the storage weeks. Conversely, the pH of KT3, ketchup fermented with *L. rhamnosus,* exhibited a decrease, starting at 3.84 after initial fermentation to 3.79 by the end of the third week. Similarly, KT4, fermented with *L. johnsonii,* decreased in pH from 3.96 to 3.69 over the same storage period. There were significant changes found for the pH levels for all the samples during storage ($p<0.05$) (Table 4), but not between the two strains.

Similar to our results, another study working on the fermentation of vegetable matrices including red cabbage, carrot, and radish also found that all fermented vegetable pH levels decreased significantly, not only during the fermentation process, but also during storage at room temperature, with final pH levels ranging from 3.70 to 3.99 (Vatansever et al., 2017). That is why controlling pH in a different period of storage for the probiotic food products helps to understand potential effects of lactic acid fermentation. Regarding research on tomato juices with *Lactobacillus plantarum* and *Leuconostoc mesenteroides* addition showed that pH levels of all samples decreased after the bacteria addition and during storage at 4°C, while a non-fermented control sample stayed stable throughout the storage period (Bah et al., 2019). It is possible that the enzymes from microorganisms can hydrolyse the substrates and produce metabolites inside of the product and this can explain the pH drop over the storage period (Nematollahi et al., 2016). The pH drop during storage is likely due to lactic acid production from carbohydrate fermentation by the probiotics. Key enzymes involved include lactate dehydrogenase, which converts pyruvate to lactic acid (Wang et al., 2021). On the other hand, the viability of probiotic strains is highly influenced by changes in pH. Most probiotics, especially those in the *Lactobacillus* and *Bifidobacterium* genera, thrive in mildly acidic environments with a pH range of 4.5–6.5. As the pH drops below this optimal range, particularly below pH 3.5, the survival of these strains decreases significantly due to disruptions in cell membrane integrity and metabolic functions (Bustos et al., 2024). It is also possible to see in our research at the end of the storage period, pH reached the lowest level as well as it also affected the viability of the strains.

These findings highlight the impact of storage on the acidity of ketchup formulations, particularly those subject to fermentation with specific lactic acid bacteria, suggesting potential implications for shelf stability.

Table 2
The viable count of both strains in the fermented ketchup fermentation and during storage.

Sample	Total count (log CFU/g)			
	After fermentation	**1 week**	**2 weeks**	**3 weeks**
KTC	nd	nd	nd	nd
KT3	9.12^{a}	8.73^{b}	8.69^{b}	8.49^{b}
KT4	9.16^{a}	8.55^{b}	8.56^{b}	8.47^{b}

Explanatory notes: KTC—control sample, KT3 ketchup mixtures with *L. rhamnosus* K3 fermentation, KT4 ketchup mixtures with *L. johnsonii* K4 fermentation, nd-not determined. Tukey HSD test shows that statistical differences in lowercase are applicable to all samples in the same row.

Table 3
Comparative analysis of probiotic viability during storage in similar matrices (log CFU/ml or g).

Study/ Product Variant	Probiotic Strains/ Matrix Type	Initial Count	Storage Conditions	Final Count	References
Beetroot Ketchup	*L. rhamnosus* K3	9.12	4°C, 3 weeks	8.49	Current Research
Beetroot ketchup	*L. johnsonii* K4	9.16	4°C, 3 weeks	8.47	Current Research
Fermented Vegetables	*L. johnsonii, L. plantarum*	9.50	4°C, 1 month	8.50	(Manowan et al., 2020)
Beetroot Juice	Lactic Acid Bacteria	9.11	4°C, 6 months	6.8	(Klewicka and Czyzowska, 2011)
Fresh Beetroot Cubes	*Lactobacillus plantarum* BL3	7.71	4 °C,3 weeks	9.16	(Barbu et al., 2020)
Dried Beetroot Chips	*Lactobacillus plantarum* BL3	7.85	4 °C,3 weeks	~7	(Barbu et al., 2020)
Freeze-Dried Beetroot	*Lactobacillus plantarum* BL3	~8	4 °C,3 weeks	~7	(Barbu et al., 2020)

Table 4
The pH values after fermentation and during storage.

Sample	pH Measurement			
	After fermentation	**1 week**	**2 weeks**	**3 weeks**
KTC	4.68 ± 0.01^{a}	4.66 ± 0.02^{a}	4.63 ± 0.03^{a}	4.65 ± 0.02^{a}
KT3	3.84 ± 0.02^{a}	3.80 ± 0.01^{b}	3.81 ± 0.05^{b}	3.79 ± 0.02^{b}
KT4	3.96 ± 0.04^{a}	3.89 ± 0.09^{b}	3.77 ± 0.03^{c}	3.69 ± 0.02^{d}

Explanatory notes: KTC—control sample, KT3 ketchup mixtures with *L. rhamnosus K3* fermentation, KT4 ketchup mixtures with *L. johnsonii K4* fermentation, Tukey HSD test shows that statistical differences different lowercase letters (a, b, c, d, e) within the same column indicate significant differences ($p < 0.05$) between samples at a specific time point, Different lowercase letters (a, b, c, d, e) within the same row indicate significant differences ($p < 0.05$) between weeks for the same sample, ± indicates SD.

3.3. Survival of L. rhamnosus K3 and L. johnsonii K4 During Simulated Digestion

It is necessary to study the survival of probiotic microorganisms in industrial processing and storage conditions, but also during gastrointestinal transit to their site of action when incorporating them into a food matrix. It is therefore imperative to conduct *in vitro* studies simulating digestion to ensure that these microorganisms survive (Bernat

et al., 2015). The validated TIM-1 Digestion System (Marteau et al., 1997) served as a crucial platform for elucidating the intricate dynamics of survival of the strains in ketchup fermented with *L. johnsonii* K4 or *L. rhamnosus* K3 over a 5-hour period. The cumulative survival expressed as percentages of bacterial intake for *L. johnsonii K4* displayed a steady increase, with a final cumulative delivery of 27 % at the end of the experiment. This progressive trend indicates a robust survival and persistence of *L. johnsonii* throughout the simulated gastrointestinal tract, suggesting its potential resilience in the human digestive system. Conversely, *L. rhamnosus* exhibited a distinctive survival pattern, with a lower cumulative percentage of intake, reaching 2.8 % after 5 hours (Fig. 2). *L. johnsonii* K4's higher survival rate in gastrointestinal conditions may be due to its robust cell wall structure and higher tolerance to acidic pH, as reported by (Zielińska et al., 2015). A study on the survival of different formulations of *Lactobacillus* probiotic strains, after a complete run on the TIM-1 system, showed up to 12 % cumulative survival (%-age of intake) (Venema et al., 2019). Similarly, another survival experiment on TIM-1 systems for probiotics showed that non capsulated cells of *Lactiplantibacillus plantarum* isolated from fermented buffalo milk survived 18.5 % cumulative survival after a complete run of upper gastrointestinal systems, including residue, while in the same conditions, *Enterococcus faecium* strains cumulative survival percentage was 15 % (Surono et al., 2018). These results showed that the survival of strains was related to the selection of specific strains, even under the same conditions.

In the investigation of beetroot ketchup samples KT3 and KT4, the cell counts (CFU) were assessed at 5-hour time complete digestion process, between 0 and 60 minutes, 60–120 minutes, 120–180 minutes, 180–240 minutes, 240–300 minutes, and in the residue. For KT3, the cell counts ranged from 6.78×10^{7} CFU at 0–60 minutes to 1.68×10^{9} CFU in the residue. In the case of KT4, higher cell counts were observed, starting from 3.78×10^{9} CFU/ml at 0–60 minutes and reaching 2.41×10^{10} CFU in the residue (Table 5). (de Oliveira et al., 2023) conducted study to investigate the effect of red beetroot on the viability of probiotic lactobacilli and showed that all strains had viable cell counts of more than 3.5×10^{10} CFU, at the end of the digestion process in static simulated digestion models. In different research on survival of *Lactobacilli* on TIM-1 upper gastrointestinal systems for 6 hours showed that probiotic as a form of powder showed 2.10×10^{8} while probiotic powder enriched with Ahiflower oil 4.14×10^{8} (Venema et al., 2020).According to (Valero-Cases et al., 2017), research on *Lactobacillus plantarum* strain in tomato juice survived during the digestion process and even improved the intestinal barrier function in *in vitro* cell cultures.

It is recommended that for foods with probiotics to have a therapeutic effect, between 10^{6} and 10^{8} CFU of viable probiotic cells should remain after the intestinal digestion stage (Wendel, 2022). Therefore, a 30 g serving of ketchup fermented with *L. johnsonii* K4 or *L. rhamnosus* K3 provides above 10^{9} CFU viable probiotic cells. This is a sufficient amount to promote consumer benefits, making the ketchup potentially probiotic. The study shows that fermentation in a non-dairy food matrix rich in nutrients and, enabled the growth of bacterial strains in the matrix.

3.4. Sensory Analysis

In this study, fermented ketchup with beetroots was prepared as an alternative functional food. That's why it is important to develop products with acceptable sensory properties. Table 4 shows the sensory characteristics of non-fermented ketchup (KTC), ketchup fermented with *L. rhamnosus* (KT3) and ketchup fermented with *L. johnsonii* K4 (KT4) that were evaluated for odour, texture and flavour attributes. The fermentation odour showed significant differences between all samples. However, *L. johnsonii* fermentation has significantly increased the intensity of beetroot and spices odour; *L. rhamnosus* fermentation affected tomato odour, fermentation odour and sweet odour, while the non-fermented sample highest intensity of another other described by panellists, "earth odour" and "bitter odour". This variation highlights the influence of fermentation on the development of distinctive profiles, which can be attributed to the metabolic activities of the bacteria strain (Zhang et al., 2023). Fermentation-induced changes in ketchup samples also influenced the product density. The density of the KT3 and KT4 changed significantly compared to KTC ($p<0.05$). This can be the reason for substrate degradation during the fermentation process and lead to changes in texture and mouthfeel, thereby affecting sensory differences (Senanayake et al., 2023). Flavour profiles also differed between selected strains. Fermented and sour flavour has the highest score in KT3, with a significant difference in other fermented samples, KT4, while KTC has the lowest scores for these attributes ($p<0.05$). These results showed that fermentation with different bacterial strains can result in different biochemical pathways leading to the generation of unique flavour compounds with the production of various metabolites, including organic acids and alcohol (Zhang et al., 2023). Overall quality scores showed significant differences, highlighting the impact of *L. johnsoni* K4 fermentation on the sensory attributes and overall acceptability of ketchup ($p<0.05$). Fermentation can improve overall quality by enhancing flavour complexity and improving textural attributes. In conclusion, the fermentation process, particularly with *L. johnsonii* K4, significantly influences the sensory characteristics and overall quality of ketchup, contributing to distinct flavour profiles, textural properties, and consumer acceptability. Similarly, in beetroot beverages *Lactobacillus casei* fermentation for 2 hours at 37°C resulted in the highest sensory acceptability, with viable lactic acid bacteria counts maintained during storage (Gamage et al., 2016). Furthermore, the optimization of fermented tomato production highlighted the importance of selecting specific lactic acid bacteria strains, such as *L. fermentum, L. plantarum, P. pentosaceus*, and *L. paracasei*, with scores to enhance sensory scores (Zhao et al., 2024). These studies collectively demonstrate the impact of lactic acid bacteria on sensory attributes in tomato and beetroot products.

PCA revealed interesting findings about the fermented samples KT3, KT4, and KTC. The KT4 sample showed an improvement in overall quality. The fermented flavour and sour flavour were dominated by KT3 and KT4. On the other hand, KTC sample had a more diverse range of flavours, including sweet and beetroot flavours (Fig. 1).KT4 has higher intensity of beetroot odour and lower intensity of fermentation odour, beetroot flavour and fermented flavour compared to other fermented sample KT3 and these attributes can be related that KT4s has highest overall quality compared to both other fermented sample and non-fermented sample.

Further investigation revealed that the sweet flavour range was higher in KTC, which could be attributed to the absence of fermentation. During the fermentation process, *L. johnsonii* and *L. rhamnosus* consumed the sugars, resulting in the production of fermented and sour and decreased sweet flavour. The PCA analysis showed that the fermented

Table 5
Cumulative CFUs as determined by microbiological cell count in the ketchup samples KT3 and KT4, and average cumulative survival during complete TIM-1 runs.

Sample	Time of Interval [minutes]					
	0–60	**60–120**	**120–180**	**180–240**	**240–300**	**Residue**
KT3	6.78×10^{7}	1.03×10^{9}	1.33×10^{9}	1.57×10^{9}	1.60×10^{9}	1.68×10^{9}
KT4	3.78×10^{9}	1.13×10^{10}	1.31×10^{10}	1.46×10^{10}	1.69×10^{10}	2.41×10^{10}

Explanatory notes: KT3 ketchup mixtures with *L. rhamnosus* K3 fermentation, KT4 ketchup mixtures with *L. johnsonii* K4 fermentation.

Table 6
Intensity of defined attributes for ketchup samples (0–10 c.u).

Attribute	Sample		
	KTC	KT3	KT4
Tomato Odour	6.06 ± 1.91[a]	6.16 ± 1.59[a]	5.85 ± 2.32[b]
Beetroot Odour	4.46 ± 1.76[a]	3.54 ± 1.79[b]	4.56 ± 1.52[a]
Fermentation Odour	2.30 ± 1.76[a]	4.29 ± 1.87[b]	3.63 ± 1.53[c]
Sweet Odour	3.44 ± 1.19[a]	3.72 ± 2.08[b]	2.72 ± 1.54[c]
Spices Odour	2.59 ± 1.80[a]	2.76 ± 1.91[a]	2.90 ± 1.78[a]
Another Odour	1.56 ± 2.09[c]	1.23 ± 1.70[b]	1.25 ± 1.50[b]
Density	4.24 ± 1.67[a]	3.40 ± 2.06[b]	3.75 ± 1.27[c]
Viscosity	3.20 ± 1.73[b]	3.13 ± 2.19[b]	3.19 ± 1.37[b]
Tomato Flavour	5.74 ± 1.96[a]	5.89 ± 1.69[a]	5.72 ± 2.26[a]
Beetroot Flavour	5.48 ± 1.64[a]	4.65 ± 1.63[a]	3.94 ± 2.03[b]
Sour Flavour	3.24 ± 0.97[a]	6.21 ± 1.55[b]	5.71 ± 1.61[b]
Fermented Flavour	2.44 ± 1.48[a]	5.14 ± 1.56[b]	4.78 ± 1.77[c]
Sweet Flavour	4.48 ± 1.83[b]	2.34 ± 0.99[c]	2.83 ± 1.59[c]
Salty Flavour	3.35 ± 1.09[a]	2.84 ± 1.16[a]	2.44 ± 1.57[a]
Spice Flavour	3.66 ± 1.82[a]	3.71 ± 2.01[b]	3.65 ± 2.12[a]
Another Flavour	1.48 ± 1.93[a]	1.63 ± 1.32[b]	1.64 ± 0.88[b]
Overall quality	6.28 ± 1.93[a]	6.23 ± 1.32[a]	7.31 ± 0.88[b]

Explanatory notes: KTC—control sample, KT3 ketchup mixtures with *L. rhamnosus* K3 fermentation, KT4 ketchup mixtures with *L. johnsonii* K4 fermentation; c.u. – conventional units; Tukey HSD test shows that statistical differences between samples are represented by means in the same row followed by different lowercase letters of. The alphabet, they differ significantly ($p<0.05$).

samples were distinct from the non-fermented samples, indicating that the fermentation process had a significant impact on the resulting sensory characteristics.

4. Conclusion

This study demonstrates that beetroot ketchup can effectively serve as a non-dairy carrier for probiotics, with high sensory acceptability and significant viability over a three-week storage period. These findings suggest potential for broader applications in functional foods. Over the storage duration, microbial counts in the fermented samples gradually decreased, yet consistently remained above 8 $\log_{10}$ CFU/ml, indicating the probiotic potential of both strains. The overall quality of developed ketchup also improved with the addition of a potential probiotic strain. The pH changes observed in ketchup underscored the impact of fermentation, raising considerations for shelf-life stability. Notably, both *L. rhamnosus* K3 and *L. johnsonii* K4 were able to survive the digestive process to some degree, although there were some differences between the strains. In particular, *L. johnsonii* K4 was able to survive up to 30 % through the gastrointestinal system. This strain also had a positive impact on the overall sensory quality of ketchup, making it better than the other samples. The ability of these probiotics to withstand the simulated gastrointestinal conditions in TIM-1 suggests their potential to reach the colon in a viable state, a critical factor for realizing their health benefits in that part of the gastrointestinal tract. The findings highlight the importance of *in vitro* digestion models like TIM-1 in assessing the behaviour of probiotics under conditions that closely simulate the complexities of the human digestive system. As consumers increasingly seek food options with potential health-promoting benefits, fermented ketchup stands as a noteworthy candidate that combines taste preferences with potential nutritional advantages.

