DOCTORAL THESIS

Diversity and stability of microbial consortia in fermented plant-based products

Maret Andreson

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Declaration:
Hereby I declare that this doctoral thesis, my original investigation and achievement, submitted for the doctoral degree at Tallinn University of Technology has not been submitted for doctoral or equivalent academic degree.

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MARET ANDRESON

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Contents

List of Publications

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Contribution to the papers in this thesis are:

I The author participated in the design of the study, experimental work and in data interpretation. She wrote the manuscript draft and participated in the editing process. Also, she handled the review and editing process for the Publication.

II The author conceptualized the study, designed the experiments, analyzed the data and wrote the original manuscript draft and participated in the editing part. Also, she handled the review and editing process for the Publication.

III The author participated in the study design, conducted the experimental work and participated in the data interpretation. She wrote the original draft of the manuscript and handled the review and editing process for the Publication.

Introduction

Humans have ingested fermented foods for centuries. Fermentation helps to make food safe for consumption, prolongs shelf-life, and improves digestibility. Additionally, it provides desirable organoleptic properties to the final product. Fermentation also removes undesirable components such as phytates and phenolic compounds. Today, the consumption of fermented foods is part of people's daily diet. The intake of fermented products supports human health, for example by strengthening the immune system.

Traditional fermented foods contain microorganisms originating from raw ingredients, kitchen utensils, and the surrounding environment. During fermentation, those microbes that are best adapted to the available substrates gradually become dominant. Over centuries, this spontaneous selection has shaped the stable mixed communities of bacteria, yeasts, and molds characteristic of traditional fermentations. The microorganisms involved in fermented food production primarily include lactic acid bacteria (LAB), acetic acid bacteria (AAB), and *Bacillus* species; and additionally, various yeast and mold species. These naturally assembled consortia underpin products such as yogurt, sourdough, wine, kimchi, and kombucha. However, these traditional fermentation methods are now used less frequently because their outcomes can be unpredictable. Modern production relies on defined starter cultures, where carefully selected strains with known technological properties are used to initiate fermentation and ensure consistent product quality.

The growing global population and novel healthy trends have increased the demand for high-protein foods. Traditional dairy and meat systems alone cannot sustainably support this rising demand due to their substantial ecological footprint. Expanding animal-based protein production requires more land for feed crops, which drives the conversion of forests, wetlands, and natural grasslands into agricultural areas. These land-use changes contribute to higher greenhouse gas emissions, reduced biodiversity, and the loss of essential ecosystem services. Growing awareness of environmental limits has placed greater emphasis on conserving natural resources and protecting ecosystems. Therefore, the development of sustainable, health-promoting fermented plant-based alternatives has become indispensable.

Transferring conventional dairy-processing methods to plant-based matrices introduces specific technological and sensory challenges. For example, Scandinavian crops (oat and pea) often have strong background flavors, which is sometimes perceived as astringent by consumers. Fermentation can offer a promising way to mitigate these sensory limitations.

For centuries, artisan fermentations have relied on well-defined cultures. Lactic acid bacteria form the core of these cultures due to their essential metabolic functions. Dairy fermentation commonly uses species from *Lactococcus*, *Lactobacillus*, *Streptococcus*, *Leuconostoc*, and related genera, forming the basis of yogurt and probiotic cultures. Plant-based fermentations, however, often rely on the same dairy-derived cultures, which perform inconsistently in non-dairy substrates and may generate undesirable aromas, especially when additives are present. Selection of autochthonous plant-associated LAB—such as *Lactiplantibacillus plantarum*, *Leuconostoc mesenteroides*, *Leuconostoc citreum*, and *Weissella cibaria*—should offer a promising strategy for improving the sensory and technological quality of fermented plant-based products. Therefore, development of novel dairy-free starter cultures is a turning point in dairy alternative production, where consortium stability and functionality can only be

improved by studying the growth dynamics, metabolism, and fermentation procedure optimization.

This thesis examines how microbial consortia form, behave, and adapt in fermented plant-based environments. One part of the work focuses on kombucha, using it as a model system to explore the structure, diversity, and stability of a naturally assembled fermentation community. Through this, the study considers how such consortia develop over time and how their composition can shift under different conditions. The second part of the thesis turns toward the development of new non-dairy starter culture candidates, concentrating on plant-associated microorganisms and their ability to adapt to new substrates. These isolates were characterized to understand their potential roles in future plant-based fermentations, and preliminary genomic insights were gathered to support their evaluation. Together, the research connects natural fermentation ecosystems with the broader goal of improving plant-based fermented products.

Abbreviations

AAB	Acetic acid bacteria
ackA	Acetate kinase
ADI	Arginine deiminase pathway
alsS, aldC	Acetolactate synthase/decarboxylase system
alsS, aldC, butA	Acetolactate pathways
AMP	Antimicrobial peptides
ANI	Average nucleotide identity
arcA	Arginine deiminase
arcB	Ornithine transcarbamylase
arcC	Carbamate kinase
ARG	Antimicrobial resistance genes
A_w	Water activity
BS	Backslopping
cysK, metC, mgl	Sulfur amino acid degradation genes
EFSA	European Food and Safety Authority
EMP pathway	Embden-Meyerhof-Parnas pathway
EPS	Exopolysaccharides
FAO	Food and Agricultural Organization of the United Nations
FDA	Food and Drug Administration
GRAS	Generally recognized as safe
HPLC	High-performance liquid chromatography
ICE	Integrative conjugative element
IMC	Isothermal microcalorimetry
LAB	Lactic acid bacteria
LC-MS	Liquid chromatography–mass spectrometry
ldhD	D lactate dehydrogenase
lip, est, fadA, fadB	Fatty acid catabolism genes
M92	Trypticase soy yeast extract medium
MALDI-TOF MS	Matrix-Assisted Laser Desorption/Ionization Time-of-Flight Mass Spectrometry
MRS	De Man–Rogosa–Sharpe
NGS	Next generation sequencing
Od1	Oat drink 1
Od2	Oat drink 2
Omics	Omic methods
pdc	Pyruvate decarboxylase
pdc, adhE	Pyruvate decarboxylation
PDA	Photodiode array

pfl	Pyruvate formate lyase
PMAxx	propidium monoazide (improved)
pox, pta	Pyruvate oxidase pathways
QDA	Quantitative Descriptive Analysis
qPCR	Quantitative real-time PCR
QPS	Qualified Presumption of Safety
Rep protein	Replication protein
rRNA	Ribosomal RNA
SCOBY	Symbiotic Culture of Bacteria and Yeast; cellulose pellicle
spp./subs.	Subspecies
TA	Titratable acidity
TCA cycle	Tricarboxylic acid cycle
UPLC	Ultra performance liquid chromatography
v/v	Volume per volume
w/v	Weight per volume
WGS	Whole-genome sequencing
WHO	World Health Organization
xpk/xfp pathway	Phosphoketolase pathway

Explanations of abbreviations used in the thesis.

1 Literature review

1.1 History of fermented foods and beverages

It is widely recognized that each community possesses a distinctive food or dietary culture that reflects its heritage and the socio-cultural aspects of its ethnicity. Each culture has its own characteristic and unique foods and beverages due to the influence of many factors such as environment, geographical location, the accessibility of plant or animal sources, and taste preference. For centuries, people have eaten either raw ingredients from nature or processed them with various techniques that help to make food more delicious, safe and easier to consume. Across the world, there are three main traditional dietary patterns, distinguished by specific main cereal. Firstly, Eastern food culture is based on cooked rice. Secondly, the Western and Australian food culture centers on wheat— and barley-based breads. Thirdly, South America and Africa stations are based on sorghum/maize porridges. [1]

The Western and Australian food culture has wheat or barley as the dominant food, followed by milk and fermented milk products such as cheese, yogurt, curd; meat and meat products for example sausages and hams; and fermented drinks like wine and beer. South American dietary culture includes fermented maize, meat and milk products, and alcoholic beverages. In Africa, fermented maize, sorghum, millets, cassava, wild legume seeds, tubers, meat and milk products, and alcoholic beverages are part of the diet [2]. Originally, the animal milk drinking cultures were Europeans, Semites, Indians, and the nomadic tribesmen of North Central Asia. In the culinary traditions of Far East Asia, the soybean—titled as the “cow of China”—is a main crop processed into soy drink, tofu (soy curd), and many traditional fermented foods, including miso, shoyu, natto, kinema, thua nao, douchi, chungkokjang, tempeh, and sufu [3].

The fermentation of raw materials of plant and animal origin has been practiced for centuries. Therefore, fermented foods and beverages are undoubtedly key components of many cultural food traditions. Fermentation makes food safe for consuming, prolongs the shelf-life and makes it more digestible and gives good organoleptic properties to the final product. Nowadays, the consumption of fermented foods is part of people's daily intake. Also, it is cost effective for industries to ensure food and beverages safety [4]. Fermentation removes undesirable components such as phytates, saponins, tannins, vicine and convicine, and phenolic components [1]. The consumption of fermented foods improves immune function, enhances vitamin bioavailability, and increases the intake of antioxidant compounds. [4]

Numerous researchers have examined the biochemistry, microbiology, and nutritional aspects of fermented foods and beverages across various regions, including Europe, Asia, Africa, North and South America. Numerous studies have shown that many microbial genera and species are associated with a wide variety of fermented foods and beverages. [2]

Traditional fermented foods harbor various microorganisms such as molds, yeast and bacteria that come from the environment. Microorganisms also originate from the ingredients (whether plant- or animal-based), as well as from utensils, containers, and the surrounding environment. These microbes become dominant through natural selection based on their ability to thrive on the substrates [4].

Global fermented foods can be classified based on the substrate into nine categories: (1) fermented milk products, (2) fermented vegetables, (3) fermented cereals, (4)

fermented legumes, (5) miscellaneous fermented products, (6) fermented roots/tubers, (7) fermented, dried and smoked fish products, (8) fermented and preserved meat products, and (9) alcoholic beverages [1], [2], [5].

1.2 Microorganisms in traditional fermented foods and beverages

For centuries, humans have relied on spontaneous fermentation to preserve food and beverages. Natural fermentation is one of the oldest methods of food processing, involving the spontaneous fermentation of raw or heat-treated substrate, or the use of the backslopping (BS) method [2]. Traditional fermented foods, which are made using spontaneous fermentation, are often symbiotic communities with bacteria and yeasts/molds. Yeast-bacteria symbiotic fermentation is used to produce foods, such as kefir, sourdough, wine, kimchi, and kombucha [2], [6], [7]. Foods produced with a symbiotic community of mold and bacteria are, for example, tempeh (*Rhizopus* spp. and bacteria) and blue cheese (*Penicillium* spp. and LAB) [7]. Sake [8] and miso [2] are fermented with a combination of mold, yeast and LAB.

During wild fermentation, the best-adapted microorganisms become dominant. One example is backslopping propagation, which involves transferring a portion of a previously fermented batch into fresh medium to initiate the next fermentation cycle. However, traditional fermentation methods are not so common nowadays due to their unpredictability. In modern production, fermentation is no longer left to spontaneous processes. Instead, manufacturers rely on defined starter cultures—carefully selected strains chosen for their predictable performance—to initiate fermentation and ensure consistent product quality [9]. These starter cultures ensure product safety, quality, and sensory consistency. Nevertheless, several artisanal practices and traditional crafts continue to employ spontaneous fermentation methods. Traditional fermented foods have a long history of consumption; therefore, these foods are safe to consume according to the European Food and Safety Authority (EFSA) [10]. EFSA have also generated a Qualified Presumption of Safety (QPS) list, where they have indicated which microorganisms are suitable to use for food production. The microorganisms involved in fermented food production primarily include bacteria, such as LAB, AAB, and *Bacillus* species, along with various species of yeasts and molds. In this work, the focus is directed to characterization of the bacteria and yeast as microorganisms responsible for the majority of food-based fermentation.

1.2.1 Bacteria—lactic acid bacteria

LAB are common microorganisms in fermented foods and beverages. They are usually non-spore-forming, gram-positive, catalase-negative, and acid-tolerant [11], [12]. The main genera include *Enterococcus, Lactobacillus, Lactococcus, Leuconostoc, Oenococcus, Pediococcus, Streptococcus*, and *Weissella* [2], [12]. Although *Bifidobacterium* and *Propionibacterium* are not true lactic acid bacteria, they are often discussed alongside LAB because of their importance in fermented foods and probiotic applications. Traditionally, LAB can be classified into three groups based on carbohydrate fermentation pathways: obligate homofermentative, facultative heterofermentative, and obligate heterofermentative [11], [13].

Obligate homofermentative LABs ferment hexose sugars (for example glucose) into lactic acid via the Embden-Meyerhof-Parnas (EMP) pathway, producing little to no ethanol. These species are not able to ferment pentoses or gluconate [14]. In homofermentative LAB, the primary metabolic route involves the conversion of a

six-carbon sugar (glucose) into two molecules of three-carbon lactic acid [15]. The main representative species are *Lactococcus lactis* and *Streptococcus thermophilus* [11]. Additionally, *Pediococcus, Lactococcus, Streptococcus*, and few *Lactobacillus* species are classified as obligate homofermentative because they ferment hexoses almost exclusively to lactic acid via the EMP pathway, whereas pentoses are not metabolized by all homofermenters [15].

In contrast, heterofermentative LAB employ a more complex pathway in which glucose is first transformed into CO_2 and a five-carbon sugar phosphate, which is subsequently degraded into lactic acid and a two-carbon end product—either ethanol or acetic acid [11]. Facultative heterofermentative LAB adapt their metabolism based on available sugars. They act as homofermenters, producing only lactic acid from hexoses, but become heterofermenters—producing lactate, acetate, ethanol, and other compounds—when fermenting pentoses or under specific nutrient-limiting conditions [14]. Some *Leuconostoc* and *Lactobacillus* species belong to the above-described group, for example, *Lactiplantibacillus plantarum*, *Lacticaseibacillus rhamnosus, Latilactobacillus curvatus* [16].

Obligate heterofermentative LABs are not able to utilize hexoses by using the EMP pathway due to a lack of fructose-1,6-bisphosphate aldolase; however, they use the phosphogluconate pathway to degrade hexoses [15], [16]. Additionally to lactic acid as the end product, these species also produce CO_2, and either ethanol or acetic acid [15]. Obligate heterofermentative LAB genera are *Leuconostoc*, *Oenococcus*, *Weissella*, and *Lactobacillus* [11].

During fermentation, LAB produce organic acids that accumulate and lower the pH of the substrate to levels that inhibit the growth of pathogenic, spoilage, and toxigenic microorganisms. In addition, they produce bacteriocins and bioactive peptides, which further contribute to food preservation and safety [12]. Many species are called “biopreservers” due to these characteristics. They are capable of altering the flavor profile of the original substrates and enhancing the nutritional quality of foods during the synthesis of volatile compounds in the fermentation process [12]. These LABs are used in various foods such as fermented milk products, fermented cereal foods, fermented vegetable foods, fermented roots and tuber products, and some alcoholic and non-alcoholic beverages.

1.2.2 Bacteria – *Bacillus* species

Another example of bacteria species that are important for food fermentation belongs to the *Bacillus* genus. *Bacillus* species are mainly present in alkaline-fermented foods of Asia and Africa [2]. *Bacillus* species are Gram-positive, rod-shape, motile, catalase-positive, and form endospores. These genus members can be aerobic to semi anaerobic bacteria [12]. *Bacillus* species usually perform in protein-rich plant-based fermented foods. They break down proteins and lipids using extracellular enzymes, enhancing digestibility and nutritional value. *Bacillus* species produce amylases, proteases, and lipases that break down raw materials, leading to the development of compounds like pyrazines and polyglutamate, which contribute to flavor and texture. *Bacillus amyloliquafacien*s, *Bacillus coagulans, Bacillus subtilis*, and *Bacillus subtilis* variety *natto* are the most well-known species.

Bacillus species' metabolism varies a lot. Most species use glucose and/or other carbohydrates as the sole source of carbon and energy; however, some species do not appear to utilize carbohydrates at all [17]. Some strains have the ability to synthesize λ-

polyglutamic acid, an amino acid polymer commonly associated with Asian fermented soybean products, which contributes to their distinctive sticky or viscous texture [12]. Some *Bacillus* species, notably *B. subtilis* and *B. coagulans*, are increasingly used as probiotics because they form spores that withstand industrial processing and acidic gut conditions.

1.2.3 Yeast

Yeasts are fungi belonging primarily to the phyla *Ascomycota* and *Basidiomycota* within the subkingdom *Dikarya* [18]. Various fermented foods and beverages are produced using yeast species, and twenty-one yeast genera have been reported to be associated with these fermentation processes. The most well-known yeast genera include *Saccharomyces, Brettanomyces, Candida, Dekkera, Hanseniaspora, Issatchenkia, Kluyveromyces, Pichia, Rhodotorula, Torulaspora*, and *Zygosaccharomyces* [2].

The most extensively studied yeast is *Saccharomyces cerevisiae*, owing to its long history of use in the production of fermented foods and beverages [19]. It is used worldwide in beer, wine, and baking industries, and its well-characterized physiology and genetic traits have made it a cornerstone of biotechnology—so much that it was long considered an exclusively domesticated species [20]. *S. cerevisiae* dominates alcoholic fermentations largely because of the Crabtree effect, which enables it to produce high ethanol levels in sugar-rich environments while repressing mitochondrial respiration, even when oxygen is present [19].

Generally, *Saccharomyces* ferments any carbon-rich substrate and produces secondary metabolites through which the growth of spoilage microorganisms is inhibited. These species exhibit a wide range of enzymatic activities, including proteolytic, lipolytic, glycolytic, pectinolytic and urease activities. However, *Debaryomyces* species produce growth factors that promote bacterial growth during sugar fermentation. *Hanseniaspora* and *Candida* species also actively participate in the fermentation process by metabolizing sugars and producing a variety of secondary metabolites, while exhibiting diverse enzymatic activities that contribute to flavor development and other fermentation characteristics [12], [21].

1.3 Fermented foods

This chapter focuses on four major categories of fermented foods—fermented milk products, fermented vegetable foods, fermented cereal foods, and miscellaneous fermented foods. Across fermented foods, the biochemical composition of the raw material determines which microorganisms dominate and which metabolic pathways are activated. Microbial fermentation is fundamentally driven by the availability of carbohydrates, proteins, and lipids, which microorganisms convert into organic acids, alcohols, peptides, and other metabolites that shape the nutritional and sensory properties of fermented foods [22]. This principle is consistent across all food-matrix categories.

In fermented milk products, the key substrate is lactose. LAB metabolizes it through glycolytic pathways to produce lactic acid. This process underlies acidification, texture formation, and flavor development, and it is an example of carbohydrate-driven fermentation [22].

In fermented vegetable foods, the main fermentation drivers are naturally occurring simple sugars (such as glucose and fructose) and fiber-associated carbohydrates [22]. Additionally, vegetables contain phytochemicals, micronutrients, and antioxidants,

which influence microbial succession and contribute to the formation of bioactive compounds during fermentation [23].

In fermented cereal foods, the dominant substrate is starch, which must be hydrolyzed into fermentable sugars before microbial metabolism can proceed. Starch degradation is as a key microbial process, enabling the growth of lactic acid bacteria, yeasts, and mixed microbial consortia [22]. Also, the role of polysaccharide-degrading enzymes is shaping microbial ecology in plant-based fermentation [11].