In conclusion, the most important achievements of our research include the investigation of a non-dairy matrix as a carrier of potentially probiotic bacteria to increase the availability and offer of these functional foods. Moreover, we have demonstrated the possibility of designing a product with very good sensory properties and health potential resulting from the viability of beneficial microorganisms both in the product and under digestion conditions in the gastrointestinal tract. Incorporating fermented beetroot ketchup into regular diets could provide a non-dairy source of probiotics, offering potential health benefits such as improved gut health and enhanced immune function. Limitations of this study include the storage period and limited strain diversity. Future research should explore different storage conditions, alternative probiotic strains, and varying fermentation substrates to expand the applicability of these findings.

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CRediT authorship contribution statement

Monika Trząskowska: Writing – review & editing, Supervision, Methodology. **Kübra Küçükgöz:** Writing – review & editing, Writing – original draft, Visualization, Validation, Project administration, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Koen Venema:** Writing – review & editing, Supervision,

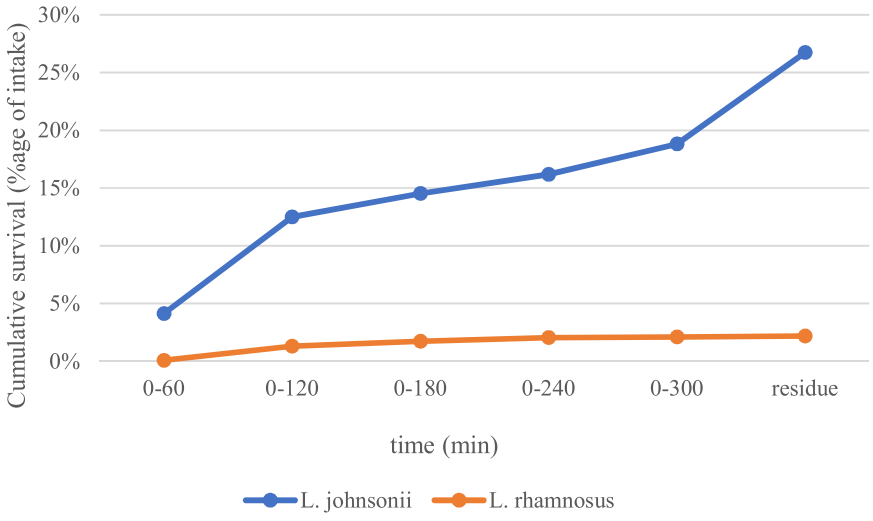

Fig. 1. Survival of *L. johnsonii* K4 *and L. rhamnosus* K3 (expressed as cumulative delivery from the ileal compartment) in the samples collected from the *in vitro* model at different time points (min).

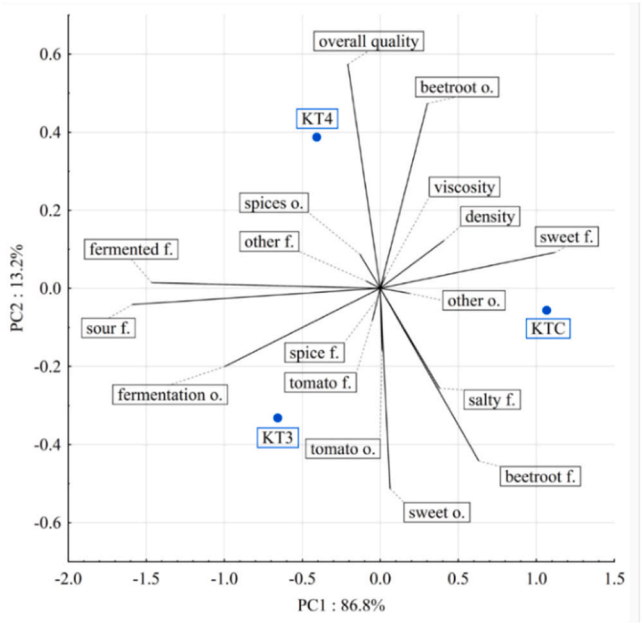

Fig. 2. The principal component analysis (PCA) analysis of sensory attributes the ketchup samples. Explanatory notes: KTC—control sample, KT3 ketchup mixtures with *L. rhamnosus K3* fermentation, KT4 ketchup mixtures with *L. johnsonii K4* fermentation.

Methodology.

Declaration of Competing Interest

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

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.fbp.2024.10.004.

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Suplementary Material

Fermented ketchup with beetroots

No	Descriptor	Definition
1.	Tomato odour	Odour characteristic of tomatoes and cooked tomatoes.
2.	Beetroot odour	Odour characteristic of beets and cooked beets.
3.	Fermented odour	Odour characteristic of fermentation.
4.	Spices odour	Odour characteristic of spices (odour of garlic, black pepper)
5.	Sweet odour	The basic differentiator does not require characterization.
6.	Different odour	Odour different than described above.
7.	Density	A feature of a product that can be felt in the mouth or visible, e.g. when pouring the product.
8.	Viscosity	Oral impression of the viscosity of the tested sample.
9.	Tomato flavor	Flavor characteristic of raw tomatoes and/or cooked tomatoes.
10.	Beetroot flavor	Flavor characteristic of raw beets and cooked beets.
11.	Fermented flavor	Flavor typical of fermented products.
12.	Salty flavor	The basic differentiator caused by salts.
13.	Sour flavor	Basic distinguishing factor caused by acids.
14.	Sweet flavor	The basic distinguishing feature caused by white sugar.
15.	Spices flavor	Flavor characteristic of spices (garlic powder, black pepper).
16.	Flavor bitter	The basic distinguishing feature does not require characteristic features (e.g. bitter flavor characteristic of grapefruit)
17.	Different flavor	Flavor different than described above.
18.	Overall quality	Impression evoked by all tested descriptors.

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I hereby represent that in the thesis Küçükgöz, K., Venema, K., Chamorro, F., Cassani, L., Donn, P., Prieto, M. A., & Trząskowska, M. (2025). Unlocking the potential of fermented beetroot ketchup: Enhancing polyphenol recovery and gut microbiota interactions. *Food Chemistry*, *463*, 141141. https://doi.org/10.1016/j.foodchem.2024.141141 my individual contribution in the development thereof involved Writing – review & editing, Writing – original draft, Visualization, Methodology, Investigation, Formal analysis, Data curation, Conceptualization.

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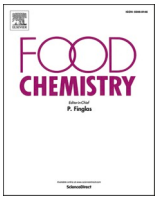

Unlocking the potential of fermented beetroot ketchup: Enhancing polyphenol recovery and gut microbiota interactions

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ABSTRACT

The study aimed to evaluate the effect of digestion and gut microbiota interactions on beetroot ketchup formulations, focusing on the release of polyphenols, bioaccessibility, and microbial interactions on gut microbiota with polyphenols. Tested ketchup samples were evaluated using the TNO Gastro-Intestinal Model 1 (TIM-1) simulated upper part of the gastrointestinal tract and the TNO Gastro-Intestinal Model 2 (TIM-2) simulated colon system. The results showed that fermentation of ketchup with *Lactobacillus johnsonii* K4, increased the release of bioactive compounds during digestion, with higher polyphenol recoveries observed in fermented samples. In particular, a fermented sample has higher recovery percentages for most of the phenolic acids, flavonoids, and betalains. However, some polyphenolic compounds were degraded during fermentation, suggesting a dynamic process of polyphenol metabolism in the gut environment. The study highlights the potential of fermented foods, such as beetroot ketchup, enriched with polyphenols and beneficial bacteria, to promote gut health and overall well-being.

1. Introduction

Tomatoes are known for their widespread culinary use and rich nutritional profile, containing bioactive compounds, including phenolics, flavonoids, and vitamins (Ali et al., 2020). These bioactive compounds have been linked to numerous health benefits and may reduce the risk of chronic diseases. That is why the consumption of tomato-based food products, such as sauces and condiments, has therefore gained popularity, due to their potential positive impact on human health (Chaudhary et al., 2018). Tomato ketchup, a widely consumed food worldwide, generally consists of tomatoes, sucrose, vinegar, salt, and different kinds of spices, along with potential texturising agents such as modified starches (Ahouagi et al., 2021). Ketchup is commonly recognised as a condiment for snacks, and it has a significant market share. Its use is expected to increase by 2.64 % annually from 2021 to 2025 (Le Thanh-Blicharz et al., 2023). Tomatoes are not only used to enhance the flavour of popular dishes but also for their health benefits.

To improve the nutritional composition and bioactive properties of ketchup sauces, the incorporation of phytonutrient-rich vegetables alongside tomatoes presents a promising opportunity for innovation and product development. An example is beetroots, which belong to a species of root vegetable that is a highly nutritious food containing various bioactive substances, such as betalains, phenolic acids, and flavonoids, all of which have been demonstrated to be effective in treating and preventing various diseases, and its use is also economically profitable (Chhikara et al., 2019; Clifford et al., 2015). Red beet is also known to have positive effects on the gastrointestinal tract as it has dietary fiber that improves intestinal peristalsis, reduces the incidence of colon lesions, and modulates the intestinal microbiota (de Oliveira et al., 2023). Red beet contains both soluble and insoluble fibers, including pectin,

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which forms oligosaccharides with different structures and properties, and it is found that these compounds can help the growth of beneficial bacteria (Gómez et al., 2016). Blending these two vegetables in ketchup formulation offers a novel approach to enhance the product's nutritional value and functional properties. The synergistic effect of combining different polyphenols from tomatoes with beetroots may provide enhanced antioxidant capacity and a broader spectrum of health benefits compared to conventional tomato-based ketchup.

Fermentation can increase the bioavailability of polyphenols and other bioactive compounds in food products and add potential health benefits. Enzymes of microbes can break down complex compounds, making the absorption of compounds easier (Yang et al., 2023). Moreover, fermented plant-based food products have become increasingly popular due to their impact as a probiotic carrier (Küçükgöz & Trząskowska, 2022). Therefore, the addition of beetroots and lactic acid fermentation to the ketchup recipe could not only enhance its nutritional value but also align with the preferences of health-conscious consumers. On the other hand, the efficacy of the polyphenol's health-promoting activities is related to their release during digestion or bioaccessibility, which is a significant limiting factor (Catalkaya et al., 2020). The fermentation process can change the chemical structure of bioactive compounds and have a major impact on their bioaccessibility in the small intestine (Dong et al., 2023). The interaction between polyphenols and the intestinal microbiota is crucial for health through the biotransformation by the intestinal microbiota (Fraga et al., 2019). It is important to note the amount of polyphenols that reach the intestine as they play a key role in this process (Santana Andrade et al., 2022).

The validated TNO *in vitro* upper digestion (TIM-1) and colon model (TIM-2) are dynamic systems that replicate the kinetic characteristics of the stomach, small intestine, and large intestine (Cárdenas-Castro et al., 2021; Venema & van den Abbeele, 2013). Their use enables screening the digestion effects and gut metabolites generated during digestion and colon fermentation to understand the biotransformation route of unabsorbed polyphenols in the small intestine.

Therefore, the purpose of this study was to analyze beetroot ketchup formulations, both fermented and non-fermented, to determine the potential health benefits for human consumption. The digestion process and gut microbiota impact the polyphenols as well as the role of fermentation in the bioactive properties of this plant-based food matrix were studied. The evaluation process involved examining bioactive compounds and antioxidant activity throughout upper gastrointestinal digestion and colon fermentation, using the TIM-1 and TIM-2 systems, respectively. The hypothesis statement for this study - the fermentation of beetroot ketchup with *Lactobacillus johnsonii* K4 increases polyphenol recovery, leading to enhanced nutritional value and potentially beneficial effects on gut microbiota composition.

2. Methodology

2.1. Preparation of Fermented Ketchup

Lactobacillus johnsonii K4 strain was obtained from the Department of Food Gastronomy and Food Hygiene at Warsaw University of Life Sciences in Poland (Zielińska et al., 2015; Zielińska et al., 2019). The strain was stored at −80 °C and then activated and incubated in MRS broth (pH 6.8 ± 0.2, Merck-110.660, Darmstadt, Germany). After that, the cells were separated from the medium through centrifugation and washed with saline to discard the growth medium. The fermentation process used a non-dairy matrix consisting of ingredients obtained from a market in the Netherlands. The control sample (KTC) contained 100 g tomato concentrate, 30 g beetroots, 1 g garlic powder, 0.3 g black pepper, 5 g sugar, and 1 g apple vinegar. For the ketchup sample with *L. johnsonii* K4 (KT4), the same ingredients were used, but supplemented with the potential probiotic *L. johnsonii* K4 at ~9 $\log_{10}$ CFU/ml. Fresh beetroots were washed, boiled, and homogenized. The mixture was then pasteurized at 72 °C for 15 min, cooled, and inoculated with a 1 ml bacterial solution containing *L. johnsonii* K4 in saline. Samples were incubated for 5 h at 37 °C, followed by immediate cooling to 4 °C and storage for 24 h before subsequent analysis.

2.2. In vitro digestion systems

The TIM-1 and TIM-2 models are advanced *in vitro* systems designed to simulate the human digestive tract. TIM-1 replicates the upper gastrointestinal tract, including the stomach, duodenum, jejunum, and ileum, allowing for the study of digestion, absorption, and bioaccessibility of nutrients and bioactive compounds such as polyphenols. It mimics physiological conditions through the addition of enzymes and digestive fluids, with samples collected from various compartments to measure nutrient absorption over time. TIM-2 simulates the human colon, focusing on fermentation processes by the gut microbiota. It uses fecal samples from healthy donors to create a standardized microbiota pool and maintains an anaerobic environment to study the production of metabolites and changes in microbiota composition. Together, these models provide a comprehensive understanding of the digestive process from ingestion to microbial fermentation in the colon.

2.2.1. In vitro model of the stomach and small intestine TIM-1

Figure 1 shows the schematic of the *in vitro* model, which has been described extensively before (Minekus, 2015). The model was set up and run according to the validated protocol for polyphenols (Ahmadi et al., 2023). Sampling made from the dialysis fluids of the jejunum and ileum were collected at 0, 1, 2, 3, 4 and 5 h, and the total fluid volume from these two compartments was carefully measured for each sample. All samples were chilled on ice until subsequent HPLC analysis. Duplicate experiments were conducted. The recovery of polyphenols for absorption was measured as the cumulative absolute amounts of polyphenols in the jejunum or ileum compartment, while the ileal efflux and residue in the total material collected behind the ileocecal valve were calculated as the amount available for the gut microbiota. Cumulative bioaccessibility is calculated by summing up the absolute amounts of polyphenols released in the ileum and jejunum dialysates per hour and dividing it by the total released absolute amounts. The total bioaccessible of polyphenols was calculated as polyphenols in the jejunal plus ileal dialysis tubes μg/l and the total amount of polyphenols available for gut microbiota was calculated by measuring the ileal efflux and residue for μg/l showed in the supplementary material (Table S1).

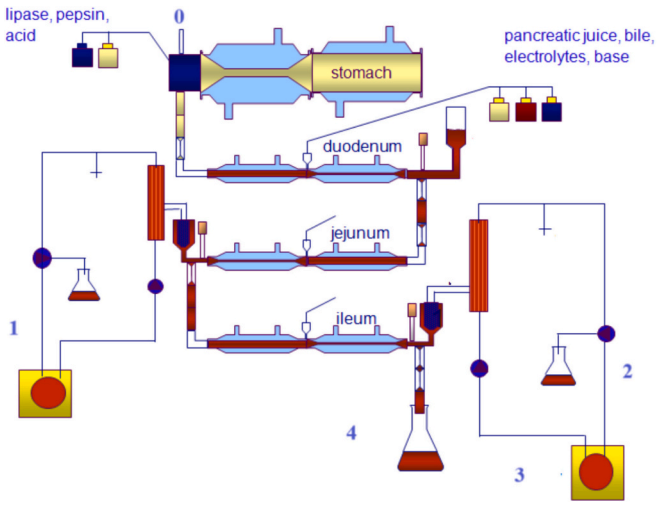

Fig. 1. Illustration of *in vitro* model for the upper gastrointestinal system. (TIM-1);
0. Ketchup meal introduced to gastric compartment, 1. Dialysate sample from jejunal compartment; 2. Dialysate sample from ileal compartment; 3. Ileal efflux; 4. Resides are collected at the end of the experiment.

The *in vitro* gastrointestinal model TIM-1 is a test system developed to mimic the digestive process in the human gastrointestinal tract. The system consists of various chambers that mimic the functions of the stomach, duodenum, jejunum, and ileum. To minimize the risk of bacterial contamination from pre-existing microflora within TIM-1, the model underwent overnight soaking (or longer) in 70 % ethanol (EtOH) before the commencement of experiments. The digestion process in the model takes five hours and involves the addition of a mixture of samples, water, enzymes, and electrolyte solutions. The pH of the mixture is carefully adjusted before it is added to the stomach part of the model. The digestion process lasted for 5 h, during which 30 g of samples (non-fermented or fermented ketchups), water, pepsin, electrolyte solution, and lipase were mixed. The pH of the mixture was adjusted to 4.5 and then added to the gastric part of the model. The testing process was monitored and controlled by sensors and computerized pumps. The TIM-1 model enables the investigation of digestive processes and the evaluation of the effects of food on the human digestive tract. Electrolyte levels, bile, enzymes, and pancreatic juice are meticulously adjusted to amounts found in a healthy adult. Mimicking the pancreatic solution involves introducing 10 % pancreatin into a small intestinal electrolyte solution containing NaCl (5 g/l), KCl (0.6 g/l), and $CaCl_2$ (0.22 g/l) at a rate of 0.25 ml per minute. Similarly, biliary output simulation entails secreting 4 % bile solution (porcine bile extract, Sigma) at 0.5 ml per minute. Each run of the TIM-1 model includes the introduction of 30 g of citrate buffer ($C_6H_5Na_3$-O_7·$2H_2O$, pH 4.5, Sigma) and roughly 5 g of a starting residue solution. This starting residue solution comprises 5 g from a prepared gastric electrolyte solution containing Lipase (37.5 mg; Rhizopus F-AP15 from Amano Pharmaceuticals), Pepsin (42.0 mg, Sigma P7012), and acetate buffer pH 5.0 (prepared using acetic acid and sodium salt trihydrate). The dialysis fluid in the jejunum is cacomposed of NaCl (5.43 g/l), KCl (0.65 g/l), $CaCl_2$ (0.37 g/l), and includes 1.55 % bile extract. Likewise, the ileum dialysis fluid shares the same composition, except for the absence of bile salts. Flow rates through the hollow fibers are standardized at 10 ml/min. Each experimental condition incorporates two replicates to ensure reliability in the experiments. During the digestive process, polyphenols were released in the jejunum and ileum compartments and passed through semipermeable hollow capillary membranes with a pore size of 0.05 μm (Spectrum Milikros 205 modules M80S-300-01P) through passive diffusion, entering the jejunum and ileum dialysis fluids.

2.2.2. *In vitro gastrointestinal batch digestion*

An *in vitro* static gastrointestinal digestion was performed on fermented beetroot ketchup with *L. johnsonii* K4 and non-fermented beetroot ketchup to collect enough material for colon fermentation following the method outlined by (Minekus et al., 2014). In brief, initially, both the fermented and non-fermented ketchup underwent an oral digestion phase using α-amylase from human saliva (A0521, Sigma-Aldrich, St. Louis, MO, USA). Subsequently, gastric digestion was simulated by adding pepsin (P-7000, Sigma-Aldrich, ≥ 250 units/mg solid) in a 0.2 M HCl-KCl buffer solution and maintaining the mixture at 37 °C for 2 h. To transition to the intestinal phase, pancreatin (P-1750, Sigma-Aldrich) was introduced and allowed to digest for 5 h at 37 °C with regular mixing.

2.2.3. *Experimental set-up and the TIM-2 in vitro model*

Fig. 2 shows the schematic of the *in vitro* model of colon TIM-2, which has been described extensively before (Venema, 2015). The model was set-up and run according to the validated protocol for polyphenols (Cárdenas-Castro et al., 2021).

Fecal samples were collected from 16 healthy individuals: 8 females and 8 males aged 23 to 50. Volunteers stated no history of gastrointestinal diseases or use of antibiotics for at least three months prior to donation. This was done following the procedure described by Aguirre et al. (2014). The resulting fecal slurry was quickly frozen in liquid nitrogen and stored at −80 °C until further use. Before inoculation, four

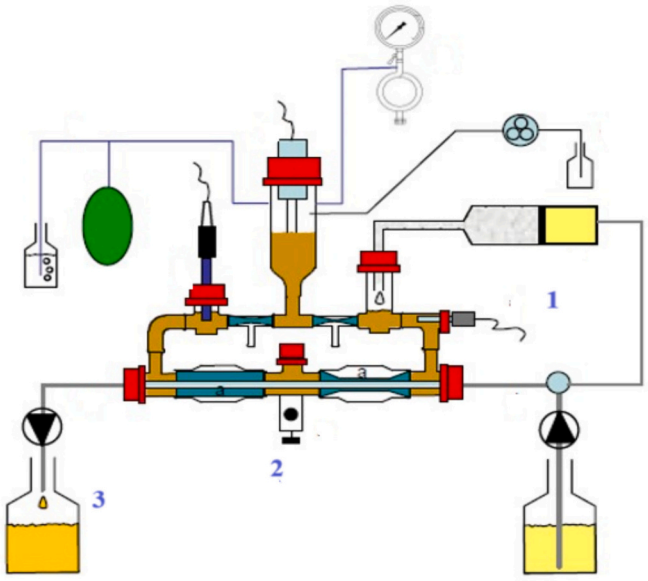

Fig. 2. Illustration of *in vitro* model for the colon system. (TIM-2); 1. Fecal sample addition; 2. Ketchup samples addition and dialysate sample collection; 3. Luminal sample collection.

30 ml tubes of fecal slurry were kept at 37 °C for one hour and then mixed with a pre-digested mixture to reach a total volume of 250 ml. Each TIM-2 unit was then given an inoculation of 60 ml of the standardized microbiota pool. Subsequently, the simulated ileal efflux medium (SIEM) was supplied to each unit at a rate of 2.5 ml/h for a 16-h adaptation period. Following this, the units were continuously supplemented with 60 g freeze-dried and pre-digested ketchup and SIEM after the starvation period. They were also supplemented with *L. johnsonii* K4 for 3 days. Three different conditions were tested: SIEM (control), SIEM +60 g freeze-dried and pre-digested ketchup, and SIEM +60 g freeze-dried and pre-digested ketchup with 0,5 ml in MRS broth (pH 6.8 ± 7.2, Merck-110.660, Darmstadt, Germany) *L. johnsonii* K4 for 3 days. Samples from both the lumen and dialysate were obtained at 0, 24, 48, and 72 h and subjected to analysis for metabolite production and microbiota composition and activity. The standardized microbiota pool was created by homogenizing fecal samples obtained from a group of healthy adults under anaerobic conditions, following the protocol described by Aguirre et al. (2014). The resulting fecal slurry was promptly frozen in liquid nitrogen and stored at −80 °C until further use. Prior to inoculation, four 30 ml tubes of fecal slurry were incubated at 37 °C for one hour and then combined with a pre-digested mixture to achieve a total volume of 250 ml. Each TIM-2 unit received an inoculation of 60 ml from the standardized microbiota pool. Subsequently, the units underwent a 16-h adaptation period with continuous supply of SIEM at a rate of 2.5 ml/h. Following this, the units were supplemented with 60 g of freeze-dried and pre-digested ketchup and SIEM after a period of starvation. Additionally, *L. johnsonii* K4 was supplemented for 3 days. Three different conditions were tested: SIEM alone (CON), SIEM +60 g of freeze-dried and pre-digested ketchup (KTC), and SIEM +60 g of freeze-dried and pre-digested ketchup with 0.5 ml of *L. johnsonii* K4 in MRS broth (KT4) (pH 6.8 ± 7.2, Merck-110.660, Darmstadt, Germany) for 3 days. Throughout the experiment, samples from the lumen were collected from the units after 24 and 48 h to simulate the passage from the proximal to the distal colon. Samples from both the lumen and dialysate were collected for analysis of metabolite production and microbiota composition and activity.

2.3. Chemical characterization by HPLC-ESI-QqQ-MS/MS

Phenolic compounds were extracted from the digestion samples according to the Cardenas protocol (Cárdenas-Castro et al., 2021), with slight modifications. Briefly, 2 ml of the digested sample and 2 ml of solvent (80 % ethanol) were mixed in glass bottles equipped with a magnetic stirrer. The solution was mixed for 1 h at room temperature. After the mixing step, the resulting solution was centrifuged at 6000 rpm for 15 min. The supernatant was then filtered using a syringe filter with a pore size of 0.22 μm. The filtered solution was stored at −22 °C until analysis. HPLC-MS/MS analysis of the phenolic profile was carried out on a Dionex Ultimate 3000UPLC+ system (Thermo Scientific, Waltham, MA, USA) coupled with a TSQ Quantis triple quadrupole spectrometer (HPLC-ESI-QqQ-MS/MS) (Thermo Scientific, USA). The separation of compounds was carried out with a Phenomenex nucleosil C18 column (5 μm, 100 Å, 4.6 mm × 150 mm.) thermostated at 40 °C. The solvents used were (A) 0.1 % formic acid in water and (B) acetonitrile. The elution gradients used were 15 % B (5 min), 15–35 % B (5 min), 35–70 % B (10 min), 70–90 % B (4 min), 90–15 % B (1 min) 15 % B (5 min), to equilibrate the column at initial conditions. A flow rate of 0.3 ml/min was used. The sample injection volume was 20 μl, and after chromatographic separation, the eluate was introduced into the triple quadrupole mass spectrometer. Mass screening was performed using a TSQ Quantis (ThermoFinnigan, San Jose, CA, USA) equipped with a working electrospray ion (ESI) source in negative/positive mode. Mass analysis was performed by selected reaction monitoring (SRM). The following parameters were used as universal conditions: sheath gas: 30 Arb; auxiliary gas: 10 Arb; ion transfer tube temperature: 325 °C; and vaporizer temperature: 350 °C. To determine the optimal conditions for identification and quantification, the SRM parameters of each compound (precursor/product ion combination, retention time, collision energy, and RF lens voltage) were optimized. Where possible, standards were used in this process and the results of previous research were taken into account for guidance. Quantification was performed using calibration curves of commercially available phenolic standards. Gallic acid was quantified using the equation ($y = 84\ x10^6x$, $R2 = 1.00$), ferulic acid quantification used the equation ($y = 2\ x\ 10^8x\text{-}2.1 \times 10^4$, $R^2 = 0.9994$), quercetin was evaluated using the equation ($y = 8\ x10^8x + 2 \times 10^6$, $R^2 = 0.9975$), the analysis of catechins used the equation ($y = 7.9\ x10^6x + 8.88 \times 10^5$, $R^2 = 0$), the quantification of betalain used the equation ($y = 4\ x\ 10^8x + 1.8 \times 10^5$, $R^2 = 0.9988$) Kaempferol was determined using the equation ($y = 2\ x\ 10^{10}x\text{-}2 \times 10^6$, $R^2 = 0.9988$). The total content of phenolic compounds was calculated as the sum of the quantifiable values. The results are expressed in mg per g of dry sample (mg/g S).

2.4. DPPH

DPPH radical scavenging activity of antioxidants and Total Polyphenol Content (TPC) were assessed according to (Cassani et al., 2022) with modifications. The evaluation of antioxidant activity involved the assessment of the ability to scavenge DPPH radicals. In this process, 50 μl of each optimal solution of each sample were dispensed into a 96-well microplate. Subsequently, 200 μl of the DPPH reagent (225 μM, initial absorbance approximately 1.4 units) was added, and the microplate was left to incubate at room temperature in darkness for 1 h. Following the incubation period, absorbance measurements were taken at 515 nm using a Synergy™ HTX microplate reader. This experimental procedure was conducted in triplicate, with the blank consisting of 50 μl of methanol. The percentage of inhibition was calculated using a standard curve of Trolox (TX) and the results were expressed as TX equivalents per ml of digested extract (μg TX equivalents/ml).

2.5. Total polyphenol content

TPC was assessed with a method involving 25 μl of three optimal extracts of each sample, all added to a 96-well microplate. Subsequently, Folin–Ciocalteu reagent (diluted at a ratio of 1:10, 125 μl) was uniformly dispensed into all wells, allowing a 3-min incubation period at room temperature. Following this, 100 μl of Na_2CO_3 solution (7.5 % *w/v*) was introduced into each well. The microplate was then placed in darkness and left to incubate at room temperature for 120 min. After the incubation period, absorbance readings were taken at 765 nm using a Synergy™ HTX microplate reader. This experimental procedure was conducted in triplicate, with the blank consisting of 25 μl of distilled water. TPC was calculated using gallic acid (GA) as standard. The results were expressed as μg GA equivalents/ml.

The extraction procedure involved placing 2 ml of the sample and 2 ml of solvent (80 % ethanol) in glass bottles equipped with a magnetic stirrer. The solution was mixed for 1 h at room temperature. Following the mixing step, the resulting solution was centrifuged at 6000 rpm for 15 min. The supernatant was then filtered using a syringe filter with a pore size of 0.22 μm. The filtered solution was stored at −22 °C until analysis. Standards for FX were purchased from Sigma. The calibration curve of the standard Fx was used to quantify Fx and its derivatives. Fx determination for HPLC; and (2) Extract dry weight (E) determination. Fx content was analyzed for each experimental condition using HPLC-DAD and expressed in mg Fx/g E dw. The HPLC equipment used included a Waters HPLC with a Waters 600 controller, pump, and photodiode array detector, Waters 717 plus auto-sampler, and an AF in-line degasser. The stationary phase was a Waters Nova-Pak C18 column (150 × 3.9 mm, WAT 088344), stabilized at 25 °C. The method used to determine Fx was previously developed and briefly described before (Lourenço-Lopes et al., 2023). Gallic Acid was quantified using the equation ($y = 84\ x10^6x$,$R^2 = 1.00$), Ferulic Acid quantification utilized the equation ($y = 2x10^8x\text{-}2.1 \times 10^4$, $R^2 = 0.9994$), Quercetin was assessed using the equation ($y = 8x10^8x + 2 \times 10^6$, $R^2 = 0.9975$), Catechin analysis employed the equation.

($y = 7.9\ x\ 10^6x + 8.88 \times 10^5$, $R^2 = 0.$), Betalain quantification utilized the equation ($y = 4x10^8x + 1.8 \times 10^5$, $R^2 = 0.9988$) Kaempferol was determined using the equation ($y = 2x10^{10}x\text{-}2 \times 10^6$, $R^2 = 0.9988$).

2.6. Gut Microbiota Composition

Microbiota composition was determined by sequencing of the V3-V4 region of the 16S rRNA gene. Briefly, DNA was extracted from the lumen samples, amplified with barcoding, pooled, and then subjected to sequencing using the Illumina MiSeq sequencing system following the manufacturer's instructions (Illumina, Eindhoven, The Netherlands). By using the Binary Base Call text-based format for storing biological sequences and their corresponding quality scores (FASTQ) pipeline (BCL2FASTQ, v.), the sequences were converted into text-based format, after quality checks (Illumina, San Diego, CA, United States of America, released version 1.8.3). Subsequently, Quantitative Insights into Microbial Ecology 2 (QIIME-2) software was employed for the analysis (Bolyen et al., 2019). The classification of sequences into amplicon sequence variants (ASVs) was conducted using the Silva database (version 132).

2.7. Statistical analysis

R software package (version 3.6.2) was used for statistical analyses. The correlation between ASVs and polyphenols was calculated using Spearman's correlation. Multiple comparisons were adjusted for using the Benjamini–Hochberg false discovery rate, and q-values (FDR-corrected *p*-values) were considered significant at $q < 0.20$.

3. Results

Understanding the transformation of polyphenols during digestion is crucial. During gastrointestinal digestion, polyphenols undergo hydrolysis and are affected by changes in pH and digestive enzymes. Some are absorbed, while others reach the colon for fermentation by the gut

microbiota, influencing its composition. Polyphenols, known for their antioxidant properties, are abundant in plant-based foods like tomatoes and beetroot. Tomatoes contain compounds such as caffeic acid, catechin, chlorogenic acid, dihydroisorhamnetin, ferulic acid, and quercetin (Erdinc et al., 2018; Lee et al., 2021). Beetroot includes betagarin, betanin, and vulgaxanthins (Nizioł-Łukaszewska & Gawęda, 2014; Sutor-Świeży et al., 2022). The specific polyphenol composition can vary based on plant variety, cultivation, and processing techniques (Ivanišová et al., 2024).