The category of miscellaneous fermented foods includes root crops, legumes, and fruit-based fermentation. For example, fruit-based fermentation such as cocoa and coffee, contain high amount of carbohydrates, which support sequential yeast-LAB-AAB activity [11], [22]. But legume fermentations are driven by proteins and oligosaccharides, which microorganisms hydrolyze into peptides and amino acids [11], [22]. Therefore, miscellaneous fermented foods' main drivers vary by substrate.

1.3.1 Fermented milk products

Fermented milk products form one of the most diverse groups of traditional fermented foods, and they are typically classified into two main categories based on the microorganisms responsible for fermentation. The first category includes products fermented predominantly by LAB, which rely on lactic acid fermentation as their primary metabolic pathway. This category comprises thermophilic LAB, typically associated with yogurt fermentation, and mesophilic LAB, which are characteristic of products such as cream cheese.

The second category, referred to as fungal–lactic fermentation, involves a cooperative interaction between yeasts and LAB in shaping the final characteristics of the product. This type of fermentation is characteristic of alcoholic fermented milks, including acidophilus-yeast milk, kefir, and koumiss, as well as mold-ripened products such as viili [2].

Historically, fermented milk products were produced using two main methods: spontaneous fermentation of raw or boiled milk, and the backslopping technique [2]. In backslopping propagation, a part of the previous batch of fermented product is used, to inoculate a fresh medium. A common example is the preparation of homemade yogurt, in which a small amount of yogurt from a previous batch is used to inoculate fresh milk. In modern dairy production, fermentation is no longer left to spontaneous processes. Instead, manufacturers rely on defined starter cultures—carefully selected strains of lactic acid bacteria are chosen for their predictable performance—to initiate fermentation and ensure consistent product quality [9].

Following the shift from traditional to controlled fermentation practices, it is also important to consider how the microorganisms involved in dairy fermentation are functionally classified. This transition helps to link the technological development of fermentation with the biological roles of the microorganisms involved. Bacterial species used in fermented milk products are generally grouped according to their roles during fermentation and product development. The first group consists of starter cultures, which initiate fermentation of the substrate and produce lactic acid along with other organic acids. Common starter species include *Lactobacillus delbrueckii* subsp. *delbrueckii*, *Lc lactis* subsp. *cremoris*, *Lc. lactis* subsp. *lactis*, *Lb. delbrueckii* subsp. *lactis*, *Lactococcus helveticus*, *Leuconostoc* spp., and *S. thermophilus* [24]. The second group consists of non-starter cultures, which contribute primarily to flavor and texture development. Some non-starter cultures are *L. plantarum, Lacticaseibacillus casei,*

Ligilactobacillus salivarius, and *Enterococcus faecium* [2]. The third group is known as protective cultures. Their role is to produce molecules (e.g. bacteriocins) that protect other cultures in the food product against spoilage microorganisms [25].

Among fermented dairy products, yogurt is widely consumed. It is valued for its nutritional quality and is produced through the coagulation of milk via the fermentation by starter cultures, such as *S. thermophilus* and *Lactobacillus delbrueckii* subsp. *bulgaricus* (*Lb. delbrueckii subsp. bulgaricus*) [2], [26]. Traditionally, it also contains probiotic bacteria, which are mainly used as non-starter cultures due to their poor growth in milk, which extends the fermentation time [27], [28]. Based on the World Health Organization (WHO) and the Food and Agriculture Organization of the United Nations (FAO), the definition of probiotic cultures is live microorganisms that, when consumed in adequate quantities, can promote the health of the host [28], [29]. The most widely used commercial probiotic bacteria are *Lactobacillus* spp. and *Bifidobacterium* spp. [13].

While yogurt represents a classic example of LAB-driven fermentation, many traditional dairy products rely on more complex microbial consortia. In particular, several fermented milks are produced through the combined activity of LAB and yeasts, resulting in distinct sensory, nutritional, and functional characteristics. Fermented milk products whose starter culture is a combination of LAB and yeast include kefir, koumiss, viili, and acidophilus-yeast milk [2], [6], [7]. The species commonly associated with these products include *Lactobacillus johnsonii*, *Lactobacillus acidophilus*, *Lactobacillus gallinarum*, *Lactobacillus amylovorus*, *Lactobacillus crispatus*, and *Lactobacillus gasseri* [1]. These mixed-culture fermentations illustrate the diversity of microbial interactions in dairy ecosystems and highlight how yeasts and LAB can work synergistically to shape product quality.

Beyond fermented milk, microbial diversity also plays a central role in cheese production, where additional microorganisms contribute to flavor, aroma, and texture during ripening. In addition to LAB, cultures such as *Brevibacterium linens*, *Propionibacterium freudenreichii*, *Debaryomyces hansenii*, *Geotrichum candidum*, *Penicillium camemberti*, and *Penicillium roqueforti* function as secondary cultures in cheese making, where they drive the development of characteristic sensory properties during the ripening process [30], [31].

As dairy fermentation technologies continue to evolve, attention has increasingly shifted from the microorganisms themselves toward the processing strategies that shape product quality. This shift has supported rapid technological development, driven in part by growing demand for value-added formulations and convenience-oriented functional ingredients. Within this technological context, the formation of sensory attributes has become a central focus, as these characteristics determine consumer perception and product identity. The sensory profile of fermented dairy products arises from a mixture of compounds produced in specific ratios during fermentation. For example, the characteristic buttery aroma of cheese and buttermilk is largely attributed to the compound diacetyl.

Achieving these sensory outcomes consistently requires precise control of fermentation conditions. For example, fermented milk products are typically mildly acidic, and their acidity is carefully regulated during production. The concentrations of lactic acid and residual carbohydrates are monitored throughout the manufacturing process to achieve the desired flavor and texture [4]. Acidification is managed by cooling the milk at the appropriate stage of fermentation, while carbon dioxide levels are

adjusted through stirring or vacuum deaeration at the end of the process [4]. Together, these technological and biochemical controls ensure that modern fermented dairy products maintain consistent quality while meeting consumer expectations for flavor, texture, and functionality.

1.3.2 Fermented vegetable foods

Perishable leafy vegetables, seasonal greens, radishes, cucumbers, and young edible bamboo shoots are often traditionally fermented food products. Most dominant species of fermented vegetables are from genera *Lactobacillus*, *Pediococcus*, *Leuconostoc*, *Tetragenococcus*, *Weissella,* and *Lactococcus* [2]. Historically, the most well-known fermented vegetable foods in Europe are olives, cucumbers, and other mixtures of vegetables such as beetroot and turnips [2], [32]. However, sauerkraut and Korean kimchi have become the most consumed foods nowadays [33], [34].

The sauerkraut is typically produced via spontaneous fermentation. Cabbages are pretreated and submerged into 0.5–3.5% salt solution, then fermented at ambient temperatures (18–20 °C) for approximately two weeks [35] to a month [36]. Traditional sauerkraut relies on the naturally occurring microorganisms present on the raw material. During spontaneous sauerkraut fermentation, the early phase is led by *Leuconostoc mesenteroides*, followed by the growth of other LAB species such as *Levilactobacillus brevis*, *Pediococcus pentosaceus*, and *L. plantarum* [35]. Among these, *L. plantarum* drives the second fermentation phase and is responsible for the high acidity characteristic of mature sauerkraut [35]. Within a week of fermentation, the microbial community shifts from less acid-tolerant heterolactic LAB to homolactic LAB species that are better adapted to the progressively acidified environment [35], [36]. The primary fermentable sugars in the cabbage are glucose and fructose. Lactic acid, acetic acid, and mannitol are the main end-products [35]. Studies have shown that *Leuconostoc* species are able to use fructose as an alternative external electron acceptor to produce mannitol [37]. However, homolactic and heterolactic LAB produce different types of organic acids. As a result, spontaneous fermentation can produce highly variable end products, leading to differences in organic acid and sugar concentrations.

LAB found in Korean kimchi are *Lc. lactis, L. brevis, L. plantarum, Latilactobacillus sakei* subsp. *sakei*, *L. curvatus, L. citreum*, *Leuconostoc gasicomitatum, Leuconostoc gelidum, Leuconostoc kimchii, L. mesenteroides, P. pentosaceus, Weissella confuse, Weissella kimchii*, and *Weissella koreensis* [4], [38].

The technology is very specific for various fermented vegetable food types. It is possible to add different additives, which influence the fermentation and microbial presence together with temperature, dynamics, time, etc. For example, salt can be added during the vegetable fermentation (dry form or brine solution) in various concentrations depending on the requested final product [4].

1.3.3 Fermented cereal foods

Fermented cereal foods are widespread in Europe, but also in America and Australia. Cereal fermentation relies on a diverse microbial community, primarily composed of lactic acid bacteria and yeasts. Their activity shapes the dough, producing its characteristic flavor, texture, and overall sensory appeal [4]. The most well-known cereal food is sourdough, which is a mixture of flour (wheat, rye, rice, etc.) and water fermented with LAB and yeasts [39]. The bacterial genera that associated with cereal fermentation are *Lactococcus*, *Lactobacillus*, *Leuconostoc*, *Enterococcus*, *Pediococcus*, *Streptococcus,*

and *Weissella*. The principal yeast responsible for cereal fermentation is *S. cerevisiae*; however, non-Saccharomyces yeast can also be found in that process. Such as *Candida, Hansenula, Pichia, Debaryomyces,* and *Trichosporon* [40].

Cereal grains represent a major global source of dietary proteins, carbohydrates, vitamins, minerals, and fiber. Nevertheless, the nutritional value of cereals and the sensory characteristics of cereal-derived products can be inferior to those of milk and dairy products. This is due to their lower protein levels, limited essential amino acids (especially lysine), reduced starch availability, and the presence of antinutrients such as phytic acid and tannins. Using fermentation, it is possible to reduce the content of these antinutrients in cereals.

Homofermentative LAB such as *Pediococcus*, *Streptococcus*, *Lactococcus,* and some *Lactobacilli* produce lactic acid as the major or sole end product of glucose fermentation. However, heterofermenters such as *Weissella* and *Leuconostoc* and some *Lactobacilli* produce lactate, CO_2 and ethanol from glucose [41].

The technological part starts with controlling the water activity (a_w) of stored grains of cereals. The a_w should be <0.6 (14% of moisture). With that a_w value, the grains are metabolically resting; therefore, components are not available for microorganisms, and the endogenous enzymes are inactivated. Fermentation is initiated when specific technological steps are applied, such as adding water, reducing particle size through milling, and carefully controlling microbial populations and enzyme activities. Water absorption raises the a_w, respiratory processes lower the redox potential, and both respiration and fermentation contribute to a decrease in pH. These changes facilitate the release of substrates through endogenous hydrolytic reactions, such as amylolysis, proteolysis, and lipolysis, and the physiological activities of intentionally added or naturally occurring microorganisms [40].

Cereal fermentation is influenced by specific key variables, and controlling these factors is fundamental to the technological practices used to produce different products with consistent, well-defined quality. These parameters are the following:

1. The cereal type determines the composition of fermentable substrates, available nutrients, growth factors, minerals, buffering capacity, and the effectiveness of any growth-inhibiting components;
2. Milling—grain particle size (before or after soaking or before and after fermentation);
3. Addition of additives to the main substrate, such as salt, sugar, hops;
4. Aeration with oxygen;
5. Water content;
6. A source of amylolytic enzymes essential for converting starch and other polysaccharides into fermentable sugars [40].

1.3.4 Miscellaneous fermented foods

Miscellaneous fermented products are a small number of important fermented products that do not fit into the standard substrate-based categories of fermented foods. This group includes foods such as vinegar, *nata*, *pidan,* tea, coffee, cacao, and others [21].

Vinegar can be produced from any sugar containing substrate or hydrolyzed starchy material through alcoholic fermentation followed by acetic acid fermentation. A wide range of vinegars are manufactured globally, such as wine, malt, fruit, grain (rice), spirit, whey, and honey vinegars. AAB such as *Acetobacter aceti* subsp. *aceti*, *Acetobacter pasteurianus*, *Acetobacter polyxygenes*, *Acetobacter xylinum*, *Acetobacter malorum*, and

Acetobacter pomorum are the predominant species involved in vinegar production [42]. In addition, various yeasts have been identified such as, *Candida lactis condensi*, *Candida stellata*, *Hanseniaspora valbyensis*, *Hanseniaspora osmophila*, *Saccharomycodes ludwigii*, *S. cerevisiae*, and several *Zygosaccharomyces* species [2], [21].

Classical black and green teas are consumed worldwide. However, fermented teas, such as *miang* of Thailand and kombucha, *puer* tea, and *fuzhuan brick* of China, are particularly popular in several Asian countries, [21]. Miang tea is produced by *Lacticaseibacillus thailandensis, Lacticaseibacillus camelliae, L. plantarum, Lactiplantibacillus pentosus, Paucilactobacillus vaccinostercus, Lactobacillus pantheris, Limosilactobacillus fermentum, Lactobacillus suebicus, Pediococcus siamensis, Enterococcus casseliflavus and Enterococcus camelliae* [42]. The *fuzhan* brick tea is fermented by fungi species from genera such as *Aspergillus, Penicillium* and *Eurotium* [43].

Kombucha is produced from green or black tea leaves with the addition of sugar and a symbiotic community of yeast and AAB. The dominant AAB species are particularly members of the *Komagataeibacter* genus—previously classified as *Gluconacetobacter* and earlier as *Acetobacter* [44], [45], [46]. Commonly reported species include *Komagataeibacter intermedius* [47], *Komagataeibacter xylinus* [47], [48], *Komagataeibacter rhaeticus* [49], *Komagataeibacter saccharivorans* [47], [48], and *K. kombuchae* [47]. In addition, LAB such as *Lactobacillus*, *Leuconostoc*, and *Bifidobacterium* have also been detected in kombucha [50]. The dominant yeasts associated with kombucha belong to the genera *Zygosaccharomyces* (e.g., *Z. bailii*), *Saccharomycodes* (e.g., *S. ludwigii*), *Candida*, *Torulaspora*, *Pichia*, *Brettanomyces/Dekkera* (e.g., *Dekkera bruxellensis* (*D. bruxellensis*)), *Schizosaccharomyces* (e.g., *Schizosaccharomyces pombe*), and *Saccharomyces* (e.g., *S. cerevisiae*) [45], [47], [51], [52], [53].

1.4 Microbial interactions within symbiotic consortia in fermented foods using kombucha and kimchi as an example

The microbial ecology of fermented foods is shaped by the structure, dynamics, and functional roles of their resident microbial consortia. Traditional fermented foods provide useful models for examining these principles, as they are composed of mixed communities of yeasts and bacteria that together form stable fermentation systems. Such consortia rely on a range of ecological interactions, including cooperation, competition, and metabolic cross-feeding, which collectively determine community function and fermentation outcomes. In this section, these interactions are further characterized by using kombucha and kimchi as illustrative examples.

1.4.1 Microbial interactions within symbiotic consortia in kombucha

Fermented foods such as kombucha serve as valuable model systems for investigating multi-species interactions. Classical kombucha are brewed most commonly from black and green tea [50], [53]. However, there are far more substrates that can be used for kombucha production, such as oolong, jasmine, and mulberry teas, rooibos, and coconut water [54]. The tea is typically supplemented with 5-10% weight per volume (w/v) of sucrose, 0–20% volume per volume (v/v) of the previous batch of kombucha liquid and 2.5% weight per volume (w/v) of kombucha biofilm to start the fermentation.

The main microbial inoculum comes from the starter culture called Symbiotic Culture of Bacteria and Yeast, which is a SCOBY. Kombucha contains mainly *Komagataeibacter* species and various yeasts. Nevertheless, the traditional fermentation occurs in an open system. The fermentation vessel is usually covered with paper tissue or gauze cloth. As a result, other environmental microbes may interact with or become part of the solution and thereby contribute to the fermentation process.

First, yeasts secrete invertase, which is an enzyme that hydrolyzes the disaccharide (mainly sucrose) into the monosaccharide's glucose and fructose. This step represents the first major point of resource sharing within the microbial community, where the released sugars become accessible to any microorganism capable of utilizing them as a carbon source. About 99% of the monosaccharides produced by *Saccharomyces* invertase diffuse into the surrounding environment before the yeast cells can take them up [54]. Most of the released sugars are consumed by nearby microbes rather than producing yeast. This pattern shows that invertase activity functions as a non-excludable public good within the community.

Although yeast-derived invertase functions as a cooperative trait, some yeast strains do not produce the enzyme and act as "cheaters" [54]. In co-culture with bacteria, invertase-producing yeast are able to outcompete "cheater" strains that do not produce invertase [55]. In other words, when yeast grows alongside bacteria, cooperative invertase-producing yeast gains a competitive advantage over non-producers. Celiker & Gore (2012) propose that this advantage arises because bacteria rapidly deplete available resources, creating an environment with very low sugar concentrations. Under these conditions, invertase-producing yeast are favored because they can retain approximately 1% of the sugars they hydrolyze, whereas non-producers receive none and effectively starve [55]. Interestingly, during kombucha fermentation, bacteria also remove sugars from the medium by converting them into the cellulose pellicle (SCOBY). This suggests that bacterial activity—by drawing sugars out of the liquid phase and into the pellicle—may similarly alter the selective pressures within the kombucha environment in a way that favors invertase-producing yeast strains [54].

After the yeast's cleavage of sucrose into its component monomers, the yeast begins to consume the remaining 1% of monomers and starts to produce ethanol. Ethanol can be harmful to both yeast and bacteria. It may modify the cellular membrane integrity, structure and function of microbes [56]. High levels of ethanol may influence the bacteria viability within kombucha, especially the AAB. These bacteria are obligatory aerobes (need oxygen to ferment) and convert ethanol to acetic acid. Under static conditions, the SCOBY can improve oxygen access for the microorganisms it contains, including yeast cells, which are incorporated into the surface biofilm. This may represent another form of cooperation between yeast and bacteria during kombucha fermentation. Additionally, some yeast strains from the *Dekkera/Brettanomyces* genus are capable of doing the same as AAB. The function of LAB in kombucha remains poorly characterized, yet LAB species are known to take part in fermented tea production and biofilm development [50].

The dominant secondary metabolites produced by kombucha consortia are acetic acid, gluconic acid, glucuronic acid and ethanol [48], [53]. However, depending on the microbial composition in kombucha, other acids can be detected, such as lactic acid, citric acid, and malic acid [45], [47], [52], [53].

1.4.2 Microbial interactions within symbiotic consortia in kimchi

Fermented kimchi is an excellent model for investigating microbial ecology of fermented foods. It is a key traditional fermented food in Korea and is widely consumed in other East Asian countries, including Japan and China. Over the past few years, kimchi has gained popularity across Europe and America, indicating the strong acceptance by health-driven consumers. Although more than 30 different vegetables can serve as the primary ingredient [33], kimchi is most commonly made from raw Napa cabbage (*Brassica rapa L*. ssp. *pekinensis*) [57]. As a central element of Korean cuisine and food culture, approximately 200 kimchi varieties are recognized, each characterized by its own flavor profile shaped by regional ingredients, fermentation conditions, and the microorganisms involved [33], [58], [59]. However, the typical kimchi is prepared by mixing brined Napa cabbage (although many other leafy and root vegetables can also be used) with red pepper powder, garlic, green onion, onion, ginger, and fish sauce [33], [59].

Fermentation is an essential step in the kimchi-making process; during this stage, the growth of pathogenic and spoilage bacteria is suppressed, and the characteristic flavor profile of kimchi develops [59].

Kimchi fermentation is shaped by multiple factors, including ingredient composition, fermentation temperature, salt concentration, oxygen availability, and pH, all of which influence the taste and quality of the final product [57], [60]. The involvement of multiple LAB genera, including *Leuconostoc*, *Lactobacillus*, *Weissella*, *Pediococcus*, and *Lactococcus*, results in batch-to-batch variability in kimchi fermentation and, consequently, in its sensory and physicochemical properties [57], [61].

Kimchi provides a tractable model ecosystem for examining how multispecies microbial communities assemble and function through intertwined processes of competition, cooperation, and metabolic cross-feeding. During early fermentation, heterofermentative *Leuconostoc* species rapidly proliferate by exploiting the relatively mild acidity and abundant sugars, producing CO_2, lactic acid, and mannitol [62]. As acid accumulates, more acid-tolerant taxa such as *Lactobacillus*, *Weissella*, and *Pediococcus* gain a competitive advantage, illustrating a clear case of environment-mediated competition in which early colonizers modify conditions in ways that favor later successional species [60]. At the same time, kimchi fermentation also depends on cooperative interactions, particularly the shared degradation of plant polysaccharides and the collective suppression of spoilage organisms. Many LAB species produce bacteriocins, organic acids, and hydrogen peroxide, which together create a protective environment that no single species could establish alone [61]. These cooperative defense mechanisms stabilize the community and maintain fermentation reliability. In addition, metabolic cross-feeding is central to kimchi's microbial ecology: sugars released by plant-cell-wall-degrading enzymes from one group of LAB become substrates for others, while metabolites such as mannitol, acetate, and amino-acid derivatives produced by early fermenters serve as growth-enhancing resources for later colonizers. This combination of competitive succession, cooperative inhibition of pathogens, and reciprocal metabolic exchange make kimchi a powerful natural model for studying how multispecies consortia self-organize, transition between ecological states, and generate emergent functional properties.

1.4.3 Comparison of kombucha and kimchi fermentation

In general, both fermented foods, kimchi and kombucha, rely on plant-derived substrates that supply sugars for microbial metabolism in spontaneous fermentation influenced by environmental conditions (Table 1). Kombucha is tea infusion fermented in the open system and is in liquid form, whereas kimchi substrates are vegetables with seasoning, and fermentation is carried out in the semi-closed system in solid state.

Both foods host multispecies microbial consortia, and the interactions among these species—competitive as well as cooperative—play key roles in structuring and stabilizing the community. However, the dominant species in kombucha are AAB and yeast, whereas in kimchi the prevalent species are LAB. In kombucha, the central metabolites that induce competition between species are sugars, whereas in kimchi the competition centers on acidification. Additionally, the cooperation mechanisms differ between the foods. In kombucha, the cooperation occurs between AAB and yeast, whereas in kimchi, the cooperation is between different LAB species.

Both yield acidic fermented foods with complex microbial communities, but the outcome is different. Kombucha´s outcome is acidic beverages and a SCOBY, whereas kimchi results in acidic and flavorful solid vegetable products. Additionally, the main acid is acidic acid, whereas in kimchi it is lactic acid.

Both systems can be modified but the influencing factors are different. Kombucha can be altered by changing the sugar concentration or source and by increasing or decreasing the oxygen availability.

Gaining deeper insight into microbial succession dynamics helps clarify the biological processes that shape quality attributes in traditional fermented foods and establishes a scientific basis for improving product uniformity through refined starter-culture design and environmental regulation. Viewing fermented foods as complex, evolving microbial ecosystems is a critical step toward transforming traditional fermentation from an experience-based craft into a controlled and standardized biotechnological process [63].

Table 1. Comparison of kombucha and kimchi fermentation.

Feature	Kombucha	Kimchi	Similarity	Differentially
Substrate	Brewed tea (different types) + sucrose + previous batch kombucha liquid + SCOBY	Raw Napa cabbage (most common); different vegetables; mixed with spices and fish sauce	Both rely on plant-derived substrates	Kombucha: tea infusion Kimchi: vegetables and seasoning
System	Open system; vessel covered with paper/gauze; environmental microbes may enter	Fermentation shaped by salt, temperature, oxygen availability, pH	Both undergo spontaneous fermentation influenced by environmental conditions	Kombucha: open system and liquid Kimchi: semi-closed and solid-state
Inoculum	SCOBY containing AAB *(*mainly *Komagataeibacter* spp.) + yeasts	Naturally occurring LAB: *Leuconostoc*, *Lactobacillus*, *Weissella*, *Pediococcus*, *Lactococcus*	Both are multispecies consortia	Kombucha: dominated by AAB + yeast Kimchi: dominated by LAB

Feature	Kombucha	Kimchi	Similarity	Differentially
Key microbial processes	Yeast invertase hydrolyzes sucrose; bacteria	Early heterofermentative LAB produce CO_2	Both show microbially driven biochemical	Kombucha: relies on yeast-AAB interactions Kimchi: LAB succession
Competition dynamics	Yeast producers outcompete "cheaters" when bacteria deplete sugars; bacteria draw some sugars into pellicle (SCOBY)	Early LAB acidify environment, enabling later LAB to outcompete them	Both exhibit environment-mediated competition where microbes modify conditions to influence community structure	Kombucha: competition centers on sugar depletion Kimchi: competition centers on acidification
Cooperation dynamics	Yeast provides public sugars; bacteria detoxify ethanol; SCOBY structure supports both	LAB together degrade plant polysaccharides; produce bacteriocins, acids, H_2O_2 to suppress spoilage organisms	Both rely on cooperative interactions that stabilize the community	Kombucha: cooperation involves yeast-AAB Kimchi: cooperation between LAB-LAB interaction
Metabolic cross-feeding	Yeast release sugars consumed by bacteria; bacteria convert ethanol	Early LAB release sugars + metabolites (mannitol, acetate, amino acid derivatives) feeding later LAB	Both depend on mutual metabolic exchange among community members	Kombucha: cross-feeding is sugar- and ethanol-based Kimchi: cross-feeding involves plant polysaccharides and LAB metabolites
Primary metabolites	Acetic acid, gluconic acid, glucuronic acid, ethanol; occasionally lactic, citric, malic acids	Lactic acid, CO_2, mannitol, acetate	Both produce organic acids that shape acidity and microbial succession	Kombucha: acetic-dominant acids Kimchi: lactic-dominant acids
Factors influencing fermentation	Tea type, sucrose concentration, oxygen availability, microbial composition, environmental microbes	Ingredient composition, temperature, salt concentration, oxygen availability, pH, LAB community structure	Both are strongly shaped by environmental parameters that influence microbial activity	Kombucha: depends on sugar + oxygen Kimchi: salt + acidification
Outcome of fermentation	Acidic beverage + SCOBY	Acidic, flavorful vegetable product	Both yield acidic fermented foods with complex microbial communities	Kombucha: drink + SCOBY Kimchi: solid vegetable food

1.5 Present methodological approaches and limitations in microbial ecology characterization

Omic methods (omics) represent one of the most adaptable and integrative frameworks in microbiology, extending beyond genomics to capture multiple biological layers that collectively describe microbial identity, activity, and ecological interactions [64]. The term omics refers to a collection of high-throughput technologies used to

comprehensively analyze different classes of biological molecules, including genomics, transcriptomics, proteomics, metabolomics, and lipidomics [63], [65]. Together, these complementary omics layers provide a comprehensive toolkit for dissecting microbial behavior in food systems, from genetic potential to metabolic output. By building on next-generation sequencing (NGS)-based genomic data, omics approaches enable researchers to connect taxonomic composition with gene expression, protein synthesis, and metabolite production, offering a multidimensional view of microbial function. Understanding which microorganisms drive wild fermentation enables accurate prediction of the resulting metabolic transformations, and this predictive capacity can be leveraged to develop new food products or reshape existing ones by improving control over industrial fermentation processes.

Recent years have seen substantial progress in the application of multi-omics technologies, providing comprehensive insights into the microbial community structure, functional potential, and active metabolic pathways of foods and fermented foods [66]. Multi-omics research integrates multiple omics layers to understand biological systems across different molecular levels [63]. Within the broader field of multi-omics, meta-omics refers specifically to community-level omics approaches used to characterize the structure, function, and activity of microbial ecosystems. Meta-omics approaches (metagenomics, metatranscriptomics, metaproteomics, metabolomics) provide comprehensive insights into shifts in microbial community composition, differentially expressed genes, protein expression patterns, and active metabolic pathways throughout fermentation [67]. Integrated multi-omics, supported by computational tools such as machine learning, enables deep exploration of the complex relationships between microbial metabolic networks and phenotypic outcomes [66].

The integration of omics approaches into the study of traditional fermentations offers a comprehensive understanding of the resident microbiota and the metabolic activities that influence the organoleptic properties of fermented foods. These approaches also deepen our understanding of the potential health benefits associated with fermented foods, while simultaneously revealing challenges linked to the absence of standardized processing in traditional production methods. These integrated insights indicate that fermentation processes can be more effectively directed and controlled, ultimately supporting the production of safer, more consistent, and higher-quality fermented foods.

1.5.1 Classical and modern methods for isolation and classification of microbes in food

Microbial detection in food relies on a combination of culture-dependent and culture-independent methods, each offering distinct advantages for characterizing food-associated microorganisms. Classical culture-dependent approaches, such as plating on selective or differential media, colony enumeration, and phenotypic characterization, remain essential for isolating viable strains and studying their metabolic capabilities [68], [69]. These methods enable detailed physiological and functional analyses but underestimate microbial diversity because many food-associated microorganisms are viable but non-culturable or require specific growth conditions [69]. The limitations of these traditional approaches become particularly evident when dealing with slow-growing, fastidious, or unculturable microorganisms that cannot be reliably recovered on standard laboratory media. Traditional identification methods based on colony morphology and phenotypic traits are also slow, labor-intensive, and

sensitive to environmental variation, which can complicate the characterization of microbial communities [70]. In addition, obtaining pure cultures often requires selective media or antibiotics, further restricting the range of detectable organisms. These constraints highlight the need for analytical tools that can detect microorganisms independently of their ability to grow *in vitro*.

To overcome the limitations of culture-dependent methods, culture-independent molecular techniques, such as PCR and quantitative real-time PCR (qPCR), allow the rapid detection and quantification of specific microbial groups directly from food matrices, thereby bypassing the need of cultivation [71]. Although these methods offer high sensitivity and specificity, their performance depends on prior knowledge of the target microorganisms and can be affected by DNA extraction efficiency, matrix-derived inhibitors, and primer bias [71]. More advanced sequencing-based approaches, including 16S ribosomal RNA (rRNA) gene amplicon sequencing and shotgun metagenomics, provide comprehensive insights into microbial community composition and functional potential, revealing taxa that cannot be cultured and predicted [71], [72]. Both 16S rRNA gene amplicon sequencing and shotgun metagenomics typically rely on NGS platforms, which enable high-throughput analysis of complex microbial communities. Although these sequencing approaches greatly expand detectable diversity, they still require careful interpretation due to amplification biases, variable gene copy numbers, and challenges in distinguishing viable cells from dead cells [72].

A recent example illustrating how sequencing workflows continue to advance is provided by Kallastu et al. (2023). They modified the NGS pipeline by incorporating the fluorescent permeable dye PMAxx to selectively detect viable cells. They further improved quantification by using spike-in controls or qPCR targeting the 16S rRNA gene V4 region, enabling estimated enumeration of living bacterial cells. This simple and reliable approach significantly enhanced the accuracy of microbiome analyses and demonstrated how targeted methodological innovations can substantially improve the quality of future sequencing-based studies.

Advances in high-throughput sequencing technologies have reshaped our understanding of food-associated microbiomes by enabling the detection of uncultured microorganisms directly from their natural environments [74]. Modern NGS platforms now support both targeted approaches, such as 16S rRNA gene amplicon sequencing, and untargeted shotgun metagenomics, providing deeper and more accurate insights into microbial communities. In addition, whole-genome sequencing (WGS) has become increasingly accessible, allowing detailed characterization of genomic variation and functional potential within microbial populations [65]. Because these sequencing strategies all rely on NGS technologies, they fall within the omics subfield of genomics, which focuses on the structure, function, and evolution of microbial genomes. Genomics therefore forms the foundational layer upon which broader omics analyses are built.

1.5.2 Modern technologies to study metabolism and characterize fermented foods

As mentioned before, the characterization of fermented foods is a combination of different approaches. While genomics focuses on microbial community composition and predicts gene functions, metabolomics provides a comprehensive biochemical fingerprint of the fermentation process by profiling the metabolites produced during microbial activity. In food fermentation research, metabolomics is essential because of four main aspects: (1) it reveals metabolic pathways which are activated by specific

microbial groups, (2) it links microbial composition to functional output, (3) it identifies biomarkers of fermentation stages or product quality, and (4) it helps to explain flavor, aroma, and texture development [75], [76]. Together, these capabilities make metabolomics a central tool for understanding how microbial activity shapes the chemical landscape of fermented foods.

Recent studies have shown that untargeted liquid chromatography–mass spectrometry (LC-MS) and nuclear magnetic resonance-based metabolomics can track changes in organic acids, amino acids, sugars, and other metabolites throughout fermentation, providing insight into microbial succession and functional shifts [77]. Such analyses help reveal how microbial communities drive biochemical transformations that ultimately influence product quality.

Another important tool for characterization of fermented foods is volatile profiling. This is a specialized branch of metabolomics that focuses on the aroma-active compounds which contribute directly to sensory perception. Volatile compound profiles are important because of three main reasons. Firstly, this method helps to match microorganisms to distinctive aroma compounds. Also, many volatiles are strain-specific and therefore enable microbial fingerprinting. Lastly, the shift in volatile profiles reflects the fermentation progress, spoilage, or errors in the fermentation process. Volatile profiling is typically performed using GC-MS to identify key aroma compounds and link them to specific microbial taxa in fermented beverages and dairy products [78]. These approaches highlight how volatilomics provides a direct connection between microbial metabolism and the sensory attributes of fermented foods.

Although modern analytical methods enable the characterization of fermented foods at the molecular level, sensory evaluation remains equally important. Ultimately, the success of a fermented product depends on consumer acceptance, as products with undesirable flavor or aroma are unlikely to be purchased. Therefore, sensory analysis serves as an essential validation step, confirming whether metabolomic and volatilomic findings translate into perceptible differences in flavor, aroma, texture, and overall acceptability [77]. Studies combining sensory evaluation with chemical profiling have demonstrated strong correlations between specific metabolites, volatile compounds, and desirable sensory attributes, reinforcing the importance of integrating molecular and sensory approaches.

Altogether, the combination of metabolomics, volatile profiling, and sensory analysis provides a comprehensive framework for understanding fermentation processes, linking microbial activity to chemical transformations and ultimately to consumer perception.

1.6 Starter culture design: dairy and non-dairy products

The development of new functional foods is increasingly directed toward sustainability, and new products are expected to have as small environmental footprint as possible. Consequently, there is growing interest in using plant materials for food applications, including the development of plant-based yogurt. However, several obstacles remain, such as the high content of antinutritional compounds in many plant substrates, and the sensory dissimilarity of plant alternatives compared with traditional dairy products. These challenges highlight the need to explore how plant-based alternatives can be made more acceptable to consumers. One promising direction is to examine the underlying technology—specifically, how starter cultures are designed and how their performance can be further improved for plant matrices. In the next subsection, this topic will be addressed in more detail.

1.6.1 History of starter culture design

The earliest fermented foods were produced through spontaneous fermentation driven by the natural microbiota of the raw material, so product quality depended on the initial microbial load and species composition. This process was later improved through backslopping, where a portion of a successful fermentation was used to inoculate a new batch. Repeated transfer enriched the best-adapted strains and unintentionally acted as an early starter-culture selection method, resulting in faster, more reliable fermentations. In less developed regions, spontaneous fermentation and backslopping continue to serve as inexpensive and reliable preservation methods [9], whereas in Western countries, fermentation relies on industrial production using commercial starter cultures, while traditionally fermented products are valued for their distinctive sensory qualities.

Early starter strains with desirable physiological and metabolic traits were typically isolated from natural habitats or from previously successful fermentations. However, several limitations must be acknowledged. Historically, the selection of commercial starter cultures was not based on a rational or systematic approach; instead, strains were primarily chosen for rapid acidification and resistance to bacteriophages [9]. As a result, these cultures often lacked flexibility in delivering specific functional or sensory attributes in the final product. This was largely because early starter cultures were maintained through daily propagation and only later became available as frozen, dried, or lyophilized preparations suitable for industrial use [9]. Since many early cultures were undefined microbial mixtures, repeated propagation likely caused shifts in community composition and the loss of individual strains. In addition, important plasmid-encoded traits could be lost during propagation or adaptation to the food matrix, reducing biodiversity and sometimes diminishing the unique characteristics of traditional products.

As fermentation practices evolved, starter cultures increasingly consisted of carefully selected strains or co-cultures that enabled the desired properties to be expressed *in situ* while maintaining natural, additive-free products [9]. These included LAB capable of degrading antimicrobial compounds, EPS, sweeteners, aroma compounds, useful enzymes, or nutraceuticals, as well as health-promoting probiotic strains [9]. This approach provided a natural alternative to chemical additives and broadened both the application range and functional flexibility of starter cultures. The addition of probiotic strains was also used to enhance the health-related value of the final product.

Using yogurt as an example, the classical yogurt starter culture consists of specific LAB strains that are selected for the functional properties described above. The main starter species are *S. thermophilus* and *Lb. delbrueckii* subsp. *bulgaricus. S. thermophilus* is known for its rapid acidification and efficient coagulation of milk proteins, which ensures short fermentation time and consistent texture—both essential for industrial yogurt production [79]. *Lb. delbrueckii* subsp. *bulgaricus* included for similar technological reasons, but its role extends further. Together these species form a mutualistic pair, in which *Lb. delbrueckii* subsp. *bulgaricus* releases amino acids that stimulate *S. thermophilus*, while *S. thermophilus* produces format and CO_2 that stimulate *Lb. delbrueckii* subsp. *bulgaricus* [80], [81]. This synergy accelerates acidification and improves flavor development.

Overall, the transition from spontaneous fermentation to carefully selected starter pairs reflect the broader shift toward controlled, predictable, and function-driven yogurt production. This development marked a turning point in dairy fermentation, where

empirical practices were gradually replaced by microbiologically informed starter-culture design. The adoption of a defined yogurt starter demonstrated how targeted microbial interactions could be harnessed to improve product quality, safety, and consistency. These advances laid the foundation for modern fermentation strategies, in which strain selection is guided by technological performance, metabolic capabilities, and desired sensory outcomes.

1.6.2 New starter culture design

Current consumption trends highlight the need to conserve natural resources and protect global ecosystems. As a result, developing sustainable, health-supporting plant-based dairy alternatives has become essential. A crucial aspect of this transition is the selection of suitable native crops that can reduce the overall carbon footprint [82]. Among Scandinavian raw materials, oats and peas are the most widely used in plant-based dairy alternatives. However, transferring conventional dairy-processing methods to plant-based systems presents several challenges [82]. For instance, these crops often have strong background flavors, sometimes perceived as astringent by consumers [83]. Fermentation offers a promising strategy to mitigate such sensory limitations.

The second category, referred to as fungal–lactic fermentation, involves a cooperative interaction between yeasts and LAB in shaping the final characteristics of the product. This type of fermentation is characteristic of alcoholic fermented milks, including acidophilus-yeast milk, kefir, and koumiss, as well as mold-ripened products such as viili [2].