Fig. 3. Cumulative bioaccessibility of phenolic acids (A), flavonoids (B) and betalains (C) during TIM-1 *in vitro* gastrointestinal digestion. KT4 - fermented ketchup with *L. johnsonii* K4; KTC - Non-fermented ketchup.

3.1. Cumulative bioaccessibility and recovery of bioactive substances during TIM-1 digestion

Figs. 3 show the cumulative absorption of phenolic acids, flavonoids, and betalains during 5 h of upper gastrointestinal digestion. The cumulative bioaccessibility is variable between KTC (non-fermented ketchup) and KT4 (fermented ketchup with *L. johnsonii* K4). The bioaccessible compounds were measured across different time intervals to understand their release and availability for absorption. KTC consistently demonstrated higher bioaccessibility percentages across most compounds compared to the KT4. However, compounds such as caffeic acid, gallic acid, p-coumaric acid, and rutin were found to have higher bioaccessibility in the fermented ketchup.

Fig. 4 illustrates the changes in bioaccessibility of antioxidant activity, and total polyphenol content during a 5-h simulation of the upper gastrointestinal system. Initially, KTC exhibited a higher bioaccessible amount of antioxidant activity and total polyphenol content, and this remained consistent at the end of the process. This could be attributed to lactic acid fermentation, which can reduce the bioaccessibility of total phenolic content and DPPH radical scavenging activity during *in vitro* digestion due to several factors, such as the binding of phenolics to fermentation metabolites, pH changes and solubility reduction, microbial degradation of phenolics and specific fermentation conditions. Altogether, these processes limit the release and availability of phenolic compounds during digestion, thus affecting their antioxidant properties (Filannino et al., 2018; Hur et al., 2014).

Figure 5 illustrates the recovery percentages of released polyphenols during digestion. KT4 has higher recovery percentages for most of the phenolic acids, flavonoids, and betalains despite KTC has higher cumulative absorption percentages. Recovery percentages are frequently even above 100 %. This result can be attributed to the transformation and release of polyphenols during digestion. They undergo various chemical changes, including hydrolysis, oxidation, and interaction with other components in the digestive system. These transformations can lead to the formation of new polyphenolic compounds or the release of bound polyphenols that were not initially quantified. As a result, the post-digestion measurements may reflect not only the original polyphenols but also additional compounds derived from the digestion process (Subbiah et al., 2024).

This shows that fermentation with lactic acid bacteria can help convert polyphenols and release more bioactive components during digestion. A higher recovery amount of polyphenols in KT4 and lower bioaccesibility levels compared to KTC also can help these compounds become available for the gut microbiota. The Supplementary material shows that for KT4 betagarin, catechin, chlorogenic acid, nariningen, betanidin, betanin, neobetanidin, vulgaxanthin III, vulgaxanthin IV, total antioxidant capacity and total polyphenol content have higher content compound available for gut microbiota, compared to the non-fermented sample. Besides, ferulic acid and gallic acid are not detected in KTC samples for gut microbiota availability, while they are present in KT4 samples.

3.2. Microbial metabolites produced during in vitro colon fermentation: Biotransformation of polyphenols

Advancing our understanding of the biotransformation processes involving phenolic compounds (PC) from various food sources that ultimately reach the colon is crucial. These compounds undergo a complex series of enzymatic reactions facilitated by the gut microbiota, leading to their transformation into diverse metabolites. Notably, the consequential biological effects on human health are primarily attributed to these microbial metabolites rather than the original PC. These transformations encompass an array of enzymatic activities, including de-esterification, oxidation, hydroxylation, dehydrogenation, decarboxylation, methylation, hydration, and deglycosylation, as elucidated (Catalkaya et al., 2020). Table 1 presents the metabolites derived from the *in vitro* colon fermentation of beetroot ketchup and the product supplemented with *L. johnsonii* K4. Across all stages of colon fermentation, compounds such as betagarin, betavulgarin, kaempferol, naringenin, betanin, and neobetanidin were consistently identified in both sample sets. Moreover, it was observed that the gut microbiota fermentation process notably enhanced the concentration of these polyphenols in both the original ketchup and the supplemented variant. However, specific compounds including catechin, chlorogenic acid, cochliophilin A, gallic acid, betanidin, vulgaxanthin I and vulgaxanthin IV exhibited degradation throughout the fermentation period in all samples. This degradation trend suggests that upon release from the bound complex polyphenols, these compounds were susceptible to microbial degradation by the colon microbiota over time. In summary, while certain polyphenolic compounds showed increases due to the fermentation process in ketchup and *L. johnsonii* K4-supplemented ketchup, the microbial activity in the colon led to the degradation of several identified compounds upon their release from the polyphenol complex.

The analysis of interactions between gut microbiota and specific compounds reveals complex interrelationships with potential implications for human health and physiology. Fig. 6 shows that the gut

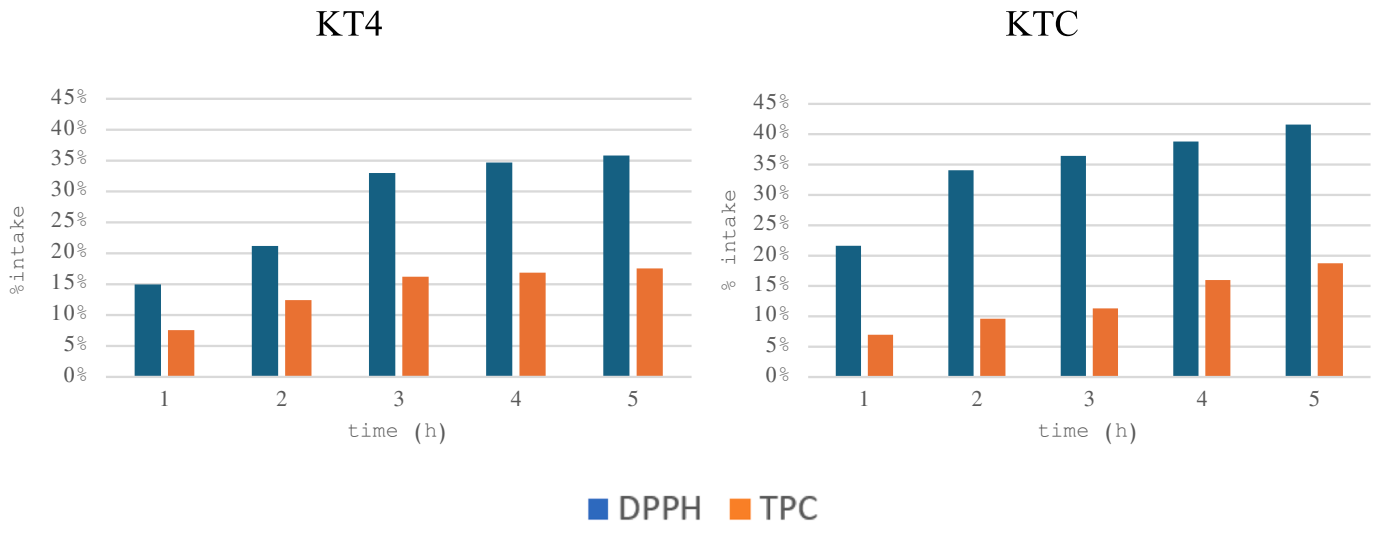

Fig. 4. DPPH radical scavenging activity of antioxidants and Total Polyphenol Content (TPC) during TIM-1 *in vitro* gastrointestinal digestion. KT4; fermented ketchup with *L. johnsonii* K4; KTC; Non-fermented ketchup.

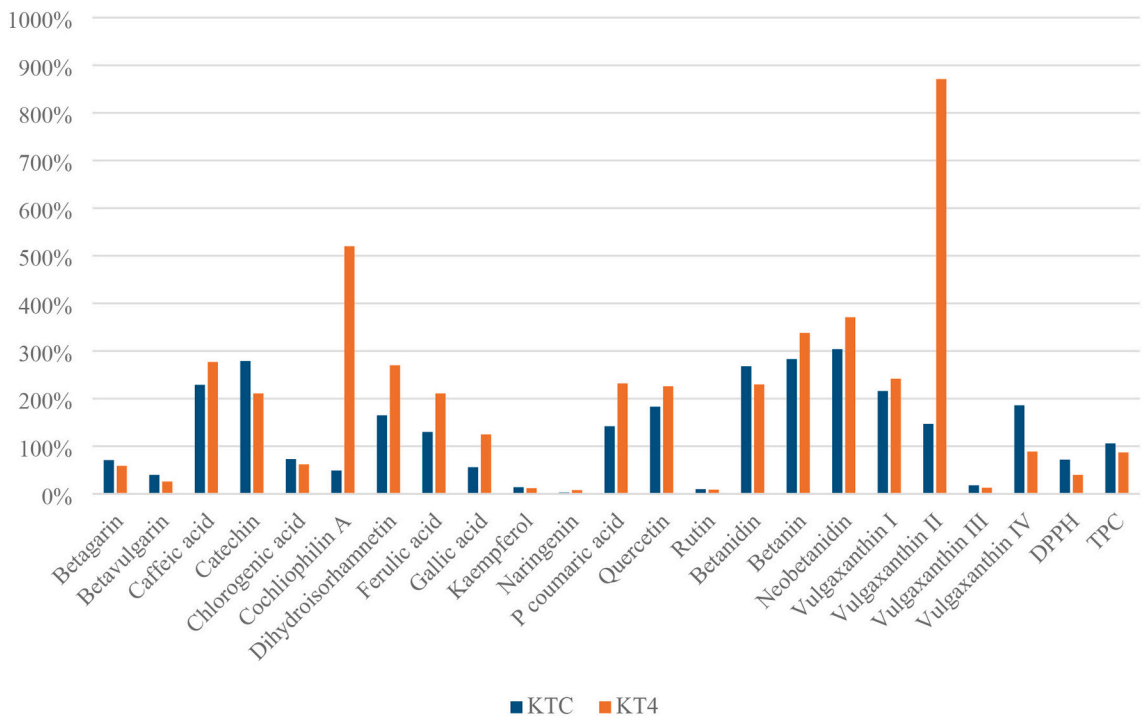

Fig. 5. Total recovery percentage of metabolites during TIM-1 *in vitro* gastrointestinal digestion. KT4; fermented ketchup; KTC; Non-fermented ketchup.

Table 1
Phenolic metabolites (ug/l) produced by gut microbiota during the *in vitro* colon fermentation.

	Time of fermentation											
	0 h			**24 h**			**48 h**			**72 h**		
Metabolites	**KT4**	**KTC**	**CON**	**KT4**	**KTC**	**CON**	**KT4**	**KTC**	**CON**	**KT4**	**KTC**	**CON**
Betagarin	4.37	5.80	3.32	1.95	2.56	2.35	5.57	10.29	6.37	8.92	10.92	10.21
Betavulgarin	1.01	0.44	0.12	0.14	0.15	0.42	0.27	0.13	0.10	0.17	0.05	0.25
Caffeic acid	28.40	102.43	n.d.	56.90	38.87	n.d.	61.53	29.97	n.d.	33.45	43.31	28.75
Catechin	1497.7	849.03	2115	706.9	434.53	192.85	1096.35	735.08	512.17	749.48	658.69	88.91
Chlorogenic acid	5.35	4.27	2.79	0.43	n.d.	0.37	1.32	0.39	0.99	0.64	0.86	n.d
Cochliophilin A	0.42	0.25	0.40	0.63	0.10	0.30	0.61	0.31	0.08	0.26	0.06	0.13
Dihydroisorhamnetin	9.95	6.02	12.72	14.14	10.13	17.52	13.23	6.40	5.90	5.55	7.04	4.24
Ferulic acid	3.51	n.d.	n.d.	n.d.	n.d.	n.d.	2.91	n.d.	n.d.	n.d.	n.d.	n.d.
Gallic acid	1.47	2.09	3.29	0.70	0.92	0.58	2.85	0.59	n.d.	0.67	0.15	n.d.
Kaempferol	0.14	0.14	0.16	0.08	0.17	0.17	0.12	0.15	0.13	0.16	0.17	0.12
Naringenin	2.62	1.24	2.06	3.52	1.96	1.96	4.58	2.92	3.90	4.29	3.12	5.35
P coumaric acid	28.73	32.71	22.42	29.33	33.41	33.41	26.62	26.57	28.97	30.66	28.47	23.71
Quercetin	4.96	3.39	4.33	5.14	5.37	4.12	3.35	4.58	5.35	3.96	5.06	4.40
Rutin	0.02	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Betanidin	118.38	130.02	101.58	35.13	42.72	65.30	66.66	89.53	83.38	62.26	88.99	56.01
Betanin	18.88	19.70	19.61	17.97	18.16	18.98	21.29	20.89	19.89	23.36	20.34	20.18
Neobetanidin	22.06	21.27	23.58	20.02	20.73	18.10	23.42	20.29	19.94	25.36	21.83	25.06
Vulgaxanthin I	35.46	41.21	40.65	26.02	27.81	64.09	33.19	29.57	47.52	38.20	27.90	28.16
Vulgaxanthin II	3.02	2.85	2.53	1.69	2.06	2.14	2.29	1.73	2.34	2.14	2.10	1.97
Vulgaxanthin III	36.12	20.31	36.58	29.32	44.69	20.12	35.62	38.62	13.97	29.14	41.19	14.89
Vulgaxanthin IV	0.49	0.51	0.28	0.10	0.11	0.20	0.16	0.00	0.12	0.14	0.14	0.04
DPPH	28.26	8.57	13.55	24.21	11.41	13.27	24.06	11.46	3.87	7.68	14.18	6.52
TPC	32.12	23.30	21.95	19.70	20.99	19.87	28.63	25.15	27.62	22.57	26.39	13.47

Explanatory notes: KT4 - fermented ketchup; KTC - Non-fermented ketchup; CON - SIEM; n.d. - not detected; DPPH (μg Troloxequiv/ml); TPC (μg Gallic acid equiv./ml).

microbiota exhibited multiple correlations between various phenolic metabolite species. Notably, Betagarin demonstrated significant positive correlations with *Prevotella 9* (q = 0.172), *LachnospiraceaeNK4A136* group (q = 0.111), *Eubacterium ruminantim* group (q = 0.172), and *Ruminococcaceae UCG014* (q = 0.172). Conversely, negative associations were found with *Bacteroides* (q = 0.172), *Parabacteroides* (q = 0.176), *Christensenellaceae R-7* group (q = 0.065), and *Defluviitaleaceae UCG-011* (q = 0.194).