Most non-dairy starter cultures currently available are isolated from the native microbiota of traditional dairy products. Product development has shown that these cultures are often ineffective at generating appealing aroma, taste, and texture profiles or at reducing off-flavors originating from plant raw materials [84]. Most commercially available non-dairy starter cultures are derived from bacterial strains originally isolated and developed for dairy fermentations, although they are typically cultivated in Kosher- and Halal-certified media to ensure their suitability for non-dairy applications.

Therefore, one promising strategy for developing starter cultures for plant fermentations is to select autochthonous strains from non-dairy sources. Autochthonous cultures are microbial strains that originate from the raw material, environment, or traditional product itself [32], [85]. In contrast, allochthonous cultures are microbial strains introduced from an external environment, meaning they do not naturally occur in the raw material or traditional product [32], [85]. Common plant-associated LAB include *L. plantarum*, *L. mesenteroides*, *L. citreum*, and *W. cibaria* [32], [86], with additional representatives from *Enterococcus* and *Streptococcus* species [32].

It is interesting that milk-origin starter cultures (such as *S. thermophilus*, *Lb. delbrueckii* subsp. *bulgaricus, Lactobacillus acidophilus, L. fermentum, L. plantarum, and Bifidobacterium bifidum*) are still used for production of non-dairy products, even though they have not demonstrated strong performance in mimicking dairy-like sensory properties in plant-based matrices. Several studies have shown that fermentation with LAB species of plant food origin [87], [88], [89] or plant beverages origin [90], [91], [92], [93], [94], [95], [96], [97], [98] reduces off-notes such as beany, earthy, astringent, which are common in legumes and cereals. Additionally, fermentation helped to obtain improved texture. However, the end-products still retain distinct plant-derived characteristics, and consumers are still able to distinguish plant-based products from

milk-based ones. To address these limitations, it becomes necessary to consider alternative microbial sources that are better adapted to plant substrates.

Historically, vegetable fermentations have relied more extensively on autochthonous microorganisms than other types of fermented foods. Selecting starter cultures from the autochthonous microbiota of vegetables and fruits is recommended, since these cultures may ensure prolonged shelf life and targeted nutritional, rheological, and sensory properties [86], [99], [100], [101], [102], [103], [104]. Di Cagno et al. (2013) suggested several aspects that need to be considered when using autochthonous LAB species for starter-culture development: pro-technological, sensory, and nutritional traits (Table 2). One of the most important aspects is rapid acidification to pH 4.5. It is also important that the selected species can consume all fermentable carbohydrates to eliminate the possibility of alcoholic fermentation by yeasts [86]. Additionally, the synthesis of EPS is an important functional trait that contributes to the smooth and creamy texture of fermented products such as yogurt. Furthermore, the article mentions that some autochthonous species may also possess probiotic potential [86]. It is also reported that some plant associated species like *Leuconostoc* spp. and *L. lactis* are able to produce diacetyl, that is the main aroma component, which gives dairy products a buttery and creamy aroma [105], [106].

Despite the characterization of numerous metabolic traits of autochthonous LAB, further studies on their physiology, genetics, and technological properties are still needed to fully exploit their potential in non-dairy applications. Given these constraints, shifting toward autochthonous, plant-adapted cultures offers a more promising route for improving fermentation performance. Such cultures are naturally better suited to plant matrices and can address sensory and technological limitations more effectively than dairy-derived strains.

Table 2. Main criteria and corresponding metabolic traits to select starters for plant-based drink fermentation. Modified and adapted from article Di Cagno et al., 2013.

Criteria	Metabolic traits
Pro-technological	Growth rate; acidification rate; salt tolerance; growth at low pH; tolerance to low pH; growth at low temperature; completeness of fermentation; malolactic fermentation; tolerance to phenols; synthesis of antimicrobial compounds; synthesis of hydrogen peroxide; pectinolytic activity
Sensory	Hetero-fermentative metabolism; synthesis of aroma compounds or their precursors
Nutritional	Synthesis of biogenic compounds; synthesis of EPS; increase of antioxidant activity; synthesis of biogenic amines

1.6.3 Rationale for non-dairy starter culture development

Although dairy starter cultures dominate commercial food fermentations, their technological performance is strongly linked to long-term domestication in milk. Milk provides a stable, protein-rich, and well-buffered environment, whereas plant substrates differ substantially in composition, containing phenolic compounds, lower protein

content, variable carbohydrate profiles, and limited buffering capacity [107]. These differences reduce microbial growth and acidification efficiency when dairy-origin cultures are applied to plant-based fermentations, resulting in weak flavor formation and limited reduction of off-notes typical of legumes and cereals.

Despite growing consumer demand for plant-based fermented products, scientific understanding of microbial behavior in plant matrices remains incomplete. Plant substrates impose conditions that differ markedly from milk, making fermentation outcomes less predictable and reducing sensory uniformity. Modern starter development also requires genomic characterization, metabolic profiling, and pilot-scale fermentation trials, which increases the complexity of creating reliable non-dairy cultures [86].

Autochthonous plant-associated LAB represent a promising alternative because they originate from environments that resemble plant-based fermentation matrices. These strains often possess metabolic traits better suited to plant substrates, including efficient carbohydrate utilization and the ability to modulate plant-derived off-flavors [86]. However, autochthonous strains remain less characterized, and their technological properties require further study before they can be reliably used in industrial applications.

Given these limitations of dairy-derived cultures, the variability of plant substrates, and the incomplete characterization of plant-associated LAB, there is a clear need to identify, evaluate, and adapt autochthonous microbial strains for use in plant-based fermentations. This study will address some of above-mentioned gaps by isolating plant-associated bacteria, characterizing their microbial composition, and assessing their suitability for fermentation in oat and pea matrices.

2 Aims of the study

I. The first aim of the study was to investigate beverage fermentation using commercial kombucha as a model system by analyzing its microbial composition, chemical parameters, and sensory properties. The work aimed to assess the stability of its naturally assembled microbial consortium and to determine the extent to which variation in community composition are driven by differences in the growth environment. (Publication I)

II. The second aim of the study was to characterize modified kombucha fermentation in novel environments, such as orange juice and coffee, using a backslopping methodology combined with continuous chemical and microbiological monitoring. Additionally, the study aimed to investigate the ability of newly introduced lactic acid bacteria to adapt to the stable kombucha consortium and to evaluate whether this approach could be used to develop new starter cultures. (Publication II)

III. The third aim of the study was to isolate novel autochthonous non-dairy starter culture candidates adapted through backslopping in plant-based drinks. The study further aimed to characterize the chemical, microbial, and growth-related changes during the adaptation process, with the objective of creating starter cultures from scratch and evaluating their suitability for different plant-based matrices. (Publication III)

3 Material and methods

The current section gives an overview of materials and methods used throughout the studies. More detailed information on the materials and methods is provided in the Publications represented in appendices (Publication I–III). Figure 1 gives general overview and embraces the experimental workflow throughout the three Publications and explains schematically how commercial kombuchas were screened, how tailored kombucha culture was backslopped in orange juice and coffee and how plant-based starter culture was developed.

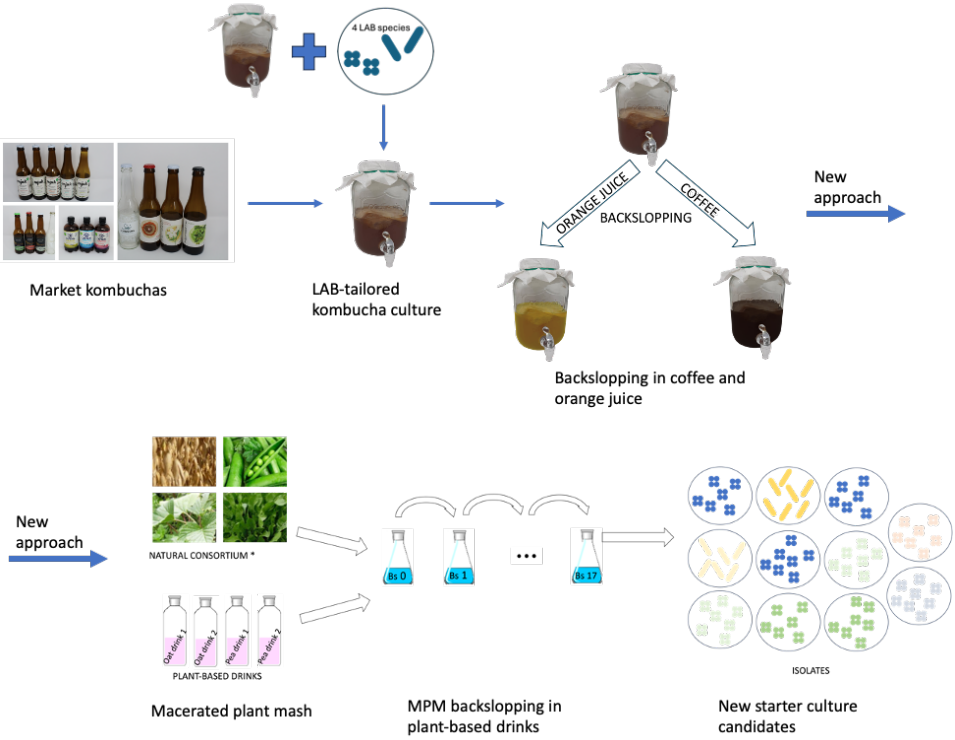

Figure 1. General workflow and experimental design of new plant-based starter culture development.

3.1 Classification of LAB species

Bothe the former and the revised *Lactobacillus* taxonomic classifications are used throughout this thesis. Since 2020, the former *Lactobacillus* genus has been reclassified into 25 genera. At the genus level, the traditional classification is retained for consistency, whereas the updated taxonomy is applied at the species level.

3.2 Collection of cultures and used strains

In Publication I, commercial kombuchas were analyzed to identify the microorganisms typically present in these beverages. Sixteen products were collected from the Estonian market in autumn 2020 (Table S 1). Most samples were Estonian craft kombuchas, but the selection also included products manufactured in Latvia and Portugal (Publication I).

The initial kombucha culture for kombucha backslopping experiments was obtained from a commercial Estonian kombucha brewery (Publication II). The added lactic acid bacterial strains obtained from the TFTAK's (Center of Food and Fermentation Technologies, Tallinn, Estonia) culture collection were used in the Publication II to create LAB-tailored kombucha culture. The strains were originally isolated from sourdough [108]. The LAB strains were *L. brevis*, *L. plantarum*, *P. pentosaceus* and *Companilactobacillus paralimentarius*.

In Publication III, the plant materials for bacterial isolates as candidates for plant-derived starter culture were collected locally. The oat florets were collected from local South Estonian farmers in September 2021. The pea pods and plant leaves (horseradish, blackcurrant) were collected from a garden located in southwest Estonia in September 2021. The collected plant materials were preserved under + 4 °C. Then, the collected floral materials were blended with tap water and crushed with Vorwerk Thermomix® TM6® blender cooker soon after the collection (Thermomix; Vorwerk & Co., Wuppertal, Germany). The ratio between solid material and tap water was 2:5 (v/v). Then, the solid parts of the floral mash were extracted through muslin cloth, and the liquid part was used for inoculation (Publication III).

3.3 Preparation of fermented drinks

Different types of media and plant-based drinks were used to study the diversity and stability of microbial consortia in fermented matrices. In the preliminary cultivation study of kombucha cultures (unpublished data), classical kombucha media were prepared. Initial kombucha fermentation was carried out in black tea supplemented with 10% sugar and incubated at two temperatures: room temperature (approximately 22 ± 2 °C) and 30 °C.

Coffee infusion and orange juice were also included in this study and later used in the backslopping experiment described in Publication II. The coffee infusion was prepared from ground Arabica beans boiled in tap water and supplemented with 10% sucrose. Pasteurized orange juice (Aura orange juice, A.Le Coq) was purchased from a local market.

In Publication III, pasteurized oat drinks (Oatly barista, Oatly AB; Tere fresh oat drink, Tere AS) and pea drinks (Vly, VF Nutrition GmbH; Dryk barista, DRYK ApS) were obtained from an Estonian grocery store.

3.4 Cultivation media

In Publication II, the LAB strains were pre-cultured in De Man–Rogosa–Sharpe (MRS) broth before being added to the LAB-tailored kombucha.

In Publication III, several bacterial agar media were used during the strain-isolation step: MRS agar (NEOGEN Culture Media) and trypticase soy yeast extract medium (M92) were used, each supplemented with 10 g/L sucrose and cycloheximide at 50 mg/L to inhibit yeast growth and support bacterial isolation, respectively.

3.5 Cultivation

In preliminary cultivation of kombucha culture (unpublished data), the Initial kombucha consortia were isolated from market kombuchas. The fermentations were carried out in black tea supplemented with 10% sugar and incubated at two temperatures for 7 days:

room temperature (approximately 22 ± 2 °C) and 30 °C in a climate chamber without aeration. The working volume was 1.5 L, using a vessel with a total capacity of 2 L. The vessels were covered with sterile paper cloths. The inoculation was carried out under the fume hood and all utilities were sterilized. The rate of inoculation was approximately 5% of the previous batch kombucha liquid, which consequently adjusted the pH to 4–4.5. The added piece of SCOBY corresponded to approximately 1/8 of the full pellicle. The same working volume, vessel size and fermentation procedures were also used in Publication II.

In Publication II, the initial kombucha consortium isolated from market kombucha and cultivated at 30 °C was used. Novel LAB strains (*L. brevis*, *L. plantarum*, *P. pentosaceus* and *Companilactobacillus paralimentarius*.) were pre-cultured at 30 °C for 12 h. Subsequently, a 1% (v/v) inoculum, corresponding to approximately 1×10^7 cells/mL of the pre-cultured LAB, was added to the initial kombucha and the mixed culture was cultivated for two weeks. This LAB-tailored culture was subsequently inoculated into orange juice and coffee infusion separately, followed by a backslopping experiment. Kombucha SCOBY was divided into eight equal sectors, and one sector was added to the fresh matrix (either in orange juice or in coffee). As the initial pH of the orange juice was 4, a piece of SCOBY was added to the orange juice without pH adjustment. In the case of the coffee environment, at first, the pH was adjusted to 4.5 with the initial batch of kombucha liquid, and thereupon, a piece of SCOBY was added.

In Publication III, 1% of macerated plant matrix was inoculated to plant-based drinks [oat drink 1 (Od1), oat drink 2 (Od2), pea drink 1 (Pd1), pea drink 2 (Pd2)] to start the fermentation and further followed by a backslopping experiment.

3.6 Backslopping

The orange juice and coffee infusion backslopping continued for five backslopping cycles. The cultivation of kombuchas in the backslopping part was performed as pointed out in the section **3.5 Cultivation**. The duration of a single cycle varied between the environments: two days for the orange-juice kombucha and four days for the coffee kombucha. The schematic figure can be seen in Publication II.

The backslopping of plant-based drinks in Publication III was conducted at 25 °C, 34 °C, and 42 °C for seventeen cycles. Samples cultivated at 25 °C were reinoculated every 48 h, and samples fermented at 34 °Cand 42 °C were reinoculated every 16 h. The reinoculation was done from the previous batch fermented plant-based drink.

3.7 Determination of pH and titratable acidity

pH and titratable acidity (TA) were measured from diluted kombucha samples by the Food and Beverage Analyzer (Mettler-Toledo International Inc., Columbus, OH, USA). All measurements were performed in sets of two replicate measurements. The pH and ORP were determined from oat- and pea-based drinks by using the Consort™ C3010 Benchtop Multiparameter Analyzer.

3.8 Determination of metabolic products

The metabolic products (organic acids, sugars, ethanol) of kombucha and fermented plant drinks were measured by a Waters 2695 high-performants liquid chromatography (HPLC) system (Waters Corporation, Milford, MA, USA) (Publications I-III). The analyzed

liquid was filtered by using 13 mm Philic PTFE 0.2 µm Non-sterile Millex-LG filters (Millipore, Darmstadt, Germany). An HPX-87H column (BIO-RAD Hercules, Hercules, CA, USA) was used, and the system was eluted isocratically with 0.005 M of H_2SO_4 at 0.6 mL/min at 35 °C. A Waters 2487 dual absorbance detector (Waters Corporation, Milford, MA, USA) and a Waters 2414 refractive index detector (Waters Corporation, Milford, MA, USA) were used for the detection and quantification of analytes. The data analysis was performed with Empower software 3 (Build 3471 FR5 SR4, Waters Corporation, Milford, MA, USA).

Free amino acid determination was performed after centrifuging the fermented plant drinks at 14,000 × g for 20 min at room temperature using a Hettich ROTANTA 460 R equipped with a fixed-angle rotor. The supernatant was passed through a 3 kDa molecular-weight cut-off filter (Amicon® Ultra-0.5, Merck KGaA, Darmstadt, Germany) and subsequently diluted with 2 parts ultrapure water before analysis. Free amino acids were derivatized with the AccQ•Fluor reagent (Waters Corp., Milford, MA, USA) following the manufacturer's instructions prior to injection. Analyses were carried out using an ultra-performance liquid chromatography (UPLC) system (Acquity UPLC; Waters Corp., Milford, MA, USA) equipped with a binary solvent manager, sample manager, and photodiode array (PDA) detector, all operated through Empower™ 3.0 software (Build 3471, Waters Corp., Milford, MA, USA). Separation was achieved on a Waters Acquity UPLC AccQ•Tag Ultra column (2.1 × 100 mm, 1.7 µm) maintained at + 55 °C. The injection volume was 1.5 µL, with amino acids eluted at a flow rate of 0.3 mL/min and detected at 260 nm. The total run time was 25 min, and data processing was performed using Empower software.

3.9 Sensory analysis

Sensory evaluation of kombuchas in Publication I was conducted by an expert sensory panel according to ISO 8586:2012. The panel consisted of eight trained assessors, who were between 25 and 48 years old (average age 34). Panelists were subjected to previous training for kombucha sensory analysis. The evaluation was carried out with two different descriptive analysis methods. Quantitative Descriptive Analysis (QDA) was conducted to compare the sensory profile of different kombuchas. Free sorting was used to map kombuchas based on similarities perceived. Assessors evaluated samples in isolated booths in a sensory room which was in accordance with ISO 8589:2007. Planning and conducting sensory tests followed the ISO 6658:2017. Kombucha samples were stored refrigerated at 4 °Cand were served at room temperature (22 °C). All sensory data were collected using RedJade (RedJade Sensory Solutions LLC, Martinez, CA, USA). QDA was conducted as individual evaluations replicated across two sessions, each lasting approximately 40–50 min. Assessors rated the intensity of all attributes on a 10-point scale with verbal anchors (0 = "none", 1 = "very weak", 5 = "moderate", 9 = "very strong"). To minimize carbonation loss—which could influence texture perception—samples were presented in sets of 3–4 and each set was evaluated immediately after bottle opening. The free-sorting task was completed in a separate session of about 15 min, during which all samples were presented simultaneously in randomized order. Assessors were instructed to group the samples freely, creating as many groups as they deemed appropriate.

3.10 Isothermal microcalorimetry study

The 48-channel isothermal microcalorimetry (IMC) TAM IV (TA Instruments, New Castle, DE, USA) and 24-channel IMC TAM III (TA Instruments, New Castle, DE, USA) were used to determine the growth of consortia during the backslopping experiment of plant-based drinks in Publication III. The data were acquired by TAM Assistant Software V2.0.105 (TA Instruments, New Castle, DE, USA) and analyzed in Microsoft Excel 2021 (Microsoft Corporation, Redmond, WA, USA).