Nariningen positively correlated with *Prevotella 9* (q = 0.050), *Lactobacillus* (q = 0.124), *Weissella* (q = 0.197), and *Parasutterella* (q = 0.197). Conversely, negative associations were observed with *Bacteroides* (q = 0.111), *Unchar.taxon-3 in Bacteroidales S24–7* group (q = 0.111), *Coprobacter* (q = 0.197), *Parabacteroides* (q = 0.132), *Butyrivibrio* (q = 0.169), *Roseburia* (q = 0.144), *Ruminococcus 2* (q = 0.120), *Sutterella* (q = 0.050), and *Anaeroplasma* (q = 0.050). Betanin displayed positive correlations with *Prevotella 9* (q = 0.019), *Methanobrevibacter* (q = 0.075), *Lactobacillus* (q = 0.161), *Weissella* (q = 0.185), *Streptococcus* (q = 0.153), *Blautia* (q = 0.161), *Coprococcus 1* (q = 0.153),

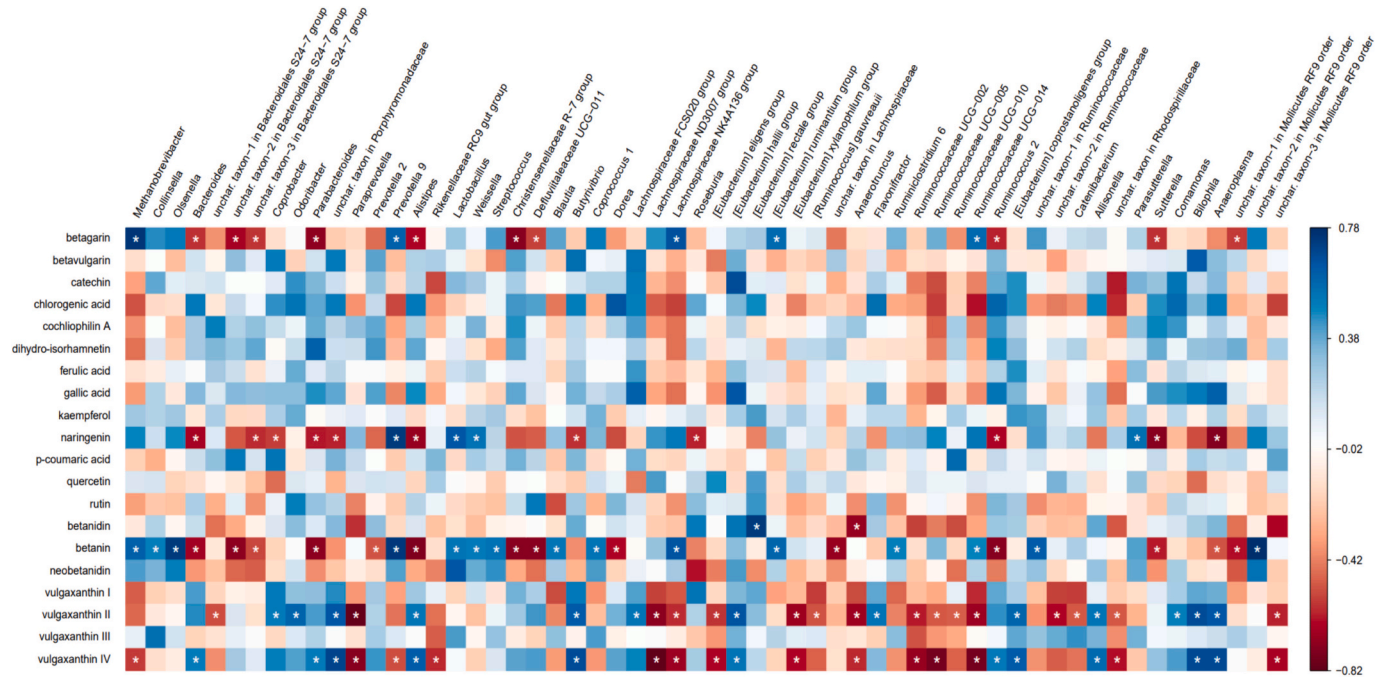

Fig. 6. Correlation between bioactive components and specific operational taxonomic unit (OTUs) at genus level. White asterisks (*) $q \leq 0.2$; blue: positive correlation; red: negative correlation. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Lachnospiraceae NK4A136 group (q = 0.054), *Eubacterium ruminantium* group (q = 0.093), *Ruminiclostridium 6* (q = 0.185), and *RuminococcaceaeUCG014* (q = 0.194), among others. Negative correlations were noted for various taxa, including *Unchar.taxon-2 in the Bacteroidales S24–7* (q = 0.019), *Parabacteroides* (q = 0.019), *Prevotella 2* (q = 0.185) groups. Moreover, vulgaxanthin II exhibited negative correlations with multiple entities: *Unchar.taxon-1 in Bacteroidales S24–7* group (q = 0.178), *Paraprevotella* (q = 0.017), *Lachnospiraceae ND3007* group (q = 0.067), *Anaerotruncus* (q = 0.067), and *Ruminococcaceae UCG-002* (q = 0.101). It presented positive associations with *Coprobacter* (q = 0.185), *Alistipes* (q = 0.173), *Comamonas* (q = 0.194), and *Anaeroplasm* (q = 0.080). Vulgaxanthin IV was positively correlated with *Bacteroides* (q = 0.198), *Parabacteroides* (q = 0.187), *Butyrivibrio* (q = 0.042), and *Ruminococcus 2* (q = 0.176). In contrast, it displayed negative associations with *Methanobrevibacter* (q = 0.121), *Paraprevotella* (q = 0.036), *Prevotella 9* (q = 0.176), and Additionally, *Unchar.taxon-3 in the Mollicutes RF9* order displayed a negative correlation with Vulgaxantin IV. This results highlights the bacteria that show positive or negative correlations with specific phenolic compounds. These interactions suggest that certain gut bacteria may thrive or be suppressed in the presence of these metabolites, potentially influencing gut health and overall physiology.

4. Discussion

4.1. Cumulative bioaccessibility and recovery of bioactive substances during TIM-1 digestion

The results show the metabolic transformations and recovery of bioactive compounds in non-fermented and fermented beetroot ketchup with *L. johnsonii* K4.

The comparison between KTC and KT4 shows differences in polyphenol composition, which could have significant health implications. It can be possible that during fermentation, *L. johnsonii* K4 produces enzymes that hydrolyse macromolecules, such as polysaccharides and polyphenols, into smaller metabolites (Yang et al., 2023). This process can lead to changes in the contents of specific phenolic compounds when comparing non-fermented samples with fermented ones. Phenolic compounds are converted into specific bioactive metabolites depending on the metabolic pathways used by specific bacterial strains. This conversion is influenced by the substrate composition, which affects the metabolic pathways and biochemical transformations during fermentation (Leonard et al., 2021; Yang et al., 2023). It is also possible to see the recovery of polyphenols during the digestion system, fermented samples had in general a much higher recovery percentage for most of the compounds at the end of the digestion process. Another investigation found changes in the red beet phenolic profile during spontaneous fermentation. There was a decrease in phenolic acids and free flavonoids, and an increase in free phenolic acids and conjugated flavonoids (Płatosz et al., 2020). The KTC was found to have a higher concentration of chlorogenic acid. Based on our findings, it was observed that the recovery percentage for chlorogenic acid was 73 % for sample KTC and 62 % for sample KT4. On the other hand, the recovery percentage for caffeic acid was 229 % for KTC and 277 % for KT4, which is greater than 100 %. This process can be explained by the fact that chlorogenic acid is an ester formed by the combination of caffeic acid and quinic acid. The breakdown and absorption of chlorogenic acid are necessary for its metabolic fate and potential effects on human metabolism (Colombo et al., 2023; Gil & Wianowska, 2017). During fermentation, kaempferol can undergo glycosylation to form kaempferol glycosides or be oxidized to form quercetin derivatives by microbial enzymes. Studies have shown that kaempferol can be converted into kaempferol 8-C-glucoside and quercetin 8-C-glucoside through metabolic enzymatic transformations, such as glycosylation and oxidation, during microbial fermentation (Huynh et al., 2018; Wu et al., 2022). Our research also found similar results: kaempferol was present in the KTC but was not detected in KT4. Additionally, quercetin recovery had a higher percentage in the KT4. During the upper gastrointestinal digestion process, low recovery percentages were observed for nariningen 3 % and 8 % for samples KTC and KT4, respectively, while quercetin showed recoveries of 183 % and 226 % for samples KTC and KT4, respectively. These results could be related In a study, the biosynthetic pathways for kaempferol and quercetin was reconstructed in *Saccharomyces cerevisiae*, with naringenin as a substrate, demonstrating the conversion of naringenin to both kaempferol and quercetin. These findings collectively indicate the diverse enzymatic transformations that kaempferol can undergo during microbial

fermentation (Tartik et al., 2023). The quercetin amount was found less in the KT4 compared to the KTC samples, and it showed a decrease after fermentation (Table S1), while gallic acid was found higher in KT4 also with a higher recovery percentage during the digestion process. Other research has also shown that after lactic acid bacteria fermentation, the content of quercetin decreased while gallic acid amounts were higher, possibly due to the metabolic pathway of the direct fission of the C-ring at the C2-O1 and C3-C4 bonds (Tang et al., 2023). Although the amounts of polyphenols may decrease after fermentation, the overall recovery of polyphenols during digestion can still be higher in fermented samples. This can be linked to the protective effects of fermentation on polyphenols, preventing their degradation during digestion. The microorganisms and acidic environment created during fermentation can help to stabilize polyphenols and protect them from enzymatic breakdown in the digestive tract. As a result, polyphenols may persist during the digestive process in fermented samples, contributing to a higher overall recovery (Yang et al., 2023).

A similar scenario also is applied to total polyphenol content and antioxidant activity; it is possible to see fermentation with *L. johnsonii* K4 increased the amount of polyphenol content and antioxidant activity (Table S1). Besides, available for gut microbiota, and bioaccessible amounts are also higher in the fermented sample. Fermentation enhances the nutritional value of the sample. Similar results are also confirmed for lactic acid fermentation applied to tomato juices (Ricci et al., 2020). These findings suggest that the fermentation process may impact the bioaccessibility of certain compounds in beetroot ketchup, potentially due to changes in the chemical composition and structure induced by fermentation. Further studies are needed to understand why fermented and non-fermented ketchup products have different bioaccessibility.

Besides, another research focuses on the fermentation of beetroot juice by probiotic *L. brevis* and *L. paracasei* resulting in increased contents of betanin, isobetanin and neobetanin, as well as of betanidine and isobetanidine, which were not found in unfermented beetroot juices. Betanidine and isobetanidine are aglycones with the ability to neutralize free radicals. Betanidin and its isomer isobetanidin were formed in fermented beetroot juice possibly as a result of the activity of β-glucosidase produced by tested probiotics (Klewicka et al., 2015).

The addition of tomato ketchup to beetroots improves their polyphenol diversity, as well as adding betalains. This can offer health benefits to consumers, such as reducing oxidative stress (Nirmal et al., 2023). Some research suggests they may also have anti-inflammatory effects and contribute to cardiovascular health by potentially lowering blood pressure and improving endothelial function. Additionally, betalains have been linked to improved exercise performance and liver health, though further research is needed to fully understand their mechanisms and health benefits (Nirmal et al., 2024).

More research is needed to understand the effects of lactic acid fermentation on polyphenols. Combining *in vitro*, *in vivo*, and clinical studies can confirm these findings and explore the health benefits of fermented foods rich in polyphenols. This understanding could lead to the development of new foods for improved health.

4.2. Microbial metabolites produced during in vitro colon fermentation: Biotransformation of polyphenols

In vitro colon fermentation of control, beetroot ketchup and the sample supplemented with *L. johnsonii* K4 resulted in alterations in metabolite profiles, highlighting the complex relationship between food components, microbe activity, and metabolite transformations. The consistent presence of betagarin, betavulgarin, kaempferol, naringenin, betanin, and neobetanidin throughout the fermentation process indicates the resilience of these polyphenolic compounds in both ketchup variants. Moreover, their increased concentration after fermentation suggests a potential role of colon microbiota in enhancing the bioavailability or bioactivity of these compounds. Conversely, the degradation of catechin, chlorogenic acid, cochliophilin A, gallic acid, betanidin, vulgaxanthin I, and vulgaxanthin IV in all samples over time after their release from the food matrix underscores their susceptibility to microbial degradation. This degradation could be attributed to enzymatic activities within the various members of the gut microbiota. While certain polyphenolic compounds showed increases due to the fermentation process in ketchup and *L. johnsonii* K4-supplemented ketchup, the microbial activity in the colon led to the degradation of several identified compounds upon their release from the PC complex. For example, in research on gut microbiota effects on chlorogenic acid, it was found that it eventually was degraded to caffeic acid through the process of microbial degradation and dihydroxylation (Tomas-Barberan et al., 2014). It is also possible to observe these kinds of transformations in our research. At the end of the fermentation process, caffeic acid levels were higher in KT4 than in control samples, possibly caused by the degradation of chlorogenic acid. According to another study on Mexican sauces, caffeic acid increased after 24 h of colon fermentation and peaked after 72 h. Research also indicates quercetin was not found at the beginning of the fermentation process and peaked during 24 h of fermentation for sauces, and during the same time of fermentation for ketchup samples as well (Cárdenas-Castro et al., 2021). The findings imply a dynamic relationship between the structural complexity of polyphenols, their association with the food matrix, and the responsiveness of colon microbiota. These dynamics influence the fate and bioavailability of polyphenols, impacting their potential health-promoting properties. The observed differences between the supplemented and unsupplemented ketchup might suggest a modulatory role of *L. johnsonii* K4 in the fermentation process. Further investigation into the specific mechanisms by which this potential probiotic strain interacts with the polyphenols and the gut microbiota could provide valuable insights into potential strategies for enhancing polyphenol bioavailability or activity in the gut.

5. Conclusion

This study highlights the role of LAB fermentation in polyphenol availability and influencing microbial metabolism in the developed beetroot ketchup. The incorporation of beetroot into tomato ketchup provides the product with additional polyphenols, broadens its polyphenol profile with betalains and offers consumers a healthier and more attractive condiment option. In the analysis, we found notable differences in the bioaccessibility, recovery, and biotransformation of specific polyphenols between non-fermented and fermented beetroot ketchup variants. Our observations show that the differential impact of fermentation, particularly with *L. johnsonii* K4, increased the recovery of most polyphenols during upper gastrointestinal digestion in the TIM-1, supporting our hypothesis. An important aspect of this work is the identification of specific microbial metabolites and their associations with bacterial taxa, providing deeper insights into the complex mechanisms underlying polyphenol-microbiota interactions. During colon fermentation, certain compounds such as betagarin, betavulgarin, kaempferol, naringenin, betanin, and neobetanidin showed higher levels in the fermented sample. This suggests that they are more resilient and may offer potential health benefits. On the other hand, certain compounds such as catechin, chlorogenic acid, cochliophilin A, gallic acid, betanidin, vulgaxanthin I, and vulgaxanthin IV were observed to be degraded in both the fermented and non-fermented samples, indicating that they are more susceptible to microbial activity. This degradation could influence the bioactivity and health-promoting properties of these compounds, although the more simple phenolic metabolite were not measured here. Our results support understanding the complex processes involved in the breakdown of polyphenols during fermentation. Through this, we can create effective methods to increase the nutritional value of fermented foods and harness their potential health benefits. This will help us advance the development of functional foods and promote better (gut) health.

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CRediT authorship contribution statement

Kübra Küçükgöz: Writing – review & editing, Writing – original draft, Visualization, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Koen Venema:** Writing – review & editing, Validation, Supervision, Methodology, Investigation, Funding acquisition, Conceptualization. **Franklin Chamorro:** Methodology, Investigation, Formal analysis. **Lucía Cassani:** Methodology, Investigation, Formal analysis. **Pauline Donn:** Methodology, Investigation, Formal analysis. **Miguel A. Prieto:** Supervision, Methodology. **Monika Trząskowska:** Writing – review & editing, Visualization, Validation, Supervision, Methodology, Conceptualization.

Declaration of competing interest

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

Data availability

Data will be made available on request.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.foodchem.2024.141141.