3.11 Metagenetic-based quantitative profiling of microbial communities

3.11.1 Microbial cell isolation, PMAxx treatment and genomic DNA extraction

Microbial cells from kombucha beverages (Publication I, II), SCOBY material (Publication I, II), and fermented oat- and pea-based drinks (Publication III) were collected aseptically and concentrated through sequential centrifugation and washing steps appropriate for each matrix. Liquid samples were clarified by low-speed centrifugation to remove larger particles, followed by higher-speed centrifugation to pellet microbial biomass. SCOBY samples were cut into small pieces with sterile tools, washed with a cold buffer, and then centrifuged to obtain microbial pellets for DNA extraction.

In Publication III, total bacterial community (viable and non-viable bacteria) was determined from the fermented oat- and pea-based drinks. The cell suspension was centrifuged at 10000 × g for 10 min at 4 °C (Thermo Scientific MicroCL 21R Centrifuge), the supernatant was aspirated, and the pellet containing the microbial cells was stored at - 20 °C until gDNA extraction. The discrimination of alive bacterial consortia was carried out starting from centrifugation at 5000 × g for 10 min at 4 °C. (Thermo Scientific MicroCL 21R Centrifuge) of cell suspension. The supernatant was aspirated, the pellet was resuspended in 400 µL sterile 0.85 % NaCl solution, and propidium monoazide (PMAxx) treatment was performed as published by Kallastu et al. (2023). After PMAxx treatment, cells were pelleted as described above and stored at - 20 °C until gDNA extraction.

The genomic DNA extraction was performed using the Quick-DNA Fungal/Bacterial Miniprep Kit (Zymo Research, USA) according to the manufacturer's instructions. The concentrations of the extracted DNA were quantified fluorometrically with the Qubit™ 3 Fluorometer (Thermo Fisher Scientific, Waltham, MA, USA) using Qubit dsDNA BR Assay Kit (Thermo Fisher Scientific).

3.11.2 Next-generation amplicon sequencing

The bacterial and fungal composition of studied kombucha beverages in Publication I and II were determined using next-generation amplicon sequencing (NGS). The v4 region of the 16S rRNA gene [109] and the ITS2 region between the 5.8S and LSU rRNA genes [110] were used for sequencing library preparation to determine the bacterial and fungal composition of kombucha(s) samples.

25 ng of extracted gDNA of kombucha samples (Publication I, II) and fermented oat- and pea-based drinks (Publication III) was taken for the amplification stage, and dual indices were used for final libraries and carried as Kazantseva et al., (2021). The NGS was carried out using the iSeq100 platform (Illumina, San Diego, CA, USA) for kombucha samples and MiSeq platform (Illumina, San Diego, CA, USA) for fermented oat- and pea-based drinks. In the iSeq platform, the iSeq 100 i1 Reagent v2 was used and applied

as 2 × 150 cycles paired-end protocol for 16S, and 300 cycles single-end protocol for ITS2 region. For the MiSeq sequencer, the MiSeq V2 Reagent Kit and 300 cycles of the paired-end sequencing protocol were used.

In all Publications, the corresponding bioinformatic analyses for bacterial identification were performed using the open-source BION-meta package [112], [113]. The Quantitative Insights Into Microbial Ecology (QIIME2 [114] was used for fungal taxonomical identification. Taxonomies of fungi were assigned with a q2-feature-classifier plug-in and classify-sklearn [115] method using the fungal ITS classifiers trained on the UNITE reference database.

3.11.3 Quantitative real-time PCR

For estimated quantification of the bacterial and yeast-species detected by the 16S and ITS rRNA amplicon sequencing in the fermented kombucha beverages in Publication I and II, a qPCR was used. Bacterial and fungal-specific gDNA concentrations were measured using qTOWER^{3}G thermal cycler (Analytic Jena, Jena Germany). Standard curves for the quantification were performed applying DNA standards from Femto Bacterial or Fungal Quantification kits (Zymo Research, Irvine, CA, USA). Specific primer pairs 515F/806R for bacterial V4 region of the 16S rRNA gene [109], and UNF1/UNF2 for fungal ITS2 region of the ribosomal gene [116] were used.

Based on the quantification data of the qPCR (Supplementary Table A.1) and according to the taxonomic proportional abundances of the sequencing results, the content of detected bacterial and yeast species of each investigated kombucha was calculated. The number of the amplified PCR products (16S rRNA or ITS2 rRNA gene copies) per volume was calculated based on the equation: Copy number=$(N_A \times m \times D)/(AS \times M)$, where N_A is the Avogadro number 6.02×10^{23} (copies/mol), m is the measured mass of DNA (g), D – dilution coefficient, AS is the amplicon size (bp) and M is the dsDNA molar mass 660 gmol/bp [117]. To estimate the cell numbers, the average copy number of 16S rRNA genes in bacteria was considered as 4.2 [118], and 150 copies for ITS2 [119].

3.12 MALDI-TOF Mass spectroscopy

Matrix-Assisted Laser Desorption/Ionization Time-of-Flight Mass Spectrometry (MALDI-TOF MS) (Bruker MALDI Biotyper, Billerica, MA, USA) was used to identify isolates from backslopped plant drinks at the species level. The analysis was conducted at Tallinn University of Technology in the Department of Chemistry and Biotechnology. Fresh colony biomass was applied to two positions on the target plate and overlaid with 1 µL of 70% formic acid, followed by the addition of the matrix solution (HCCA, Bruker Daltonics, Billerica, MA, USA). Each MALDI run was preceded by calibration using the bacterial test standard (BTS; Bruker Daltonics, Billerica, MA, USA) to ensure measurement accuracy. Microbial identifications were obtained by matching the acquired mass spectra against the Bruker MSP database using Bruker Compass software (Bruker Daltonics, Billerica, MA, USA) under default settings. Identification scores of 2.0 or higher were considered indicative of species-level identification.

3.13 Data visualization and statistical analysis

Statistical analyses and data visualization were performed in R studio (Publication I—version 4.1.0; Publication II—version 4.0.2; Publication III—version 4.3.3 R Foundation for Statistical Computing, Vienna, Austria) using established packages for multivariate

analysis, graphical representation, and data handling. Sensory and compositional datasets were explored through principal component analysis, analysis of variance, and distribution-based visualizations, while free-sorting data were evaluated using correspondence-based multivariate methods. Microbial, chemical, and sequencing-derived datasets were visualized using grammar-of-graphics approaches, supported by tidyverse tools for data manipulation and import. Additional multivariate analyses, including PCA for amino acid profiles and ordination methods such as NMDS for microbial community dissimilarities, were conducted using specialized R packages designed for ecological and sensory data interpretation.

3.14 Genome analysis

3.14.1 Taxonomic profiling

Raw Illumina paired-end whole-genome sequencing reads were processed and taxonomically classified using the nf-core/taxprofiler pipeline (v2.0.0, https://nf-co.re/taxprofiler/2.0.0). Pre-processing quality control was evaluated using FastQC. Short-read trimming and quality filtering were performed using fastp. Taxonomic profiling of the quality-filtered reads was executed using Sylph, querying against the GlobDB database (release r226).

3.14.2 Genome assembly and annotation

De novo assembly of the isolated bacteria was conducted using the nf-core/bacass pipeline (v2.5.0,https://nf-co.re/bacass/2.5.0) operating in short-read mode). The filtered reads were assembled into draft genomes using Unicycler. An initial structural and functional annotation of the assembled scaffolds was carried out within the nf-core/bacass pipeline using Bakta. To ensure the highest accuracy and leverage updated databases, the resulting scaffolds were subsequently reannotated using a newer standalone version of Bakta (v1.12.0) supplied with the full database version 6.0 [120].

3.14.3 Functional screening

Reannotated assemblies were further analyzed for functional genes using nf-core/funcscan pipeline (v3.0.0, https://nf-co.re/funcscan/3.0.0/). Gene prediction was carried out using Pyrodigal. The assemblies were screened across three primary functional categories:

- Antimicrobial Peptides (AMPs): Putative AMPs were identified using a combinatorial approach with ampir, amplify, and macrel. The outputs from these tools were parsed and aggregated using AMPcombi.
- Antimicrobial Resistance Genes (ARGs): A comprehensive screening for ARGs was conducted utilizing multiple tools: fargene, RGI, AMRFinderPlus, DeepARG, and ABRicate. To ensure consistency across the disparate tool outputs, results were standardized using hAMRonization and argNorm.
- Biosynthetic Gene Clusters (BGCs): Secondary metabolite pathways and BGCs were predicted on contigs larger than 3,000 bp. The screening utilized antiSMASH, DeepBGC, and GECCO.

4 Results and discussion

This thesis is based on three publications that focus on understanding the development of microbial consortia during natural and controlled fermentation. As the food industry must meet stringent high-quality standards, starter cultures need to be carefully selected to ensure the consistency and quality of fermented products. To improve our understanding of microbial interactions within fermentation consortia and to support the development of starter cultures better suited to plant-based matrices, this work investigated microbial community composition, microbial interactions, and the resulting chemical changes during fermentation. The main findings are summarized in the following sections, while detailed results and discussions are presented in Publications I-III.

4.1 Communities in different plant-based matrices (Publication I, II, III)

4.1.1 Microbial population of market kombuchas (Publication I)

To better understand the complex interactions between bacteria and yeast and their correlations with the chemical and sensory properties of established fermented foods, kombucha was chosen as a model system. Sixteen different kombuchas were selected from the Estonian market, representing products manufactured in different European countries.

An NGS-based metabarcoding approach was used to characterize the microbial composition of the studied kombuchas, thereby revealing the similarities and unique characteristics of commercially available products. All studied commercial kombucha products differed in starter culture composition; however, products from the same producer often exhibited similar microbial profiles across different matrices (Figure 2A). Kombuchas from manufacturers S, M, and H formed distinct groups, but K1–K2 and C1–C3 did not, which was confirmed by their difference in bacterial composition (Figure 2B). It can be assumed that every manufacturer has its own unique starter culture, which is used for different products. The differences in microbial composition, however, can reflect various technological processes, effects of matrix and additives, and shelf life after production.

More precisely, the highest proportion of bacteria in S1 and S2 beverages belonged to *Zymomonas mobilis* (Figure 2B). The dominant species in kombuchas M1-M5 were *Lactobacillus*, while *Komagataeibacter* and *Bacillus* were in H1-H3 beverages. K1 and K2 kombuchas included mainly *Komagataeibacter* and *Gluconobacter* species. The most diverse bacterial composition across the one manufacturer was in C1-C3 products, where the common bacterial pattern was not detected. Thus, all three kombuchas had totally different major bacteria – *Pseudomonas putida* in C1, *B. coagulans* in C2, and *Gluconobacter oxydans* in C3 beverage. All mentioned species except *P. putida* are commonly found in kombucha [44], [50], [53], [54]. *P. putida* is a soil- and plant-associated bacterium, found mainly in soil and rhizosphere environments, such as plant roots and environmental surfaces [121]. This species detection most likely reflects environmental contamination.

It was observed that commercial kombuchas showed a distinctive pattern in the bacterial composition – LAB dominated in green tea and AAB in black tea beverages. Coton et al., (2017) also showed that LAB species are dominantly found in green tea

kombucha. Villarreal-Soto et al., (2018) also demonstrated that acetic acid bacteria such as *K. rhaeticus* prevail in black tea kombucha.

Yeast communities in the analyzed kombuchas were profiled using ITS2 NGS metabarcoding. Overall, the yeast communities displayed lower taxonomic diversity than the bacterial communities. All identified yeasts belonged primarily to the genus *Dekkera*/*Brettanomyces* (Figure 2C). Kombuchas H, K, M and S had different proportions of *Dekkera anomala* and *D. bruxellensis* in their composition. The S1 beverage along with dominant *D. bruxellensis* contained a low portion of *Zygosaccharomyces parabailii*. Similarly to the bacterial composition, C group products stayed apart and were more unique regarding the yeasts presented. Moreover, they differed even inside the manufacturing group. C1 product along with dominant *D. anomala* had in the composition *Saccharomyces* spp. C2 had two dominant species *H. valbyensis* and other *Saccharomycetales spp.*, and C3 was dominantly represented by *Issatchenkia spp*.

All studied kombuchas differed by their microbial composition, chemical parameters, and thereby sensory characteristics. The presence of AAB and LAB in kombucha and their production of organic acids determine the level of pH in the beverage. According to the Food and Drug Administration (FDA) Food Code model, the pH of commercial kombuchas must be in the range of $2.5 \leq pH < 4$ [122], [123]. Low pH values are required to decrease the contamination risk. Also, the pH must be ≥2.5 to prevent damages caused by drinking acidic drink [122], [123], [124]. The kombuchas in this study were in the required range (pH 2.8–3.7) and therefore according to this criterion were safe to consume.

The absence of a commonly recognized kombucha definition and uncertain technological characteristics allows high diversity in market kombuchas. This permits the appearance of a beverage with a kombucha label but omits its possible beneficial qualities, as in the case of several drinks we studied. The FDA is elaborating to set the variance limits for kombucha safety plan [123], and the Kombucha Code of Practice has stated the product standards and safety requirements [125]. The Kombucha Code of Practice has defined that kombucha is made from tea leaves and coffee and fermented with symbiotic consortia of bacteria and yeasts. Despite that, no official definition of kombucha has been declared yet by the European Food Safety Authority or FDA.

The results from Publication I showed that market kombuchas contained mainly AAB and LAB, however, there were also uncommon species such as *Pseudomonas* spp. and *Bacillus coagulans*. The chemical analysis demonstrated significantly elevated concentrations of ethanol. AABs use ethanol to produce acetic acid [48], [53], [126], [127], [128]. However, high concentrations of ethanol might decrease the LAB population due to toxicity [54]. These findings highlighted key aspects that motivated us to further investigate the kombucha culture. In the next step, the preliminary cultivation of kombucha was performed.

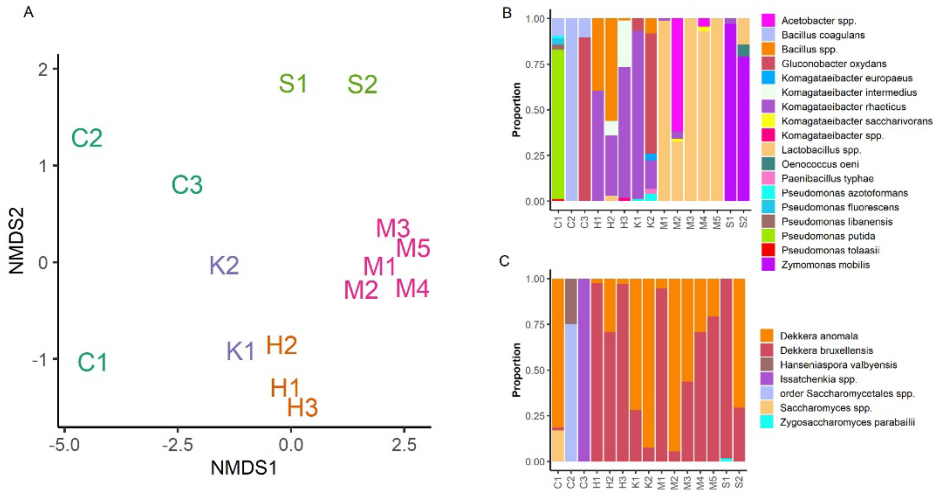

Figure 2. Microbial composition of commercial kombuchas. Non-metric multidimensional scaling of kombucha by 16S metabarcoding results is presented in plot A. Relative abundances of microbial composition according to 16S metabarcoding for bacteria are graphed in plot B, and ITS2 NGS for yeasts in plot C. The data obtained and modified from Andreson et al., (2022).

4.1.2 Preliminary cultivation of kombucha culture (unpublished data)

To test various commercial kombucha potential and culture stability, a preliminary experiment was carried out in laboratory conditions. Four different market kombuchas (K1, S2, H1, M1) from Publication I were selected to evaluate kombucha culture formation and sensory properties. The fermentation was carried out under conventional kombucha settings, using black tea supplemented with 10% (w/v) sucrose and incubated at room temperature (around 22 ± 2 °C). This experiment showed that samples M1 and H1 were unable to initiate fermentation, as no SCOBY formation or decrease in pH was observed. These findings suggest that the product may have undergone post-fermentation processing, such as pasteurization, which rendered the microbial community non-viable.

Both K1 and S2 commercial kombucha beverages contained viable microorganisms capable of initiating fermentation. K1 formed a firm SCOBY more rapidly, whereas S2 produced a fragile SCOBY and required a longer fermentation time compared to K1. Additionally, S2 exhibited slightly unpleasant sensory characteristics, which were associated with sulfur-like off-flavor. According to the 16S metabarcoding analysis, S2 contained *Z. mobilis* (Figure 2B). This bacterium is capable of producing hydrogen sulfide (H_2S) through the assimilatory sulfate reduction pathway [129]. This has been experimentally demonstrated in wild-type strains and is further supported by genomic pathway analysis [130]. Moreover, H_2S is a well-known contributor to sulfurous off-flavors in fermented beverages [131], which is consistent with the sensory characteristics observed in S2.

It has been shown that changing the cultivation temperature of kombucha can alter its species composition [48], [50]. For example, increasing the temperature of the kombucha environment from the usual room temperature to 30 °C may lead to an increase in LAB and a slight decrease in AAB [48]. This finding indicated that the effect of temperature on species composition in kombucha should be tested next.

In the next step, the effect of a 10 °C difference in fermentation temperature on the microbiological composition of the K1 and S2 kombucha culture was evaluated. Both the SCOBY and the kombucha liquid obtained after cultivation at 20 °C and 30 °C were analyzed using an NGS-based metabarcoding method (Figure S 1, Figure S 2). According to the results, at both temperatures, the dominant bacterial species in K1 were *K. rhaeticus* and *Komagataeibacter europaeus*, with *K. rhaeticus* comprising ~96% of the community (Figure S 1). In the beverage fermented at 30 °C, *K. rhaeticus* (87%) and *K. intermedius* (9%) were detected, while *K. europaeus* was not detected. Thus, the temperature change did not alter the dominant genus. LAB species disappeared or were present below the detection threshold. All detected species were from the *Komagataeibacter* genus.

In the temperature study, *D. bruxellensis* was the dominant yeast species in K1 kombucha (Figure S 2). It accounted for 87% of the yeast community in the kombucha cultivated at 20 °C, 88% in the SCOBY grown at 30 °C, and 98% in the corresponding fermented beverage. In the community cultivated at 20 °C, additional yeasts were detected: *Z. parabailii* (8%), *S. pombe* (2%), and 3% of yeasts belonging to the genus *Zygosaccharomyces*. Of these, only *S. pombe* (1%) was also identified among the yeasts detected in the kombucha community cultivated at 30 °C. At 30 °C, two additional yeast taxa appeared: *D. anomala* (6%) and yeasts from the genus *Saccharomyces* (2%).

For S2 classical kombucha, temperature had a stronger effect on community composition (Figure S 1). The bottled product was dominated by *Z. mobilis* (95%), but the SCOBY grown at 20 °C contained only 3% of this species and was instead dominated by *K. rhaeticus* (95%) (Figure S 1). At 30 °C, the community shifted again, with *K. intermedius* (56% in the SCOBY; 47% in the liquid) and *K. rhaeticus* (41% in the SCOBY and 48% in the liquid) become dominant. Small proportions of unclassified *Komagataeibacter* (2%) and *G. oxydans* (1%) were also detected. *Z. mobilis* decreased further and was found only at 1% in the beverage at 30 °C.