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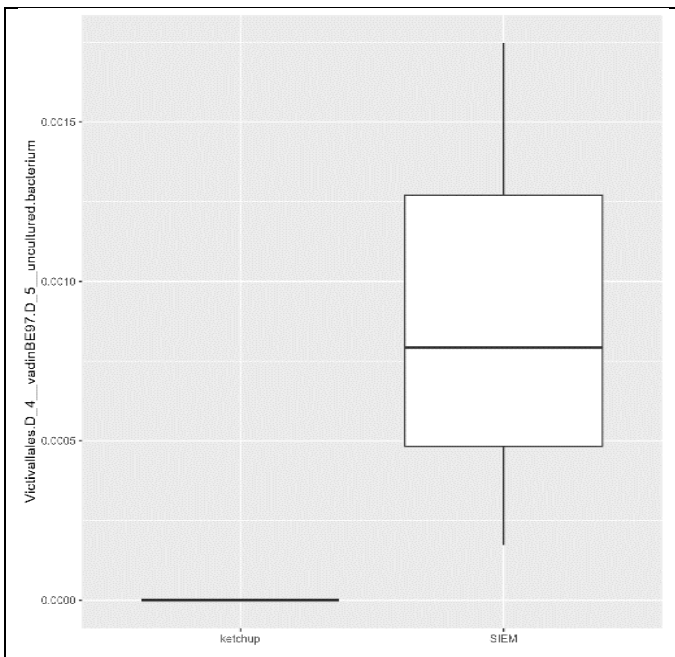
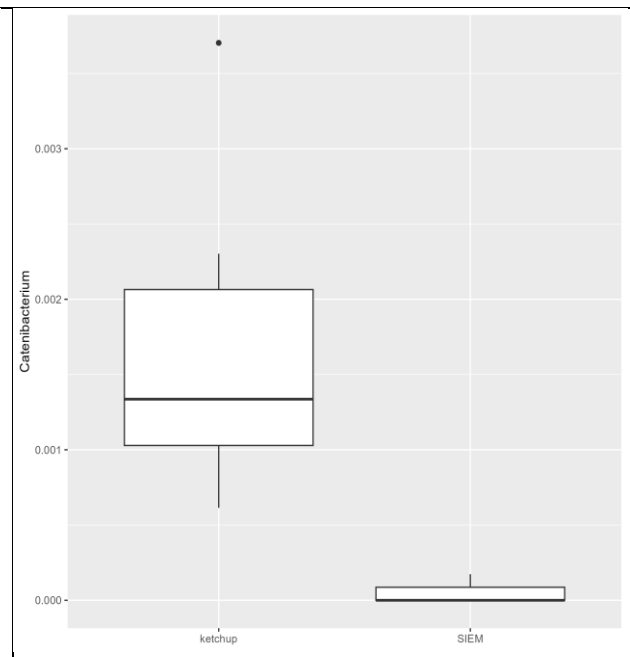
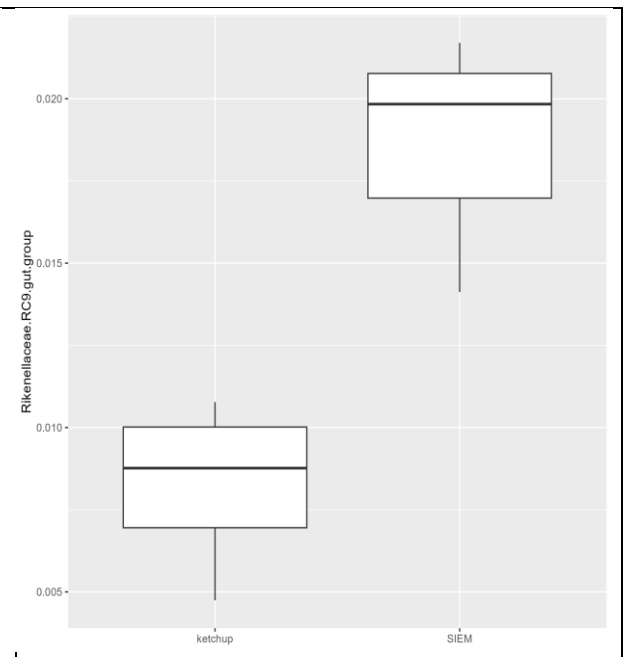
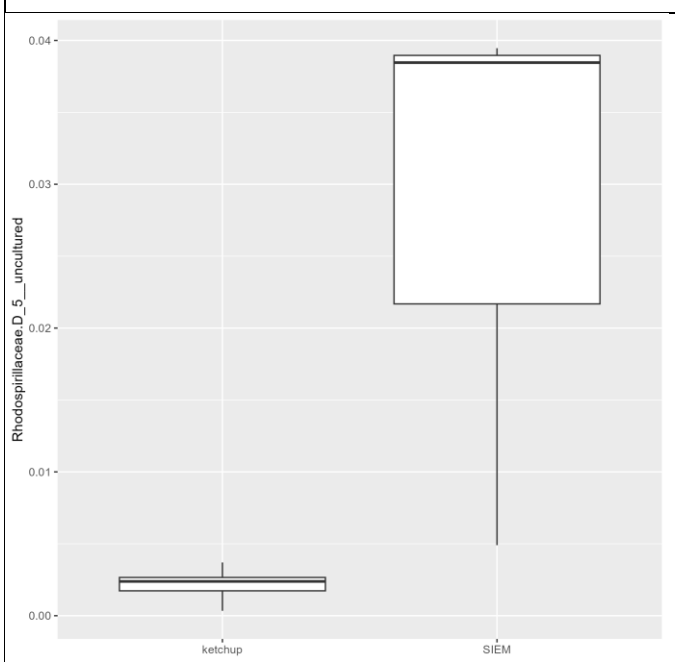
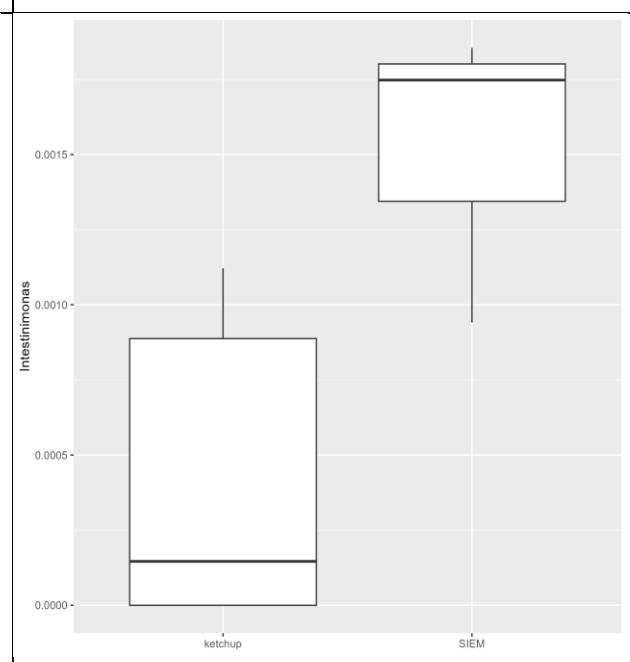
Supplementary Table 1: The relative abundance of significant difference taxa at the end of gut microbiota intervention with addition of ketchup and standard ileal efflux medium (SIEM), ($q \leq 0.2$)

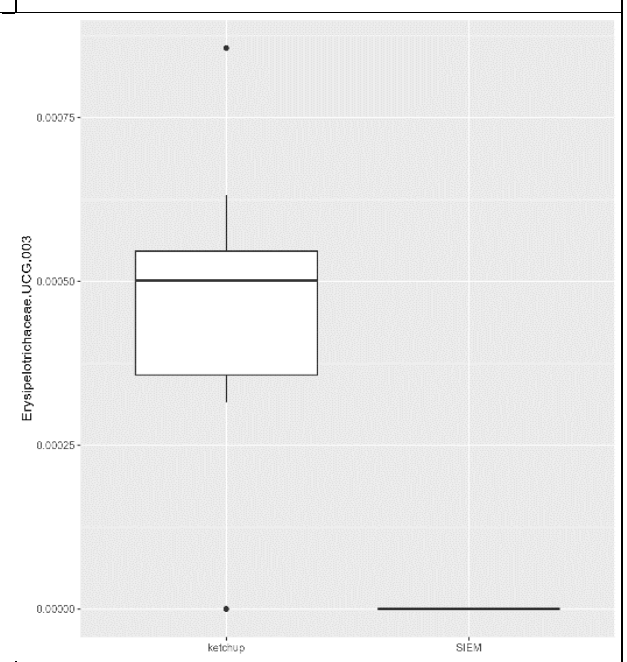


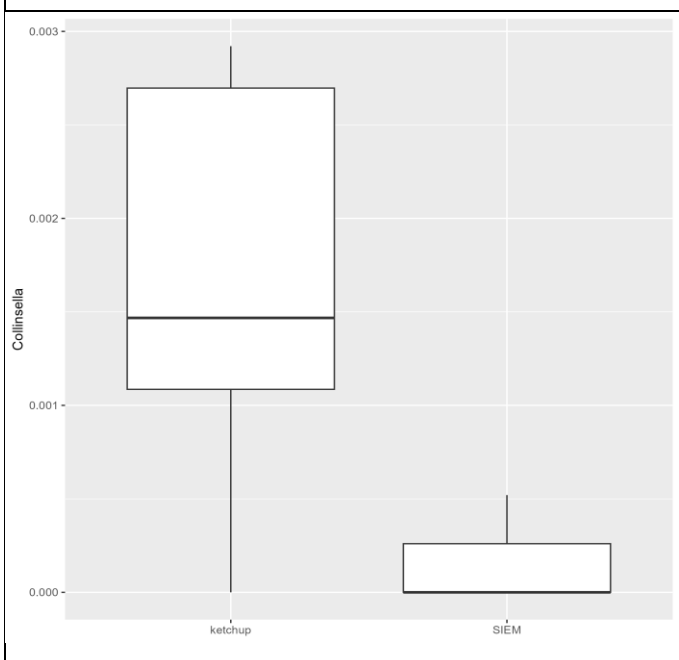


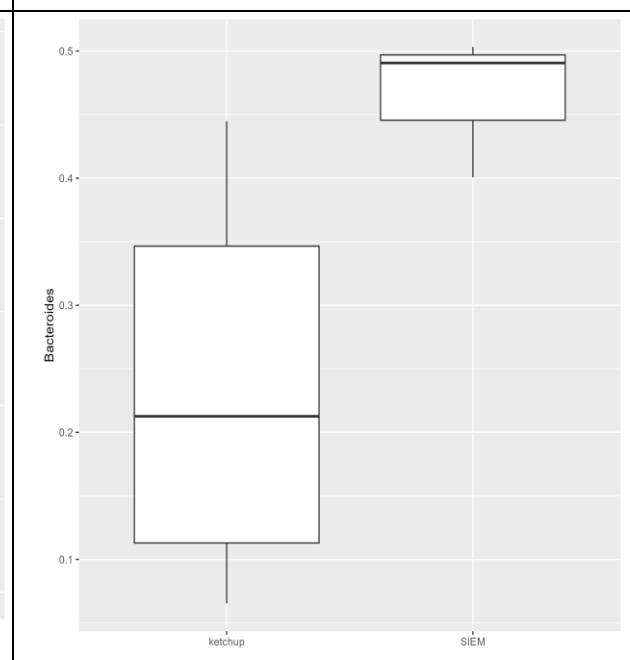


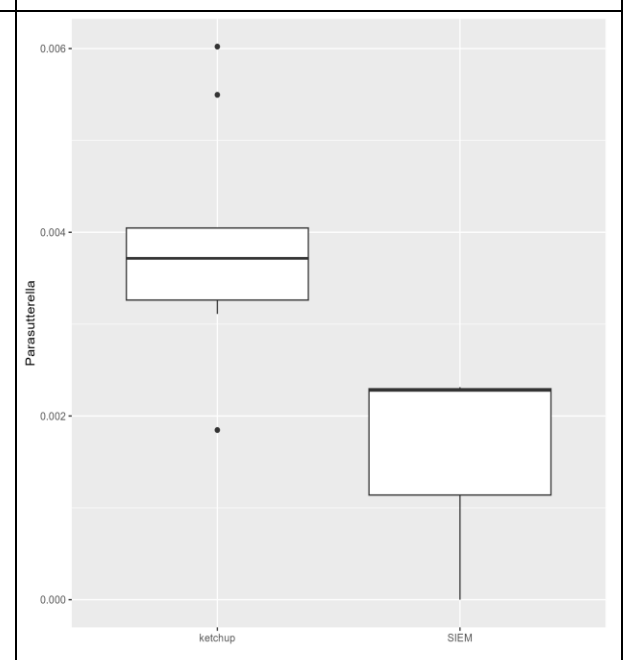












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Co-authorship statement

I hereby represent that in the thesis Küçükgöz, K., Venema, K., & Trząskowska, M. (2024b). Gut microbiota modulatory capacity of fermented ketchup in a validated in vitro model of the colon. *Food Research International*, *192*, 114801. https://doi.org/10.1016/j.foodres.2024.114801 my individual contribution in the development thereof involved Writing – review & editing, Writing – original draft, Visualization, Validation, Project administration, Methodology, Investigation, Formal analysis, Data curation, Conceptualization.

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Signature

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Gut microbiota modulatory capacity of fermented ketchup in a validated *in vitro* model of the colon

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ABSTRACT

This study aimed to evaluate the effects of fermented beetroot ketchup enriched with *Lactobacillus johnsonii* K4 and non-fermented beetroot ketchup on pooled fecal microbiota from healthy adults in *in vitro* colon model. The research focused on how these products influenced the composition and functionality of the gut microbiota, as well as metabolite production, using the validated dynamic *in vitro* colon model, TNO Intestinal Model (TIM-2). After an initial starvation phase, a single 60 g dose of predigested and freeze-dried ketchup was introduced into the model. The potential probiotic strain *Lactobacillus johnsonii* K4 was added over three days. A carbohydrate mixture of standard ileal effluent medium (SIEM) served as the control. Our analysis identified 21 bacterial taxa that were significantly modulated (q-value < 0.2) when comparing ketchup samples to control samples. Notably, the ketchup samples led to an increase in butyrate-producing taxa, including *Faecalibacterium*, *Blautia*, *Ruminococcaceae, Ruminiclostridium 6*, and *Anaerostipes*. Conversely, there was a reduction in potentially pathogenic genera *Desulfovibrio* and *Escherichia-Shigella.* Distinct profiles of short-chain fatty acids (SCFA) were observed among the fermented ketchup, non-fermented ketchup, and control samples. Non-fermented ketchup resulted in higher proportions of acetate, propionate, and butyrate compared to the other interventions. This may be related to the fermentation with lactic acid bacteria in fermented samples with lower levels of substrate for SCFAs production. However, fermented ketchup sample has higher relative abundance of beneficial bacteria like *Lactobacillus, Weissella* and *Dorea* in gut microbiota. These findings suggest that beetroot ketchup, can positively influence gut microbiota composition and function, highlighting its potential benefits for human health.

1. Introduction

Studies focused on the composition and activity of the gut microbiota discovered a close relation to different aspects of human health, including immune function, metabolism, and even neurological processes (Ogunrinola et al., 2020).Therefore, scientists have become interested in dietary factors that can positively impacts on gut microbiota (Nova et al., 2022). Fermented food products are commonly used for their potential probiotic effects, improved nutritional qualities and sensory aspects. Lactic acid fermentation is one of the most frequent fermentation types. This process includes the conversion of sugars into lactic acid by lactic acid bacteria, such as *Lactobacillus* and *Leuconostoc* spp., which can significantly influence the chemical composition of the food product (Soemarie et al., 2021). Fermented food products could have an impact on gut microbiota modulation, which in turn has significant implications for human health.

A recent longitudinal study of a subset of American Gut Project participants found differences in microbiota composition and faecal metabolome among fermented food consumers vs. non-consumers (Taylor et al., 2020). These products led to an increase in beneficial members of the gut microbiota such as lactic acid bacteria and bifidobacteria (Patel et al., 2023). These microorganisms help the fermentation of non-digestible carbohydrates and produce SCFAs as metabolic by-products (Morrison & Preston, 2016). SCFAs, specifically acetate, propionate, and butyrate, are important for maintaining gut health. They can serve as an energy source for the cells in the colon, regulate

Abbreviations: SCFAs, Short-chain fatty acids; ASVs, Amplicon order modifications; RA, Relative abundance; SIEM, standard ileal effluent medium; *L*, Lactobacillus; rRNA, ribosomal ribonucleic acid.

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electrolytes, and contribute to maintain a slightly acidic pH in the gut, inhibiting the growth of harmful bacteria (den Besten et al., 2013). As indicated, consuming fermented food products induces changes in the gut microbiota composition, with regular intake enhancing the abundance of beneficial bacteria, promoting a balanced and diverse gut microbiota. This shift is crucial because imbalances in gut microbiota have been linked to various diseases, including inflammatory bowel diseases and metabolic disorders (Zhang, 2022). Furthermore, fermentation can increase the bioavailability of polyphenols and other bioactive compounds of food products as enzymes produced by microorganisms break down complex compounds (Yang et al., 2023).

In recent years, fermented non-dairy food products have gained special popularity because their food matrix can be a potential carrier of probiotics and act as an alternative to animal-based probiotic foods and sustainability (Küçükgöz & Trząskowska, 2022). For example, red beetroots, commonly found in Central and Eastern Europe have a rich nutrient profile, including sugars, phenolic compounds, carotenoids, flavonoids, and betalains. Besides, red beetroots are classified as "functional foods" due to their health-promoting properties (Chhikara et al., 2019). Another study investigated the effects of red beetroot as a dietary supplement in weaned pigs. The findings suggest that red beetroot has potential as a natural alternative for improving gut microbiota and metabolite production though optimal dosing and composition require further exploration (Adekolurejo et al., 2023).

The earthy flavour of these foods prevents consumer acceptance, so fermentation methods are being explored in order to enhance their sensory appeal and nutritional value (Casciano et al., 2022). Moreover, the food matrix properties of tomatoes help the growth of microorganisms for fermentation as it is a natural source of sugar, vitamins, polyphenols and flavonoids (Ricci et al., 2020). Furthermore, lactic acid bacteria can be part of the tomato fruit's natural microbiota, and many authors have examined the potential use of autochthonous bacterial strains in fermentation. The selection of bacterial strains has also been crucial to developing fermented tomato products with a suitable volatile profile, which directly impacts their taste (Pereira et al., 2023).

The effect on gut microbiota composition and metabolites can be determined with *in vitro* experiments, clinical trials or animal-based assessment. A validated model that has been extensively used to assess the diversity and composition of the microbiota in the colon is the dynamic TNO *in vitro* model (TIM-2). This dynamic model of the proximal colon contains components such as dialysis system, temperature control, and peristaltic movements to regulate and closely simulate conditions in the large intestine, which are validated, and computer controlled. Specifically, the dialysis system prevents microbial metabolites from accumulating, which would otherwise inhibit the microbiota, allowing the model to realistically mimic the human proximal colon (Venema, 2015).

This research explores the multifaceted effects of the developed beetroot ketchup (fermented or not), focusing on its potential to modulate the gut microbiota composition and SCFAs production by utilizing the TIM-2 simulated digestion system. These findings will contribute to our understanding of the role of fermented foods in promoting gut health and may have broader implications for human health.

2. Methodology

2.1. Preparation of fermented ketchup

L. johnsonii K4 was sourced from the Department of Food Gastronomy and Food Hygiene, Warsaw University of Life Sciences, Poland (Zielińska et al., 2015, 2019). The strain, stored at −80 °C, was activated, incubated in MRS broth (pH 6.8 ± 7.2, Merck-110.660, Darmstadt, Germany), and separated from the medium through centrifugation and washed with saline to discard growth medium. The fermentation process utilized a non-dairy matrix with tomato concentrates, beetroots, garlic powder, black pepper, white sugar, and apple vinegar obtained from a Netherlands market. In the non-fermented sample (KET), ingredients comprised 100 g tomato concentrate, 30 g beetroots, 1 g garlic powder, 0.3 g black pepper, 5 g sugar, and 1 g apple vinegar. For the ketchup sample with *L. johnsonii* K4 (KT4), the same ingredients were used, supplemented with the probiotic *L. johnsonii* K4. Fresh beetroots underwent washing, boiling, and homogenization. The mixture was pasteurized at 72 °C for 15 min, cooled, and inoculated with a 1 mL bacterial solution containing *L. johnsonii* K4 in saline. Samples were incubated for 5 h at 37 °C, followed by immediate cooling to 4 °C and storage for 24 h before subsequent procedures and analysis.

2.2. In vitro gastrointestinal batch digestion

Fermented beetroot ketchup with *L. johnsonii* K4 and non-fermented beetroot ketchup described above were transferred to an *in vitro* static gastrointestinal digestion according to (Cárdenas-Castro, Venema, et al., 2021) to gather all the required digested substrate for the colon systems in a batch. Firstly, oral digestion phase with with α-amylase from human saliva (A0521, Sigma-Aldrich, St. Louis, MO, USA) was applied. After that pepsin (P-7000, Sigma-Aldrich, ≥ 250 units/mg solid; 0.2 mL of a 300 mg/mL solution in 0.2 M HCl-KCl buffer) was added to the mixture to simulate gastric digestion for 2 h at 37 °C. To stop gastric digestion and start the intestinal phase, pancreatin (P-1750, Sigma-Aldrich) was added and incubation was continued for 5 h at 37 °C with mixing regularly. After completing the intestinal phase, the combined insoluble indigestible fraction and soluble indigestible fraction were dialyzed process for 72 h bythe (D9527-30.48 m avg. flat width 43 mm, 14000 Da, Sigma-Aldrich) and the total indigestible fraction (TIF) was collected and freeze-dried (FreeZone 6) and stored at −20 °C.

2.3. Collection and preparation of fecal samples

The microbiota pool technique used for the sample preparation as specified by (Aguirre et al., 2014). Stool samples were collected from 16 healthy individuals: 8 females and 8 males aged 23 to 50. Volunteers stated no history of gastrointestinal diseases or use of antibiotics for at least three months prior to donation. Stool samples were collected, combined and homogenized. Experiments performed in anaerobic conditions. After accumulating fecal samples snap frozen in nitrogen and stoked at −80 °C until before use. Before the inoculation, four 30 mL tubes of frozen samples were melted for individual period at 37 °C and then mixed with pre-lowered dialysate to have a total capacity of 250 mL. In the Netherlands, collecting fecal samples from individuals without prior screening or interventions does not require ethical approval. However, all donors provided informed consent.

2.4. Experimental set-up and the TIM-2 in vitro model

Each TIM-2 unit received an inoculation of 60 mL of the standardized microbiota pool, as detailed in Section 2.3 and the unit was filled with pre-reduced dialysate until 125 ml.The microbiota was adapted to the model conditions using simulated ileal effluent medium (SIEM), which replicates the indigestible portion of a high fiber diet that can reach the colon and undergo fermentation described in (Cárdenas-Castro, Venema, et al., 2021). SIEM consisted of the following components (g/L): 9.0 g pectin, 9.0 g xylan, 9.0 g arabinogalactan, 9.0 g amylopectin, 43.7 g casein, 74.6 g starch, 31.5 g Tween 80, 43.7 g bactopepton, 0.7 g oxbile, 4.7 g K2HPO4··3H2O, 8.4 g NaCl, 0.009 g FeSO4··7H2O, 0.7 g MgSO4··7H2O, 0.8 g CaCl2··2H2O, 0.05 g bile, 0.02 g haemin, and 0.3 g cysteine·HCl, along with 1.5 mL of a vitamin mixture. Subsequently, SIEM was supplied to each unit at a rate of 2.5 mL/h for a 16-hour adaptation period. Following this, after a 2 h starvation period, one unit was continuously supplemented with 60 g freeze-dried and predigested ketchup and SIEM, while another was in addition also supplemented with *L. johnsonii* K4 for 3 days. A third unit was only fed with SIEM, leading to three different conditions: i) SIEM (control), ii) SIEM+60 g freeze-dried and predigested ketchup, and iii) SIEM+60 g

freeze-dried and predigested ketchup with 0,5 mL in MRS broth (pH 6.8 ± 7.2, Merck-110.660, Darmstadt, Germany) *L. johnsonii K4* for 3 days. Samples from both the lumen and dialysate were obtained at 0, 24, 48, and 72 h and subjected to analysis for metabolite production and microbiota composition.

2.5. SCFA production

The produced SCFAs were measured by Gas chromatography–mass spectrometry *(8890 GC System, Agilent Technologies*), according to Chiu (Chiu & Kuo, 2020). Firstly, samples collected from lumen and dialysate during *in vitro* colon fermentation were centrifuged for ten minutes at 14.000 rpm. After that supernatant from 150 µL lumen sample was transferred to a glass-GC-vial containing 550 µL of internal standard solution including deionized water, formic acid, 2 mg/mL 2-ethyl butyric acid, and methanol.The temperature settings for the injector port, oven, flame ionization detectors, and mass spectrometer detector were 250 °C, 200 °C, 275 °C, and 225 °C. A constant carrier gas flow rate of 1.2 mL/min was maintained over the column. A calibration curve was used to determine concentrations. Cumulative amount of SCFAs was calculated by adding the amounts produced in the lumen and dialysate at different time points, along with their mean and range numbers.

2.6. DNA extraction

DNA was extracted from 250 µl of inoculate and luminal samples, which had been thawed at room temperature, using the Qiagen DNA extraction kit (Qiagen, Leiden, the Netherlands) following the procedure outlined by (Stolaki et al., 2019).The purity and yield of the extracted DNA were evaluated with a Nanodrop ND-1000 spectrophotometer (Nanodrop Technologies, Wilmington, USA). The DNA concentration was then adjusted to 20 ng/µl for use as a template in subsequent 16S rRNA gene PCR amplification.

2.7. Gut microbiota composition

The composition of gut microbiota was determined by sequencing the V3-V4 domain of the 16S rRNAs. The DNA samples obtained from the lumen samples was carried out following the producers's pact (Illumina, Eindhoven, The Netherlands). By using the Binary Base Call text-based format for storing biological sequences and their corresponding quality scores (FASTQ) pipeline (BCL2FASTQ, v.), the sequences were converted into text-based format, after quality checks (Illumina, San Diego, CA; released version 1.8.3). Subsequently, the Quantitative Insights into Microbial Ecology 2 (QIIME-2) program (Bolyen et al., 2019) was appropriated for study. The Silva table was used as the remark 16S ribosomal RNA table to categorize the sequences into ASVs taxonomically.

2.8. Statistical analysis

R software package (version 3.6.2) was applied for statistical analyses. Different indexes were computed to indicate alterations in bacteria composition, and the abundances of microbial species within the total microbial community were presented as RA (Relative Abundance). Non-parametric Kruskal-Wallis study was used to discover dissimilarities in variety affluence with various sample variations. The correlation between RA of ASVs and microbial metabolites was calculated using Spearman's correlation. Multiple comparisons were adjusted for using the Benjamini–Hochberg false discovery rate, and q-values (FDR-corrected p-values) were considered significant at $q < 0.20$.

3. Results

3.1. Changes in microbiota composition

Different alterations in microbiota compositions between control and ketchup added samples were observed. There were 21 taxa with significant differences (q-value < 0.2) between ketchup and control samples and there was not significant difference between KT4 and KET. Therefore, results were grouped as ketchup added samples and control. Notably, several taxa associated with gut health (butyrate production) exhibited significant increases (Fig. 1). *Blautia* (q = 0.093) was significantly higher in ketchup-added samples than in control samples. Similarly, *Faecalibacterium* (q = 0.093) and *Ruminiclostridium 6* (q = 0.093), *Ruminococcaceae UCG-013* (q = 0.173), *Ruminococcus gauvreauii group* (q = 0.190), Ruminococcaceae (q = 0.093) and *Coprococcus 1* (q = 0.190) exhibited significantly higher abundance in ketchup samples, indicating a distinct microbial profile induced by ketchup consumption. Interestingly, *Eubacterium ruminantium group* (q = 0.194) was found significantly higher in ketchup-added samples, indicating a unique response to ketchup supplementation, as it was absent in the control. *Anaerostipes* (q = 0.150) was also significantly higher in abundance in samples with ketchup, with no corresponding presence in the control samples Moreover, *Escherichia/Shigella* (q = 0.093) was not detected in ketchup-added samples, signifying its absence in the presence of ketchup and was significantly higher abundance in the control samples, where it thrived without the inhibitory effects of ketchup (Fig. 1). Similarly, *Desulfovibrio* (q = 0.093) exhibited significantly higher abundance in control samples than in ketchup-added samples. The reduction of *Desulfovibrio* in ketchup-added samples suggests that ketchup might inhibit the growth of this bacterial group. The absence of *Escherichia/Shigella* and *Desulfovibrio* in ketchup-added samples may be due to antimicrobial properties or unfavorable conditions for pathogenic bacteria created by components of ketchup. Additionally, it can be possible that ketchup could lower the pH, creating an environment less favorable for pathogenic bacteria. These findings highlight the interactions between ketchup and gut microbiota, elucidating specific taxa that are either promoted or inhibited in the presence of ketchup, showing the impact of ketchup consumption on the gut microbial community.

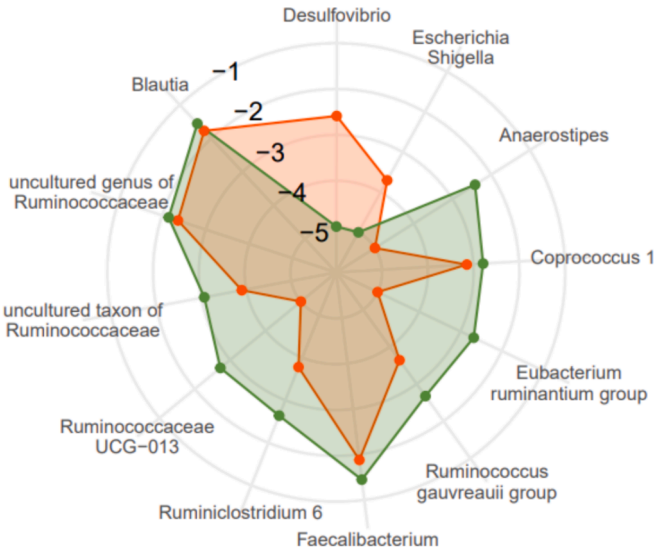

Fig. 1. Relative abundance of selected bacteria species (several butyrate producers and the two potential pathogenic genera) after supplementation with standard ileal efflux medium (control; red points) or 60 g predigested ketchup (green points), Log10 relative abundance (plotted as − 5 when taxon was absent).

Fig. 2 provides a separate view of specific taxa that in three different samples without significant differences *Lactobacillus* demonstrated an increase, particularly in fermented ketchup samples with *L. johnsonii K4*, followed by ketchup-supplemented samples, indicating a potential role of ketchup in fostering the growth of lactic acid bacteria. In addition, *Weissella* has higher RA in fermented ketchup sample while it was not found in non-fermented and control sample. The relative abundance of *Dorea* was higher in fermented ketchup samples than in control samples at the end of the experiment.

In the analysis of relative abundance, other significant differences for several bacterial taxa were also observed (shown in the supplementary material) between the control (SIEM) and predigested ketchup samples. *Victivallales* (q = 0.01), *Rikenellaceae* (q = 0.09), *Rhodospirillaceae (D-5-uncultured)* (q = 0.09), and *Bacteroides* (q = 0.173) exhibited significantly higher relative abundances in the control group compared to samples with ketchup supplementation. Additionally, *Parasutterella* (q = 0.173) and *Catenibacterium* (q = 0.09), *Intestinimonas* (q = 0.130) *Anaerostipes* (q = 0.150) *Erysipelotrichaceae UCG-003* (q = 0.150), and *Collinsella* (q = 0.173) were significantly higher in samples with ketchup, underscoring the impact of ketchup supplementation on the relative abundance of these bacterial taxa. These findings highlight the specific alterations in the relative abundance of these bacterial taxa in response to ketchup consumption, showed the complex interactions between ketchup and the gut microbiota composition. However, besides composition, the activity of the microbiota is also important, which is highlighted in the next sections.

3.2. Production of SCFA

In this work, the colonic fermentation of pre-digested ketchup, predigested ketchup with *L. johnsonii* K4 supplementation or SIEM as a control led to the production of different profiles of organic acids. The samples supplemented with ketchup during colon simulation have a higher abundance of acetate, propionate and butyrate than the control sample (Fig. 3). Table 1 presents the cumulative production of acetate, propionate, butyrate, and total SCFAs after supplementation with fermented ketchup, non-fermented ketchup, and control (SIEM) in TIM-2 at 72 h. The acetate: propionate: butyrate molar ratios exhibited distinct profiles for each sample. Non-fermented ketchup resulted in mixtures containing the major proportion of acetate, propionate, and butyrate. In contrast, SIEM was identified as the least butyrate-producing substrate. The cumulative SCFA concentrations at 72 h highlight the variability in fermentation outcomes associated with different interventions, underscoring the impact of substrate composition on the metabolic profile in the TIM-2 system.

Numerous intricate cross-feeding mechanisms contribute to the synthesis of SCFAs. To explore these interactions, Spearman correlations were conducted between SCFA concentrations and specific taxa at the genus level.The remarkable correlations are shown in Fig. 4. Among the major SCFAs, acetate exhibited significant correlations with all taxa in Fig. 4, except with the *Christensenellaceae R-7* group, *Clostridiales* family, and *Eubacterium rumiantium* group. *Blautia* and *Ruminoclostridium 6* were positively correlated with both acetate and butyrate production, while *Bacteroides* displayed a negative correlation with these two SCFAs. Interestingly, propionate production did not exhibit significant correlations with any specific taxa.

4. Discussion

4.1. Gut microbiota composition

This study provides a detailed analysis of the interactions between ketchup consumption and gut health by examining the change in composition of gut microbiota resulting from predigested ketchup supplementation.