For yeasts (Figure S 2), temperature had a clear impact on community structure. *D. bruxellensis* remained the dominant species at both temperatures, but its proportion increased substantially with heat: from 73% at 20 °C to over 90% at 30 °C (92% in the SCOBY and 98% in the beverage). As *D. bruxellensis* expanded, the relative abundance of other yeasts declined. At 20 °C, *S. pombe* (19%) and *Z. parabailii* (4%) were still present at notable levels, along with a small fraction of other *Zygosaccharomyces* species. At 30 °C, however, these taxa were detected only at very low levels—*S. pombe* at 6% in the SCOBY and 2% in the beverage, and *Z. parabailii* at just 1% in the SCOBY. No additional *Zygosaccharomyces* species were detected.

Two main conclusions can be drawn from this temperature variation experiment. First, cultivation at 20 °C and 30 °C had a measurable effect on the communities of both K1 and S2 kombuchas cultures. However, this effect was relatively minor in K1 but considerably more pronounced in S2, likely reflecting differences in the initial composition and stability of the microbial consortia. Second, the results support the view that the kombucha community is a living, dynamic and cooperative ecosystem. The appearance, disappearance, and re-emergence of specific taxa under different cultivation conditions suggest that the relative abundance of individual species continuously fluctuates in response to environmental factors. Consequently, species may become dominant or be suppressed depending on the fermentation conditions, causing their abundance to rise above or fall below the detection threshold of NGS-based metabarcoding.

The aim of this experiment was to determine whether fermentation temperature could increase the abundance of the LAB species. However, the LAB population remained below the detection threshold at 30 °C in this experiment. The results also demonstrated that a 10 °C change in fermentation temperature did not substantially alter the composition of the core kombucha microbiota. Species belonging to the genus *Komagataeibacter* and *D. bruxellensis* remained dominant under both temperature conditions. In both kombucha cultures, LAB species either disappeared or remained below the detection threshold. Therefore, the next objective was to increase the LAB population by inoculating selected LAB strains and then evaluate their effect on the microbial consortium and the sensory properties of the fermented beverage. Furthermore, because S2 kombucha exhibited an unpleasant sulfur-like off-odor, K1 was selected for the subsequent experiments.

4.1.3 Creation of LAB-tailored kombucha culture (Publication II)

Kombucha is a stable ecological community of microorganisms. To better understand the ecological interactions within the established cultures, as well as the cooperative and cross-feeding potential of its members, the commercial kombucha starter was challenged with LAB.

A tailored kombucha culture was created by inoculating four LAB species into the culture derived from the commercial kombucha culture K1, which had been cultivated at 30 °C in black tea. Throughout the remainder of this thesis, this consortium is referred to as the initial kombucha culture. The introduced LAB species had previously been isolated from sourdough and included *L. brevis, L. plantarum, P. pentosaceus* and *C. paralimentarius.*

After two weeks of fermentation, LAB-tailored kombucha was sensorially different. The initial kombucha had strong acidic taste and odor, whereas the LAB-tailored kombucha exhibited a milder acidic profile resembling lactic acid. Therefore, the addition of LAB influenced the sensory characteristics of kombucha by making it more consumer friendly. Nevertheless, the TA and pH values did not have a significant difference between the LAB-tailored and initial kombucha.

The 16S and ITS2 results showed a variation between these two kombuchas. In bacteria composition, *K. rhaeticus* dominated in LAB-tailored kombucha, while the initial kombucha contained two other species like *K. europaeus* and *K. intermedius*. In yeasts, the LAB-tailored kombucha had more than one dominant species, however, *D. bruxellensis* prevailed in initial kombucha.

Additionally, a small stability test was performed in the four-month period. Although added LAB species were detected at the beginning of the experiment, at the end, they were not found in LAB-tailored kombucha using 16S metabarcoding. To increase the detection of introduced species, the samples were analyzed by qPCR with strain-specific primers at five sampling points (Figure 3). LAB abundance was relatively high at the first sampling point, with values between 10^4 and 10^5 cells per milliliter of kombucha. However, the abundance of LAB species decreased by 1–3 log in the second sampling point and stayed below the detection threshold until the end of the fourth month. Interestingly, tailored kombucha culture affected consortia composition, decreasing bacterial diversity and significantly shifting the yeast profile of initial cultures. Nevertheless, the modified LAB-tailored kombucha culture was used further in backslopping experiments due to the preferred sensory parameters.

The experiment showed that the introduction of LAB species into a mature SCOBY altered the microbial community structure by changing the relative abundances of the resident microorganisms. However, the introduced LAB strains gradually disappeared over time. These findings further support that kombucha is a stable yet dynamic microbial ecosystem, in which microorganisms interact through cooperative relationships and cross-feeding while continuously adapting to environmental stimuli. Despite this ecological flexibility, modifying the core microbiota of kombucha remains challenging. Although LAB can potentially adapt to novel fermentation matrices, the sourdough-derived strains used in this study were not well suited to the kombucha ecosystem, reflecting the fundamental ecological differences between sourdough and kombucha fermentations. Therefore, an alternative strategy was adopted for further experiments.

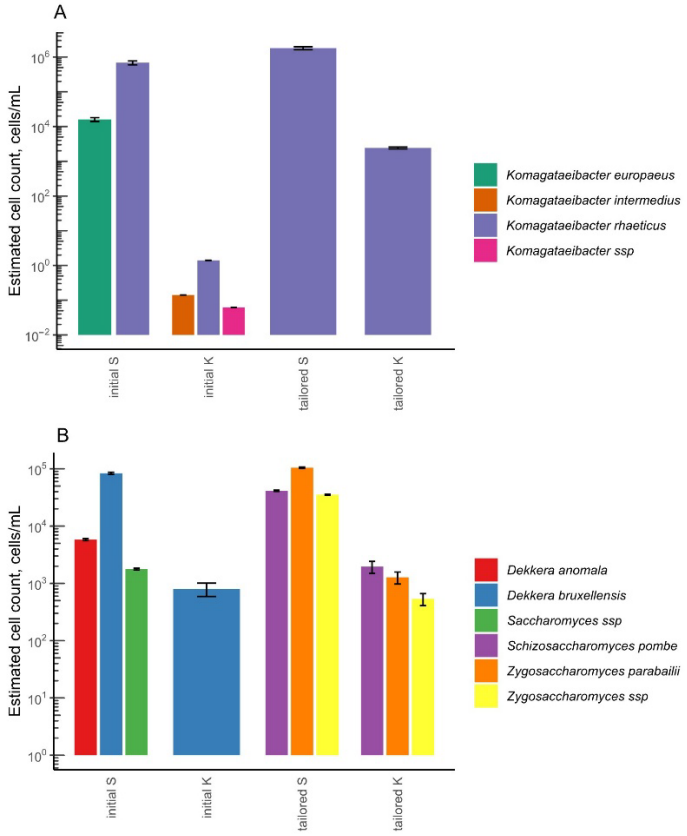

Figure 3. Microbial composition of initial and tailored kombucha at the end of the backslopping process according to 16S metabarcoding for bacteria (A) and ITS2 NGS for yeast (B) graphed as normalized abundances. The abbreviation S stands for SCOBY, and K for kombucha liquid. Modified data were obtained from Andreson et al., (2023).

4.1.4 Microbial population of macerated plant mass (Publication III)

As interest in plant-based fermented foods has grown, research has increasingly focused on developing non-dairy starter cultures, as microbial species isolated from dairy products often do not perform effectively in plant-based matrices.

Developing effective starter cultures for plant-based fermentations requires microorganisms that are able to grow, adapt, and remain stable in plant-derived substrates. Earlier research has shown that many allochthonous microorganisms struggle to establish themselves in new environments, particularly when transferred into plant-based matrices. These findings highlight the importance of identifying autochthonous microorganisms naturally associated with plant substrates, as they are more likely to adapt, persist, and perform effectively under these conditions.

Previous studies have shown that autochthonous species have more potential to modify the initial composition than allochthonous species [86], [132], [133]. For example, Di Cagno, Surico, Paradiso, et al., 2009 showed that tomato juice fermented with autochthonous species had a shorter lag phase, higher total antioxidant activity during storage, and a positive odor effect due to the synthesized volatile compounds than allochthonous species. Therefore, isolation and investigation of autochthonous species could lead to more stable culture and preferred sensorial attributes in the end-product. This was the new approach that was tested in further experiment. With this rationale, the next stage of the work focused on generating a starter culture derived directly from plant materials rather than introducing microorganisms originating from ecologically unrelated environments.

In the frame of the next study, autochthonous bacteria from plant materials, including oat florets, pea pods and leaves from horseradish and blackcurrant, were isolated. The selection of pea pods and oat florets was done because the new matrix that was planned to use was pea drink and oat drink. Horseradish and blackcurrant leaves were selected due to their long historical use in spontaneous fermented foods because of their aroma notes. Additionally, the reason for selecting plant ground parts was to avoid obtaining too many soli bacteria.

Two types of commercial plant-based beverages – oat and pea—were selected as matrices for evolutionary adaptation due to the relevance of these crops to the Nordic market and their potential for future applications. Both conventional and PMAxx-treated 16S metabarcoding were employed to differentiate total and viable microbial communities accordingly (Figure 4). The initial microbial composition was characterized following maceration of the plant materials.

The composition of the total and viable microbiota in the mash differed slightly, although the majority of the initial consortia were represented by regular plant inhabitants. The largest proportion of microbiota was formed by environmental inhabitants such as *Curtobacterium flaccumfaciens* followed by *Sphingomonas* spp., *Frondihabitans australicus*, and *Pantoea agglomerans* (Figure 4). Thus, *C. flaccumfaciens* is a well-known bean pathogen [135], whereas *Sphingomonas* spp are common inhabitants of plant tissues and have been reported to promote plant growth and suppress plant pathogens [136]. Interestingly, *Pantoea ananatis* and *Neorhizobium galegae* were detected only in the PMAxx-treated sample and were absent from the sample analyzed by the standard sequencing methodology, showing the importance of differentiating between total and viable microorganisms, as only viable microorganisms actively contribute to ecosystem function and can subsequently be isolated for potential technological applications. The initial LAB population accounted for less than 0.1% of the

total microbial community. In the PMAxx-treated sample, *Enterococcus* spp. Represented the dominant viable LAB, whereas *E. faecium* prevailed in the untreated sample representing the total microbial consortia. Additionally, *Lactobacillus delbrueckii*, *Lactobacillus* spp., and *Ligilactobacillus aviarius* were detected among the viable bacterial population.

In summary, the initial microbial consortia consisted predominantly of viable environmental bacteria, whereas LAB species represented only a minor fraction of the community, with *Enterococcus* spp. Being the dominant LAB. In the subsequent adaptation phase, the macerated plant mash was propagated through 17 consecutive backslopping cycles in two plant-based matrices (oat and pea drinks) to enrich the LAB population and promote the selection of microorganisms adapted to these substrates.

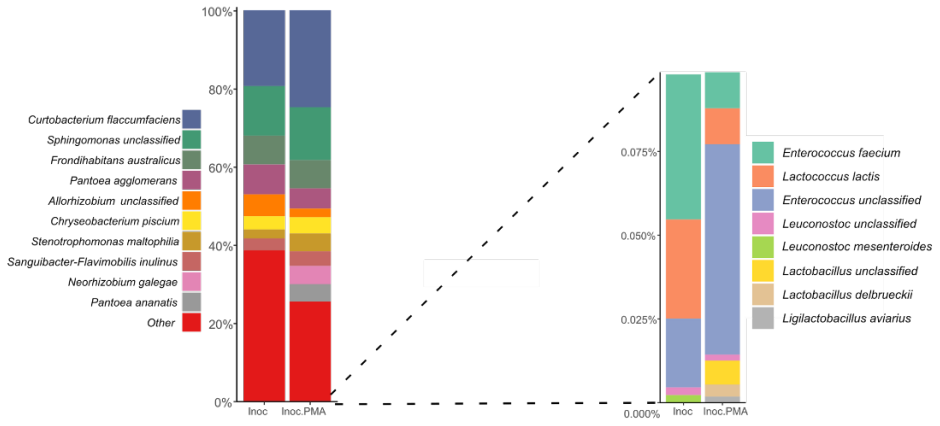

Figure 4. Bacterial composition of the inoculum. The results of standard DNA sequencing methodology (Inoc) and DNA sequencing methodology modified with PMAxx treatment (Inoc.PMA) for detection of viable bacteria species are demonstrated. From the total of detected live species less than 0.1 % of LAB are zoomed in on the right side of this figure. Obtained from Andreson et al., (2024).

4.2 Backslopping propagation influence on microbial composition and dynamics of coffee and orange-juice kombuchas (Publication II)

To adapt and evolve established and tailored consortia, as well as newly isolated bacterial strains for plant-based food matrices, a continuous backslopping approach was used under different experimental conditions using two sets of matrices: orange juice and coffee infusion, and pea and oat drinks. During these experiments, changes in microbial community composition, chemical characteristics, and sensory properties were monitored. The effect of the fermentation matrix and temperature provided valuable insight into the dynamics of consortia development, adaptation and stabilization.

4.2.1 Chemical composition and sensory evaluation of orange-juice and coffee kombucha

As described in **section 4.1.3** (Publication II), the LAB-tailored kombucha culture was previously developed. Next, this LAB-tailored culture was further examined to determine how backslopping propagation in different plant-based matrices influences the microbial

composition of kombucha. The culture was propagated through sequential backslopping cycles in two untraditional matrices—coffee and orange-juice—and the resulting microbial and chemical dynamics were monitored. These analyses were complemented by sensory evaluation to provide an integrated assessment of how matrix-dependent propagation shapes the development of the LAB-tailored kombucha culture.

As mentioned in **section 1.4.1**, the classical kombucha is brewed from black or green tea leaves; however, it is also permitted to use different plant-based beverages to brew kombucha and still label the product as kombucha. Therefore, from now on, we used labels coffee kombucha and orange-juice kombucha.

A preliminary optimization study was performed before the backslopping experiment of orange-juice kombucha. The fermentation of LAB-tailored kombucha was monitored over a period of five days. pH, titratable acidity, and sensorial characterization were performed (data not shown). After more than two days of fermentation, the orange-juice kombucha became sensorially intolerable because of the obtained rotten and strong acidic taste. Therefore, the duration of backslopping cycle of orange-juice kombucha was selected as two days. The same preliminary study was also carried out with coffee, to understand the exact day (based on sensorial acceptance) when to do the backslopping. The sensory properties of fermented drinks became unpleasant after the fourth day. Therefore, the duration of one BS cycle for coffee kombucha was four days. The whole experiment continued for five backslopping cycles.

The classical pH of natural orange-juice was 3.99 ± 0.02 (Figure 5A). TA values of orange-juice kombucha rose rapidly cycle by cycle. The highest TA values were detected at the fourth and fifth BS cycles. Therefore, it was a trend.

The coffee infusion pH at the beginning was 5.69 ± 0.04, and it was further adjusted to around pH 4.7–4.9 (Figure 5B). The lowed detected pH of coffee kombucha was 4.02 ± 0.01. The same trend in TA values was exhibited as in the case of orange-juice kombucha. In both environments, the later backslopping cycles ended faster. Therefore, these results refer to faster adaptation by the kombucha microbes.

The dominative acid in orange-juice kombucha was citric acid throughout the experiment. This came from the matrix mainly, because it is detected that orange juice contains citric acid around 9.7-15.1 g/L [137]. Acetic acid showed the greatest increase throughout the backslopping cycles (0.04 ± 0.00 to 3.08 ± 0.02 g/L) (Figure 6A). The same tendency was seen in coffee kombucha, where the acetic acid increased from 0.32 ± 0.01 to 4.86 ± 0.02 g/L (Figure 6B). Ethanol concentration increased throughout the backslopping cycles in orange-juice kombucha (Figure 6A). The highest ethanol concentration was 23.10 ± 0.04 g/L at the end of experiment. This may indicate shift in consortia towards yeasts, but to conclude that the experiment lasted a short time. However, the coffee kombucha ethanol concentration changes at BS timepoints were different. The ethanol concentration rose to 8.58 ± 0.28 g/L after the first cycle, then decreased to 5.91 ± 0.48 g/L after the second, with similar levels in cycles two and three (Figure 6B). By the end of the fifth cycle, ethanol increased again to 10.32 ± 0.32 g/L, which remained about two-fold lower than in the orange-juice kombucha. Fructose and glucose in orange-juice kombucha initially represented roughly half of the sucrose concentration. A shift in carbohydrate utilization occurred between BS 1.2 and BS 3.1, after which sucrose declined more rapidly than the monosaccharides. By the end of the fifth cycle, sucrose was nearly depleted, while residual fructose and glucose remained. However, coffee kombucha showed only minor changes in sugar concentration compared to orange-juice kombucha, in which the balance of sugars shifted at the end

of the backslopping cycles. The most likely explanation is that sugars were converted to ethanol by yeast, as indicated by the high ethanol level at the end of the last BS cycle.

During backslopping in orange juice, the following organic acids were produced: gluconic acid, glucuronic acid, lactic acid, and succinic acid (Figure 6A). In contrast, backslopping in coffee infusion resulted in the formation of formic acid, gluconic acid, and succinic acid (Figure 6B). Gluconic and glucuronic acids are characteristic organic acids of kombucha fermentation [44], [45], [47], [48], [50], [51], [52], [53], [54]. *Komagataeibacter* spp. produces gluconic acid when oxidizing glucose. During fermentation, these AAB utilize fructose and glucose to form a cellulose network as a secondary metabolite [138], [139], while enzymatic oxidation of glucose at carbon 1 and 6 leads to the formation of gluconic and glucuronic acids [50], [139]. Other organic acids detected in orange-juice and coffee-infusion kombucha are also commonly reported in the literature [45], [50], [53]. Overall, both environments show an acceleration of the fermentation rate during backslopping (Figure 6), indicating that the species adapt and evolve in response to environmental change.