Several of the taxa that are stimulated are known for their beneficial roles in gut health, fermentation of dietary fibers, and production of SCFAs, particularly butyrate, indicating a potential positive influence of ketchup on gut microbial functionality. The observed substantial increase in these butyrate-producing bacteria, including *Faecalibacterium*, *Ruminococcaceae*, and *Ruminococcus gauvreauii* group, signifies a potential enhancement of gut health due to ketchup consumption. The elevation of these butyrate producers in response to ketchup supplementation underscores the potential positive impact of predigested ketchup on gut mucosal health, since butyrate has been shown to increase the barrier function of the colonic gut wall, as well as induce an increase in mucus production (Genua et al., 2021; Singh et al., 2023). In another study, fermented tomato was found to promote the growth of beneficial bacteria such as *Bifidobacterium* and *Akkermansia, Roseburia*, *Coprococcus*, *Oscillospir*a, and *Ruminococcus*, all known for their beneficial properties within the gut microbiome (Wei et al., 2024). In another research, beet-derived pectin and pectin-derived oligosaccharides have emerged as potential prebiotics, showing modulatory effects on gut microbiota composition. Specifically, they have been noted to increase populations of beneficial bacterial species like *Faecalibacterium prausnitzii* and *Roseburia intestinalis* (Gullón et al., 2013). *Faecalibacterium* is known for its positive impact on gut health and has been associated with various health benefits (Maioli et al., 2021). *Blautia* contributes to fermentation of dietary fibers and the production of short-chain fatty acids (SCFAs) and with these features it supports gut health, the immune

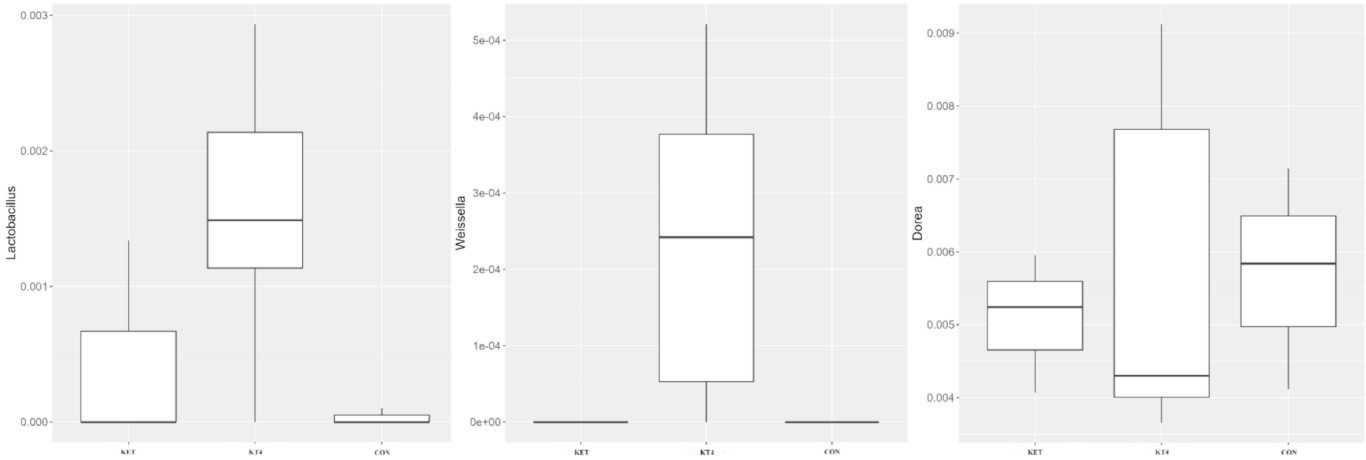

Fig. 2. The relative abundance of *Lactobacillus*, *Weissella* and *Dorea* at the end of gut microbiota intervention with addition of KT4; fermented ketchup; KET; Non-fermented ketchup and CON; (standard ileal efflux medium (SIEM).

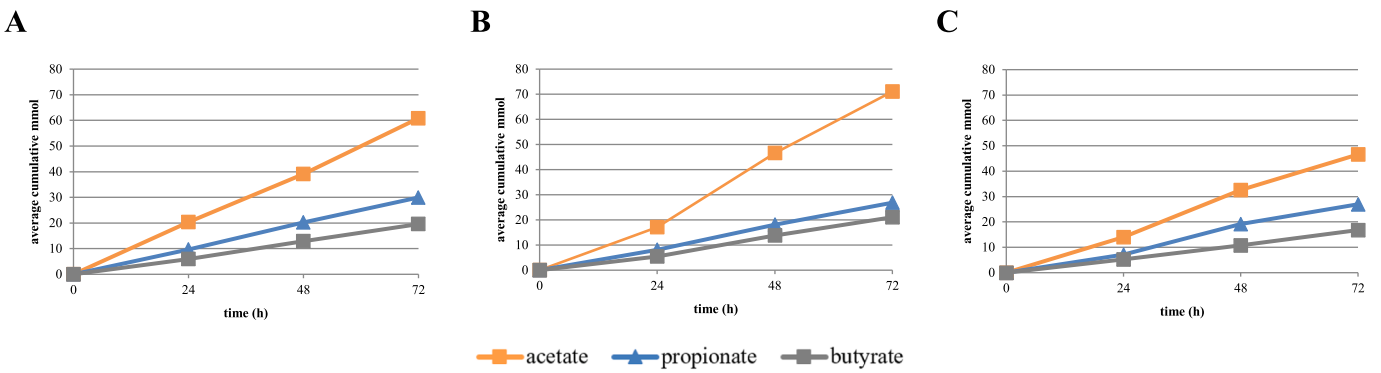

Fig. 3. Cumulative production (mmol) of the SCFAs; acetate, propionate, and butyrate at 24 h, 48 h, and 72 h after supplementation with (**A**) KT4; fermented ketchup; (**B**) KET; Non-fermented ketchup, (**C**) CON; (standard ileal efflux medium (SIEM).

Table 1
Cumulative production (mmol) of acetate, propionate, butyrate and total SCFAs present in these metabolites after supplementation with KT4, KET and CON *in vitro* dynamic fermentation in the TIM-2 system.

Intervention	Acetate	Propionate	Butyrate	Total SCFAs
KT4	60.83	29.95	19.57	124.86
KET	71.08	26.85	21.07	133.90
CON	46.58	26.96	16.80	107.69

KET; non-fermented ketchup, KT4; ketchup with *L. johnsonii K4* fermentation, *CON;* SIEM.

system, and metabolic processes (Liu et al., 2021) Conversely, *Desulfovibrio*, a hydrogen sulfide (H_2S) producer associated with gastrointestinal disorders, was reduced in ketchup samples. Elevated levels of *Desulfovibrio* and H_2S have been linked to inflammatory bowel diseases (S. B. Singh & Lin, 2015). *Escherichia-Shigella*, which contains potential pathogenic species, was completely absent in ketchup samples.

Fermented ketchup samples enriched with *L. johnsonii* K4 exhibited an enhanced presence of *Lactobacillus* in the gut microbiota, alongside increased relative abundance of *Weissella* and *Dorea*. Notably, *Lactobacillus* and *Dorea,* as symbiotic bacteria, displayed reduced levels of secretory immunoglobulin A, versus potential gut pathogens, as highlighted in studies (Krishnamurthy et al., 2023). *Weissella* species are also known for their beneficial effects due to their roles in food fermentation, where they contribute to flavour development and preservation. Certain *Weissella* strains also show potential as probiotics, offering benefits such as antimicrobial activity and promoting gut health through the production of bioactive compounds like prebiotics (Abriouel et al., 2015).

Similarly, in other research, administration of fermented beetroot juice in animal studies positively modulated cecal microbiota and metabolic parameters. It notably increased populations of beneficial bacteria like *Lactobacillus* spp. and *Bifidobacterium* spp., while decreasing harmful bacterial groups such as *Clostridium* spp. and *Enterobacteriaceae.* This led to increased short-chain fatty acid contents in feces, indicating potential benefits on gut health (Klewicka et al., 2015). Some studies have investigated the effects of beet consumption on gut microbiota composition, suggesting a prebiotic-like effect. Prebiotics are selectively utilized substrates by microorganisms in the gut microbiota, providing various health benefits to the host (de Oliveira et al., 2021). Pectin and pectin-derived oligosaccharides have been identified as prebiotics due to their ability to modulate gut microbiota composition, particularly by increasing the populations of *Faecalibacterium prausnitzii* and *Roseburia intestinalis* (Gullón et al., 2013).

Tomato consumption was shown to positively affect the gut microbiota composition. Studies using piglets as a model organism have found that short-term tomato consumption can lead to a microbial shift, including a higher ratio of *Bacteroidetes* and *Firmicutes* with higher alpha-diversity, indicating a shift to a more desirable gut microbiota phenotype (Goggans et al., 2022). In a rat model of non-alcoholic fatty liver disease, tomato juice intake affected alterations in certain intestinal bacterial groups, such as increasing the abundance of *Lactobacillus* and improving metabolic symptoms (García-Alonso et al., 2017). Moreover, the use of sugar beet with pectin having higher fractions of rhamnogalacturonan and neutral sugars (at 7.5 g) in a fermentation model using a TIM-2 colon model resulted in increased populations of specific bacterial species. These included *Oscillospira*, *Blautia*, *Dorea*, *Ruminococcus*, *Coprococcus*, *Lachnospiraceae*, and *Clostridiales* within the phylum *Firmicutes*, as well as *Paraprevotella*, *B. uniformis*, *B. ovatus*, *P. distasonis,* and *Prevotella* within the phylum *Bacteroidetes* (Larsen et al., 2019). The observed decrease in *Intestinimonas* in ketchup-supplemented samples, in contrast to increased *Anaerostipes, Erysipelotrichaceae UCG-003,* and *Collinsella*, signifies the specificity of ketchup's impact on the gut microbiota composition. Conversely, the higher abundance of *Anaerostipes* and *Collinsella*, recognized as butyrate producers and contributors to gut health, reflects a favorable shift in the microbial community

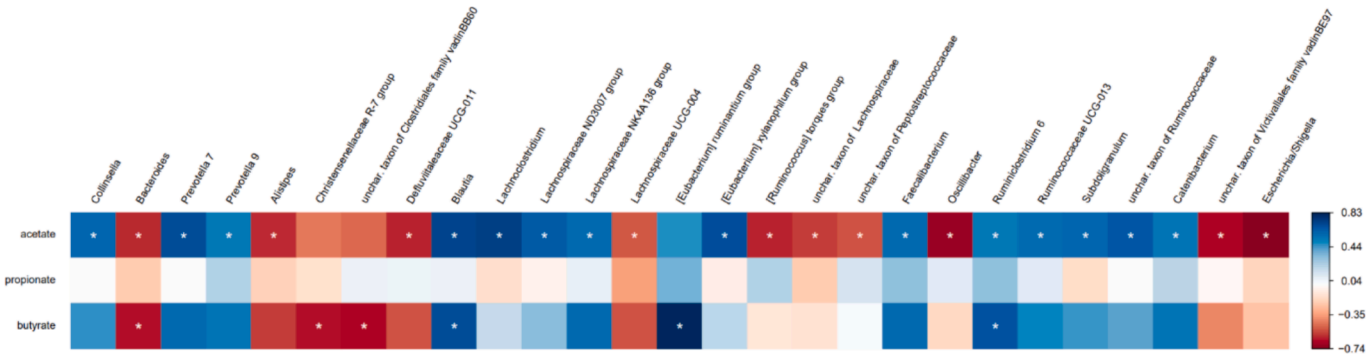

Fig. 4. Correlation between metabolite production and ASVs at genus level. White asterisks (*) $q \leq 0.2$; blue: positive correlation; red: negative correlation.

composition (Bailén et al., 2020; Qin et al., 2019).

4.2. SCFAs analysis

SCFAs such as acetate, propionate, and butyrate have gained attention due to their potential health benefits. Acetate, recognized as a significant energy source for the liver and a contributor to adipose tissue lipogenesis, has been associated with regulating appetite, enhancing fat oxidation, and improving metabolism, particularly in overweight individuals (González Hernández et al., 2019). Propionate, acting as a precursor for gluconeogenesis and exerting satiety signaling effects alongside immune-modulatory properties, holds promise for impacting metabolic homeostasis and regulating inflammation (Pang et al., 2023). Meanwhile, butyrate, a crucial SCFA produced during the fermentation of dietary fibers, is known for its anti-inflammatory characteristics, and fortifying the intestinal barrier (Liu et al., 2018). Moreover, butyrate, identified as the preferred energy source for colonocytes, exhibits multifaceted benefits, including anti-cancer properties by prompting the differentiation of transformed cells (Bekebrede et al., 2021).

The correlations observed between specific microbial taxa and butyrate production underscore the important role played by microbial communities in synthesizing SCFAs. The diversity of functions attributed to these SCFAs shows that the observed variability in SCFA production from our study might have impacts for health outcomes. Based on an *in vitro* dynamic fermentation system (TIM-2), molar ratios of acetate, propionate, and butyrate were determined after 72 h of the introduction of fermented ketchup, non-fermented ketchup, and control substrates. Fermented ketchup produced lower SCFA compared to non-fermented ketchup since the potential probiotic strain included in the fermented ketchup most probably consumed fermentable nutrients so that lower fermentation substrates were available to the microbiota. As a result, non-fermented ketchup produced a well-balanced mix with considerable amounts of acetate, propionate, and butyrate, whereas the control substrate (SIEM) produced the least amount of butyrate (Fig. 3). These findings underscore the substantial influence of substrate composition on the metabolic profile within the TIM-2 system. A study administering beetroot fermented juice observed increased fecal SCFA content in rats, coupled with decreased activity of α-glucosidase and β-glucuronidase. These changes suggested a reduction in post-prandial hyperglycemia and the enterohepatic circulation of toxic compounds, indicating positive effects on cecal microbiota and metabolic parameters in rats consuming the beet juice fermented by probiotic *L. casei* and *L. brevis* (Klewicka et al., 2015).

In another research con TIM-2 colon systems, fermentation of SIEM, raffinose, and tomato varieties, including both regular and husk varieties, resulted in varied SCFA production profiles over 24 and 48 h. Acetic acid was the predominant SCFA produced across all substrates, with significantly higher levels observed in tomato substrates compared to SIEM and raffinose, while propionic and butyric acid levels varied between substrates and fermentation times (Cárdenas-Castro, Zamora-Gasga, et al., 2021).

In our research, correlations between specific taxa and SCFA concentrations showed while acetate did not display significant correlations with certain taxa, we identified associations between *Blautia* and *Ruminoclostridium 6*, positively linked to acetate and butyrate production. Conversely, *Bacteroides* exhibited a negative correlation with these SCFAs. Additionally, propionate production did not exhibit significant correlations with specific taxa, highlighting the complexity of SCFA synthesis and microbial contributions. Similar investigations exploring the impact of beetroot consumption revealed correlations between SCFAs and genera such as *Blautia*, *Collinsella*, *Lachnobacterium*, and *Lactobacillus*, with an inverse association noted with *Ruminococcus*, further underlining the diverse contributions of different microbial groups to SCFA production (Wang et al., 2023).

5. Conclusion

Tomatoes are popular all around the world for their flavor and health benefits. Incorporating beetroot into tomato-based ketchup sauces can increase nutritional value and improve gut microbiota composition. This presents opportunities for product development and innovation. Our study explores the impact of fermented beetroot ketchup enriched with *L. johnsonii* K4 and non-fermented beetroot ketchup on the gut microbiota. The examination of microbiota composition showed important changes induced by ketchup addition, which was performed using the TNO Intestinal Model (TIM-2) to closely simulate colonic fermentation. A significant increase in beneficial taxa like *Faecalibacterium*, *Blautia*, *Ruminococcaceae*, *Ruminiclostridium 6*, and *Anaerostipes*, known for their butyrate production, proves health effects of tomato-based ketchup with beetroot. Conversely, reducing potentially pathogenic species like *Desulfovibrio* and *Escherichia-Shigella* in ketchup-added samples indicates inhibitory effects on these microbes, further underlining the potential health benefits. On the other hand, fermented ketchup samples enriched with *L. johnsonii* K4 effectively modulate gut microbiota composition by promoting the presence of beneficial bacteria like *Lactobacillus, Weissella,* and *Dorea* which are associated with enhanced gut health compared to non-fermented or control samples.

Moreover, the production of SCFAs demonstrated that ketchup supplementation led to higher concentrations of acetate, propionate, and butyrate compared to the control. These SCFAs play crucial roles in maintaining gut health and function, acting as an energy source and regulating gut pH to inhibit the growth of harmful bacteria. Additionally, the correlation between specific bacterial species and metabolites further emphasized the intricate interplay within the gut microbiota, giving further insight into potential associations with human health. The findings highlight ketchup's capacity to promote beneficial bacteria, inhibit potentially harmful species, and influence the production of SCFAs. We can gain a better understanding of the role of plant-based foods in promoting gut health and potentially impacting human well-being if we examine these dynamics.

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CRediT authorship contribution statement

Kübra Küçükgöz: Writing – review & editing, Writing – original draft, Visualization, Validation, Project administration, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Koen Venema:** Writing – review & editing, Supervision, Resources, Project administration, Investigation, Formal analysis, Data curation, Conceptualization. **Monika Trząskowska:** Writing – review & editing, Supervision, Conceptualization.

Declaration of competing interest

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

Data availability

Data will be made available on request.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.foodres.2024.114801.

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Supplementary Table 1: The relative abundance of significant difference taxa at the end of gut microbiota intervention with addition of ketchup and standard ileal efflux medium (SIEM), ($q \leq 0.2$)