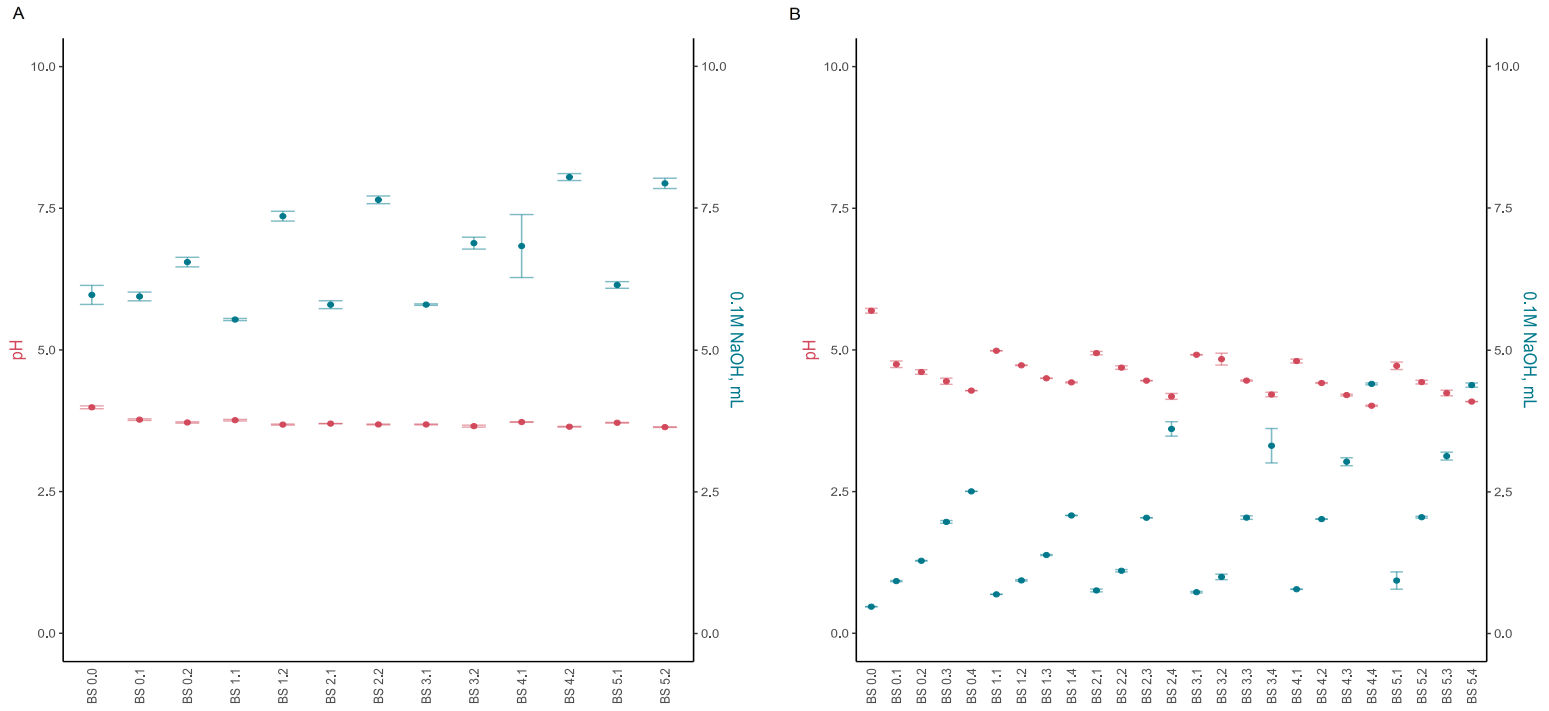

Figure 5. The pH (red dots) and titratable acidity (blue dots) values of orange-juice kombucha (A) and coffee kombucha (B) during backslopping cycles. Titratable acidity units are mL of 0.1 N NaOH. The abbreviation BS stands for backslopping. The first number shows the backslopping cycle and the second number states the day of the backslopping cycle. The obtained data are modified from Andreson et al., (2023).

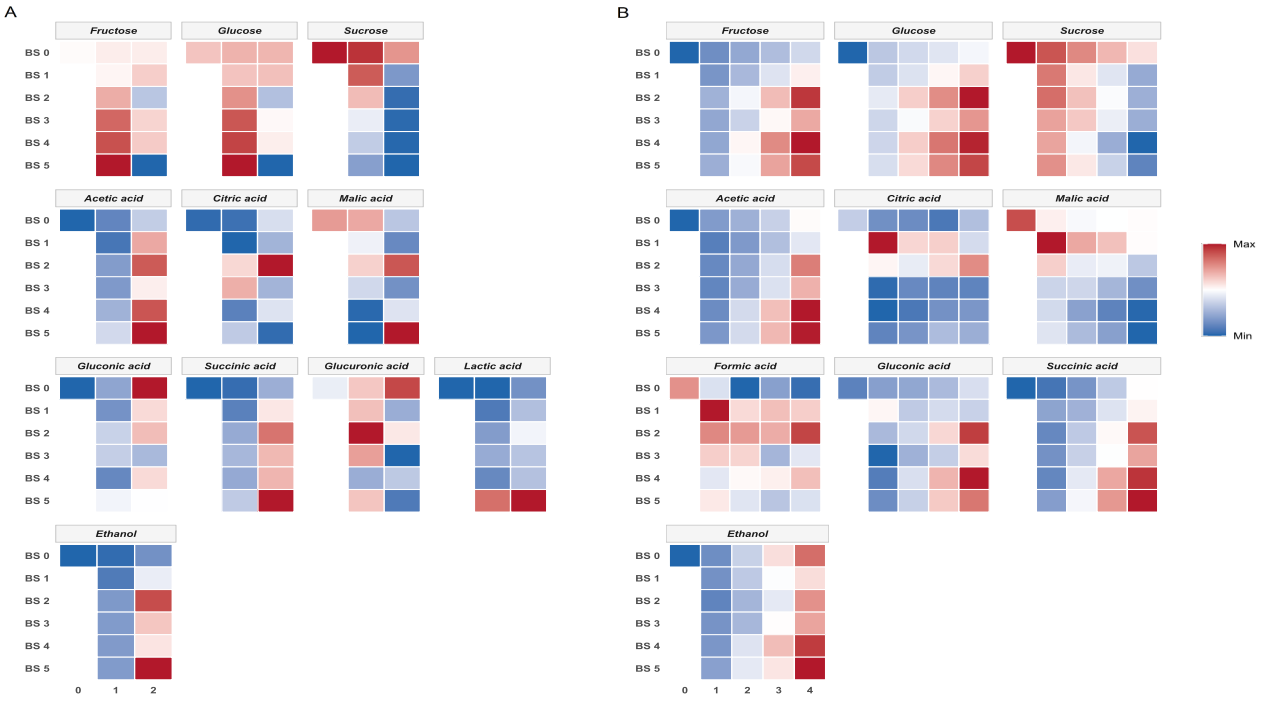

Figure 6. Heat plots to present organic acids, sugars and ethanol changes during backslopping cycles. Heat plot A demonstrates orange-juice kombucha backslopping, and heat plot B is for coffee kombucha. Red indicates increased levels, whereas blue indicates decreased levels of the detected metabolites relative to Day 0. The abbreviation BS stands for backslopping. The first number shows the backslopping cycle and the second number states the day of the backslopping cycle. Bar chart figure with concentrations is in Andreson et al., (2023).

4.2.2 Microbial composition of orange-juice and coffee kombucha

The microbial composition of orange-juice kombucha was detected at each backslopping cycle using only the SCOBY, as it served as the inoculum transferred to each fermentation batch during the backslopping process. On the contrary, both the fermented liquid and the SCOBY were transferred during coffee backslopping process, and therefore both fractions were analyzed to characterize the microbial communities in their respective ecological niches. The SCOBY and the fermentation liquid represent distinct ecological environments and harbor different microbial communities. The BS0 cycle corresponds to the initial adaptation phase, during which the microbial consortium was transferred from the original black tea matrix (BS0.0) to either orange juice (BS0.2) or coffee infusion (BS0.4).

In orange-juice kombucha, two dominant bacterial species—*K. rhaeticus* and *K. intermedius*—were present throughout the experiment (Figure 7A). *K. rhaeticus* dominated the early backslopping cycles, when total cell counts were lowest ($1.81 \pm 0.18 \times 10^6$ cells/mL), whereas *K. intermedius* increased steadily and became the prevailing species in later cycles ($1.12 \pm 0.12 \times 10^7$ cells/mL). Low-abundance *Komagataeibacter* reads were also detected across several stages. By the end of fermentation, *K. intermedius* was the dominant species in the orange-juice kombucha.

The sequencing results revealed that the coffee kombucha (Figure 7B) contained the same two major bacterial species during the backslopping as in the orange-juice kombucha. *K. rhaeticus* occurred only from point BS 0.0 to BS 1.4 in both the SCOBY and the liquid, and it dominated in the kombucha liquid at all backslopping points (BS 0.0 to BS 5.4), although total bacterial counts in the liquid remained low. In contrast, the bacterial cell counts in SCOBY remained relatively stable throughout the experiment, reaching $1.32 \pm 0.07 \times 10^5$ cells/mL at BS 5.4, similar to BS 0.4.

Additionally, the SCOBY consistently contained approximately twice as many bacterial cells as the liquid. This is likely due to the aerobic nature of acetic acid bacteria, which rely on oxygen to oxidize sugars into organic acids. In addition, these species produce a dense cellulose matrix that forms a thick, spongy structure capable of physically retaining a higher density of bacterial and yeast cells than the surrounding liquid. Being positioned at the air–liquid interface, where oxygen availability is greatest, further supports their accumulation within the SCOBY.

The highest SCOBY counts were observed for *K. rhaeticus* at BS 0.4 ($1.62 \pm 0.23 \times 10^7$ cells/mL) and for *K. intermedius* at BS 3.4 ($9.03 \pm 0.77 \times 10^6$ cells/mL). *K. rhaeticus* dominated early in the process, while *K. intermedius* increased during later backslopping cycles, and the two species reached parity by the end. Total SCOBY cell density declined over the course of the experiment, reaching $2.25 \pm 0.10 \times 10^6$ cells/mL at BS 5.4. Overall, the coffee backslopping process stabilized both the bacterial composition and overall abundance.

The results showed two yeast genera throughout the experiment in orange-juice kombuchas (Figure 8A). At BS 0.0, *Z. parabailii* dominated, followed by *S. pombe,* and *Zygosaccharomyces spp*. The lowest cell count of yeast, similar to the bacterial cell load at BS 0.0, was measured as $1.81 \pm 0.05 \times 10^5$ cells/mL. Total yeast abundance peaked at BS 0.2 ($3.35 \pm 0.62 \times 10^7$ cells/mL), while species proportions remained similar. From BS 2.2 onward, *S. pombe* became dominant with the cell count of $4.10 \pm 0.31 \times 10^6$ cells/mL and increasing further by BS 5.2. Although yeast numbers rose

again toward the end (1.10×10^7 cells/mL), they remained slightly lower than at the beginning.

The coffee kombucha results showed that it contained the same two yeast genera as the orange-juice kombucha (Figure 8B). At the beginning of BS experiment in black tea (BS 0.0), the kombucha liquid and SCOBY contained different dominant yeast species. The *Z. parabailii* prevailed in SCOBY but *S. pombe* in the kombucha liquid. In general, the *S. pombe* was dominant species in kombucha liquid throughout the backslopping cycles. Interestingly, the total cell count of yeast in SCOBY was roughly two- to three-fold higher than in kombucha liquid with minimum cell count in as BS 4.4 ($2.29 \pm 0.65 \times 10^2$ cells/mL). Throughout the backslopping experiment, the yeast community in coffee kombucha liquid and SCOBY decreased consistently. Final counts were $6.42 \pm 0.26 \times 10^5$ cells/mL and $1.18 \pm 0.06 \times 10^3$ cells/mL, correspondently.

To complement the bacterial results, the yeast community dynamics were also examined across both matrices. Overall, both backslopping environments showed clear shifts in species dominance over time. Comparing the bacterial composition of the orange-juice and coffee backslopped LAB-tailored kombucha with the initial kombucha, the original SCOBY contained two species: *K. rhaeticus* and *K. europaeus*. In contrast, the orange-juice backslopped LAB-tailored kombucha comprised three species: *K. rhaeticus*, *K. intermedius*, and *Komagataeibacter* spp. Similar to our observations of single-species dominance, Tolu et al., 2022 reported the same pattern in backslopped sourdough. These findings indicate that, despite environmental modification, the core microbial community tends to persist within the matrix [140], [141].

In this study, the microbial composition shifted dynamically across the backslopping cycles. Several initially detected bacterial species were under the detection limits during intermediate cycles but reemerged at later stages in coffee kombucha. This pattern showed that microbial populations did not change in a steady direction but shifted back and forth over the cycles as conditions in the environment changed [142]. These fluctuations indicate that microbial succession in kombucha is inherently non-linear and reflects continuous ecological re-balancing in response to substrate composition and metabolic by-products.

Dominant bacteria also differed by substrate: *K. rhaeticus* prevailed in the initial tea kombucha, *K. intermedius* dominated in orange juice, and *K. rhaeticus* regained dominance only at the end of the coffee fermentation. For yeasts, LAB addition markedly altered the community structure. While *D. bruxellensis* dominated the initial kombucha inoculum, *Zygosaccharomyces spp.* became predominant in the LAB-tailored version (Figure 3). In contrast to bacteria, yeast communities in orange juice and coffee did not revert to the initial composition. These patterns suggest that microorganisms present at low abundance in the starter do not disappear but persist at sub-detectable levels and can proliferate when conditions become favorable. Thus, backslopping plays a key role in shaping microbial consortia by selectively promoting or suppressing specific taxa depending on the environment.

An additional trend in the coffee kombucha was that ethanol levels increased over the cycles. This rise was associated with higher acetic acid production. This phenomenon could be explained by the fact that both AAB and yeast species are active in the kombucha, where yeast is continuing of producing ethanol and AAB converts yeast-produced ethanol into organic acids. That relationship was less evident in the orange-juice kombucha. ITS2 metabarcoding data showed that *S. pombe* was initially abundant, decreased slightly, and then increased again by the end of backslopping,

suggesting adaptation to the orange-juice matrix. In both orange-juice and coffee kombucha, ethanol concentrations eventually reached levels considered inhibitory for AAB, limiting their ability to convert all ethanol to acetic acid. *Komagataeibacter* species tolerated and adapted to these elevated ethanol conditions, whereas the supplemented LAB did not. This highlights that metabolic stressors, in this case particularly ethanol accumulation, act as strong selective force that shape the final consortia composition by favoring ethanol-tolerant taxa to prevail.

Saad et al., (2025) demonstrated similar phenomena of different kombucha cultures. However, they analyzed mother and daughter SCOBY as well as the liquid at each cycle during three backslopping cycles. Therefore, the presence of mother SCOBY might have had less influence on the final microbial population than in Publication II, where there was only one SCOBY. Even though the approach of Saad et al., (2025) was different, the main conclusions aligned with those of the Publication II—despite methodological differences, both studies identify *Komagataeibacter* spp. as the dominant acetic acid bacteria, yet the patterns of succession diverge. Dynamic and non-linear yeast succession, with species disappearing and reappearing across cycles demonstrated in both studies.

Together, the two studies demonstrate that backslopping is a powerful ecological filter that reshapes microbial consortia by selectively promoting taxa best adapted to the prevailing environment. Both studies show that microbial succession is dynamic rather than linear, with low abundance taxa persisting at sub-detectable levels and re-emerging when conditions become favorable. However, the drivers of microbial change differ: the present study highlights matrix driven selection in orange juice and coffee, while Saad et al., (2025) emphasizes biofilm structural selection between mother and daughter pellicles. These complementary perspectives illustrate how both biofilm structure and substrate composition contribute to the long-term evolution of kombucha microbial communities.

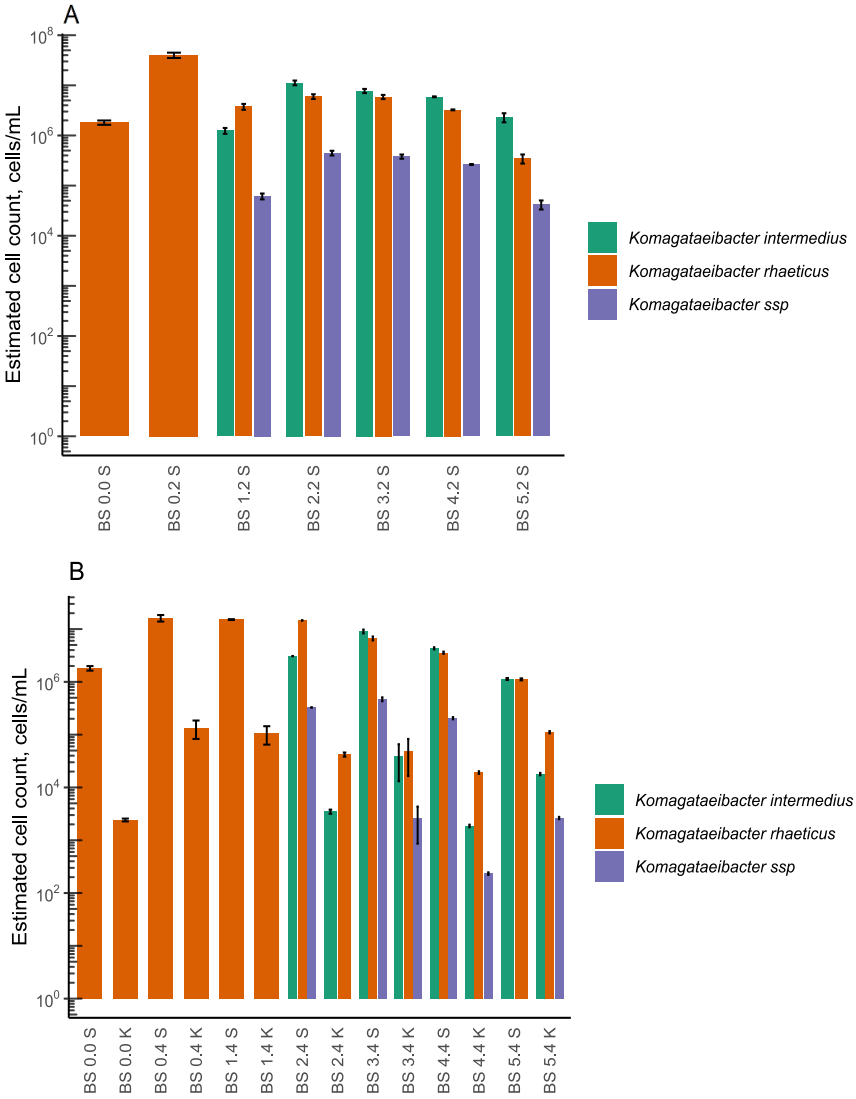

Figure 7. Bacterial composition of orange-juice (A) and coffee (B) kombucha during backslopping cycles according to 16S metabarcoding. The graphs are presented as normalized abundances. The first number shows the backslopping cycle and the second number states the day of the backslopping cycle. Obtained data are modified from Andreson et al., (2023).

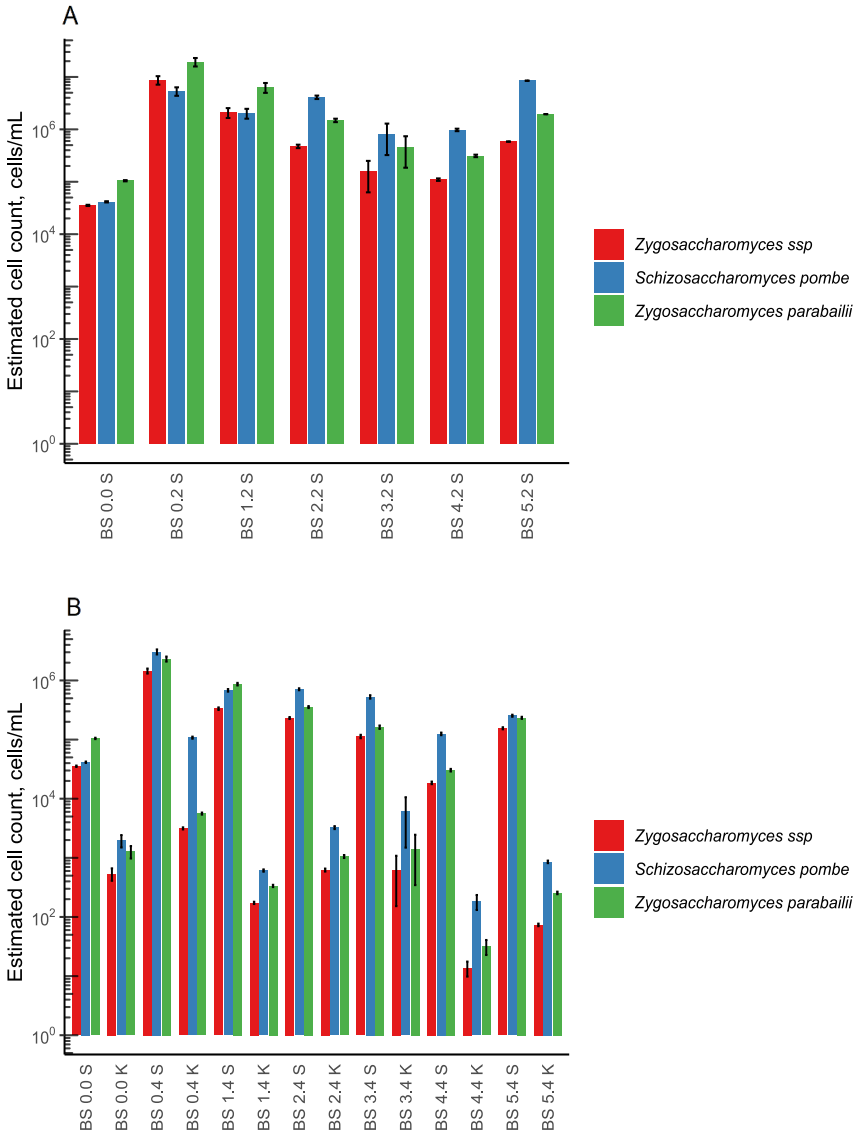

Figure 8. Yeast composition of orange-juice (A) and coffee (B) kombucha during backslopping cycles according to 16S metabarcoding. The graphs are presented as normalized abundances. The first number shows the backslopping cycle and the second number states the day of the backslopping cycle. Modified and obtained from Andreson et al., (2023)

4.3 Backslopping propagation influence on microbial composition and dynamics of pea-oat base fermented drinks (Publication III)

As described in **section 4.1.4** (Publication III), the macerated plant mash was prepared and its microbial composition characterized. The results showed that the consortium was dominated by typical soil/plant inhabitants, while the population of LAB remained below 0.1%. This indicated that LAB were still present in the matrix, but the community was primarily composed of soil-derived species.

Based on previous findings demonstrating that kombucha culture composition can be modulated through backslopping propagation, the macerated plant mash was subsequently used to inoculate plant-based matrices (oat and pea drinks). Even though backslopping is a commonly used inoculation procedure based on the addition of a small part of previously fermented product into a fresh similar environment [108], this technology is not often used for bacterial strain adaptation and novel complex starter culture generation. A 17-cycle backslopping experiment was conducted to enrich the LAB population in the initial consortium, with the aim of increasing their abundance or potentially enabling them to outcompete the originally presented environmental-derived bacteria.

To further diversify and stabilize the adapted microbiota, the backslopping was carried out under different temperature regimes: 25 °C, 34 °C and 42 °C. A temperature of 25 °C was selected to enrich the environment by mesophilic LAB species that are incredibly important for producing food that require gentler, medium-heat fermentation (like cheese) without requiring specialized, high-heat equipment. Also, these bacteria often can produce aromatic molecules such as diacetyl, which is the main aroma component that gives dairy products a milky and creamy flavor [106]. The lower temperature effectively inhibits the activity of the enzyme diacetyl reductase. This enzyme typically reduces and degrades the desired diacetyl into acetoin, which is a less aromatic compound with a much weaker buttery note and therefore reduces the overall flavor intensity of the product [143]. By cultivating at 25 °C, the degradation is minimized, allowing the LAB to retain and accumulate higher concentrations of diacetyl [144]. 34 °C was an optimum chosen to enrich the microbiota by faster growing mesophilic strains with faster acidification rate, while 42 °C was chosen to isolate thermophilic species [135].

The backslopping experiment was performed and consortia stability was measured by isothermal microcalorimetry (IMC). IMC is a unique method with high sensitivity which measures the heat production or absorption by metabolically active microorganisms in hermetically sealed ampoules [145], [146], and thereby describes fine changes in their composition. The analysis began with the microbial composition of the macerated floral mass together with the biomass from the plant-based drinks at the zero time-point. The plant-based drinks were not sterilized because sterilization requires very high pressure and temperatures (at least 140 °C), and because allowing beneficial strains naturally present in the drink matrix to join the consortium may enhance the sensory profile of the final product. The subsequent numbers refer to each BS cycle. All the consortia curves were different by their shape, indicating unique cooperation and interaction between the members. According to IMC, consortia Od1-25 and Od2-25 achieved stability by the 8th cycle (Figure 9A), while the pea drinks stabilized four cycles earlier. At 34 °C, according to IMC, all plant drinks reached stability by the fourth cycle (Figure 9B). For consortia cultivated at 42 °C, stability was also observed on the fourth cycle, except for Pd1-42, which settled on the eighth cycle (Figure 9C). Therefore, depending on the matrix and temperature, stabilization took various time and each consortia had its own unique growth curves.

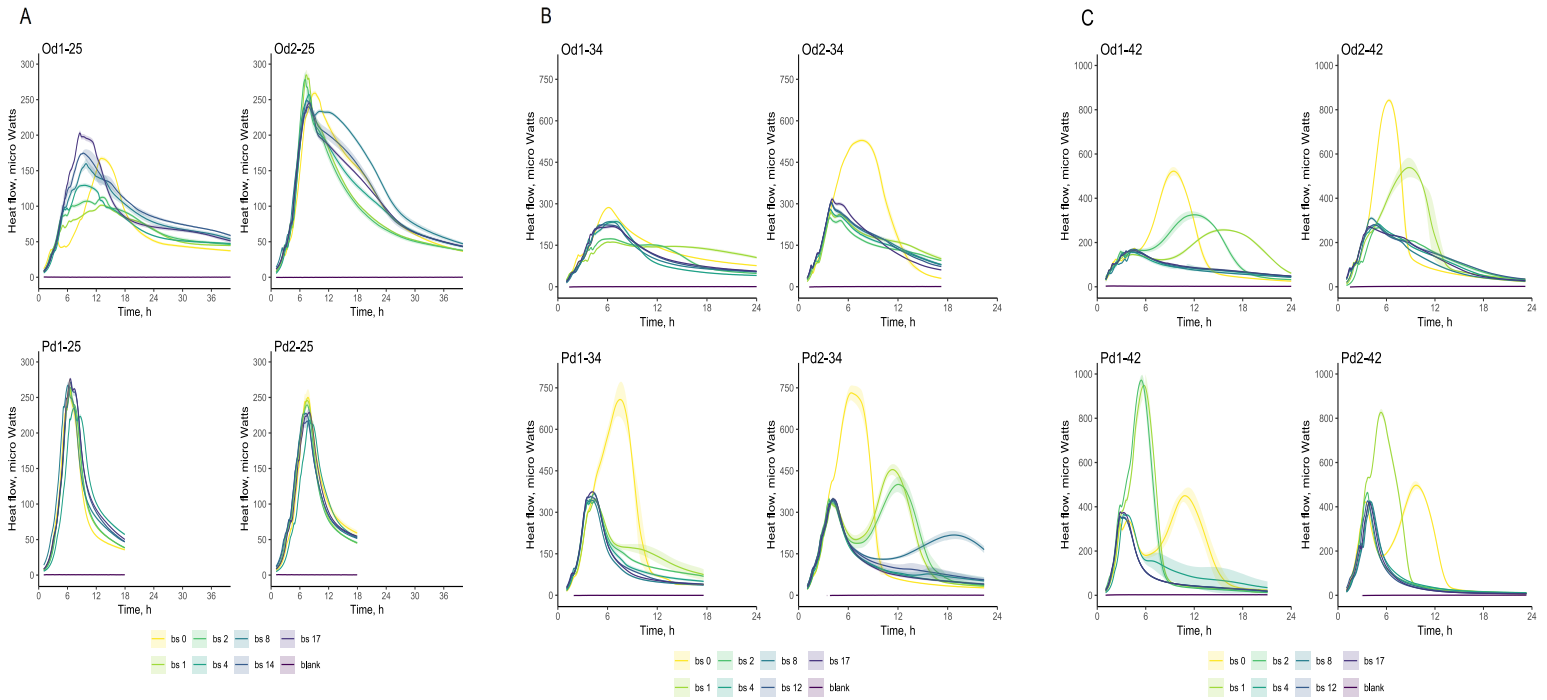

Figure 9. Microcalorimetry growth curves during backslopping cycles in all environments at 25 °C (A), 34 °C (B) and 42 °C (C). Graphs names are indicating: Oat drink 1 cultivated at 25 °C (Od1-25), Oat drink 2 cultivated at 25 °C (Od2-25), Pea drink 1 cultivated at 25 °C (Pd1-25), and Pea drink 2 cultivated at 25 °C (Pd2-25), Oat drink 1 cultivated at 34 °C (Od1-34), Oat drink 2 cultivated at 34 °C (Od2-34), Pea drink 1 cultivated at 34 °C (Pd1-34), and Pea drink 2 cultivated at 34 °C (Pd2-34), Oat drink 1 cultivated at 42 °C (Od1-42), Oat drink 2 cultivated at 42 °C (Od2-42), Pea drink 1 cultivated at 42 °C (Pd1-42), and Pea drink 2 cultivated at 42 °C (Pd2-42). Modified and obtained from Andreson et al., (2024).

Dynamical changes in the relative abundance of viable bacterial communities during the backslopping cycles at different fermentation temperatures were monitored by 16S rRNA metabarcoding (Figure 10, Figure 11, Figure 12). The composition of the viable microbiota was influenced by both the fermentation temperature and the specific formulation of the plant-based matrix. Notably, even commercially available beverages based on the same plant source promoted the growth of various bacterial species, highlighting the influence of product formulation on microbial community development. Thus, during fermentation and backslopping at 25 °C, *Lc. lactis* initially dominated in Oat Drink 1 at 25 °C (Od1-25), but from the 8th BS cycle onward, *L. pseudomesenteroides* and *L. mesenteroides* began to prevail, with the latter becoming dominant by the end of the experiment (Fig. 10). Oat Drink 2 at 25 °C (Od2-25) displayed greater initial diversity than Oat drink 1, though *Lc. lactis* was slightly more abundant. By the middle of the experiment (8th BS cycle), *L. pseudomesenteroides* and *L. citreum* started to prevail, with *L. pseudomesenteroides* ultimately overtaking the consortia.

The pea samples were less diverse than the oat samples, showing minimal changes in bacterial composition. *Lc. lactis* dominated throughout the experiment in both Pea Drink 1 (Pd1-25) and Pea Drink 2 (Pd2-25) samples at 25 °C. The only notable difference appeared in *Enterococcus faecalis* (*E. faecalis*) abundance, which showed a slight increase in Pd1 at the 8th BS cycle, while in Pd2, this increase occurred earlier at the 4th Bs cycle. By the 17th BS stage, the bacterial microbiota had stabilized, with *Lc. lactis* continued to dominate in the pea matrices, while more complex consortia developed in the oat drinks.

Engels et al. (2022) investigated the fermentation of different plant-protein emulsions derived from soy, pea, chickpea and mung bean using 151 LAB strains of both dairy and non-dairy origin at 30 °C. They found that *Lc. lactis*, *L. plantarum* and *L. mesenteroides* were the most viable species across all matrices. In contrast, *Streptococcus* strains and *Lacticaseibacillus* strains showed poor survival and limited acidification capacity in all tested matrices. In the current study, the predominance in pea samples (Pd1-25, Pd2-25) is also by *Lc. lactis,* followed by *Leuconostoc* species. This pattern is consistent with the ability of certain LABs to adapt efficiently to plant-protein substrates, although the competitive outcome is also influenced by the fermentation temperature used in each system. Also, *Lc. lactis* may thrive in low-buffer, plant-protein environments and acidify them rapidly because its metabolism is optimized for fast pH reduction under weakly buffered conditions, allowing it to outcompete slower acidifiers [148]. *Leuconostoc* species grow well in legume matrices and contribute heterofermentative metabolism because they efficiently utilize plant-derived carbohydrates and generate CO_2, acetate, and aroma-active compounds that support their growth in these substrates [149]. Their metabolic activity is also favored at moderate temperatures, which may explain why *Leuconostoc* species gradually increase in abundance during backslopping at 25 °C, particularly once early acidification by *Lc. lactis* has modified the environment in their favor.

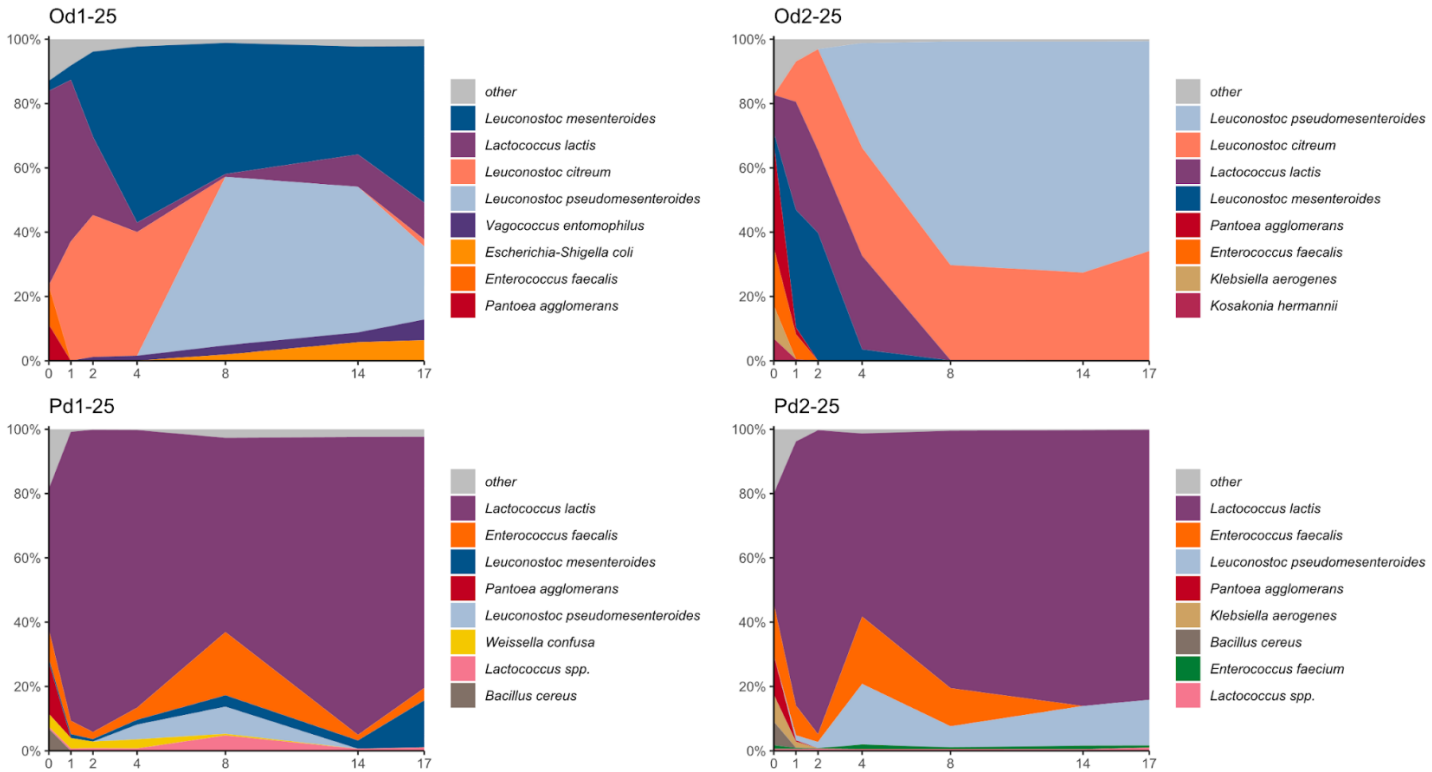

Figure 10. The dynamics in the microbial composition according to 16S rRNA NGS from backslopping over 17 cycles at 25 °C*. The 0 signifies the macerated floral mass composition together the microbial biomass from the plant-based drinks and further numbers the backslopping points. Graphs names are indicating Oat drink 1 cultivated at* 25 °C *(Od1-25), Oat drink 2 cultivated at* 25 °C *(Od2-25), Pea drink 1 cultivated at* 25 °C *(Pd1-25), and Pea drink 2 cultivated at* 25 °C *(Pd2-25). Obtained from Andreson et al., (2024).*

The bacterial distribution during backslopping at 34 °C (Figure 11) differed markedly from that at 25 °C. New species such as *Escherichia–Shigella coli* (*E. coli*) and *Vagococcus entomophilus* became more abundant, while *Lc. lactis* still prevailed in the oat-drink matrix. For Od2, Pd1, and Pd2, stable consortia formed during the first backslopping cycles, whereas Od1 continued to change even after the 12th BS cycle. In Od1, *E. coli* showed slightly higher abundance mid-experiment, but *Lc. lactis* again became dominant toward the end. Od2 showed comparable early dynamics, and from the 2nd BS cycle onward, *Lc. lactis* remained dominant. The pea samples displayed different dominant species than the oat samples. In Pd1-34, *B. cereus* and *E. faecium* were most abundant at the beginning, but by the 8th BS cycle, *B. cereus* had decreased, *Lc. lactis* had increased, and *E. faecium* remained high. At the end, *E. faecium* still dominated, followed by *Lc. lactis, E. coli*, and *W. confusa*. In Pd2-34, *E. faecalis* dominated throughout the experiment.

Molina et al., 2024 fermented different plant-based drinks (soy, pea, oat, and potato) with *Lc. lactis* and *L. pseudomesenteroides* at 30 °C. They discovered that coculture fermentation had many benefits, such as faster acidification than in monoculture fermentation and reduction of plant off-flavors in all plant-based drinks. Additionally, they detected a synergistic interaction between the strains, where *L. pseudomesenteroides* broke down the proteins and *Lc. lactis* used amino acids. In the current study, the presence of these species was also demonstrated in oat samples. This synergistic interaction may explain why in Publication III the oat samples did not show significantly higher free amin acid concentrations than the pea samples (Figure. 7 in Publication III).

The patterns observed in the current study align mechanistically with the synergistic interaction described by Molina et al., 2024, where *L. pseudomesenteroides* enhances protein degradation and releases amino acids that support the growth and metabolic activity of *Lc. lactis*. In our oat samples, the co-occurrence of these species suggests that similar cross-feeding may have taken place, stabilizing the consortia and promoting the dominance of *Lc. lactis* even at elevated fermentation temperatures. This synergy could also help explain why the free amino acid concentrations in oat and pea matrices remained comparable, as the metabolic cooperation between the strains may have balanced amino-acid release and consumption across different substrates.

In general, the samples fermented at 42 °C in all environments were less diverse than samples fermented at 25 °C and 34 °C, except the sample Od2 (Figure 12). In the Oat drink 1 fermented at 42 °C (Od1-42), the two most abundant species – *E. faecium* and *E. coli* were prevalent throughout the experiment. *E. faecium* dominated at the beginning and end of the experiment, and *E. coli* in the middle. The Od2-42 had a highly diverse composition. *L. lactis* seems to have prevailed at the beginning of the experiment. Three species – *E. faecium*, *Lb. johnsonii*, and *E. coli* were most abundant from the 4th BS point till the end of the experiment. In the Pea drink 1 fermented at 42 °C (Pd1-42), *E. coli* was most abundant in the beginning. Interestingly, *E. faecium* was the only species detected in the sample from the 8th BS point till the end.

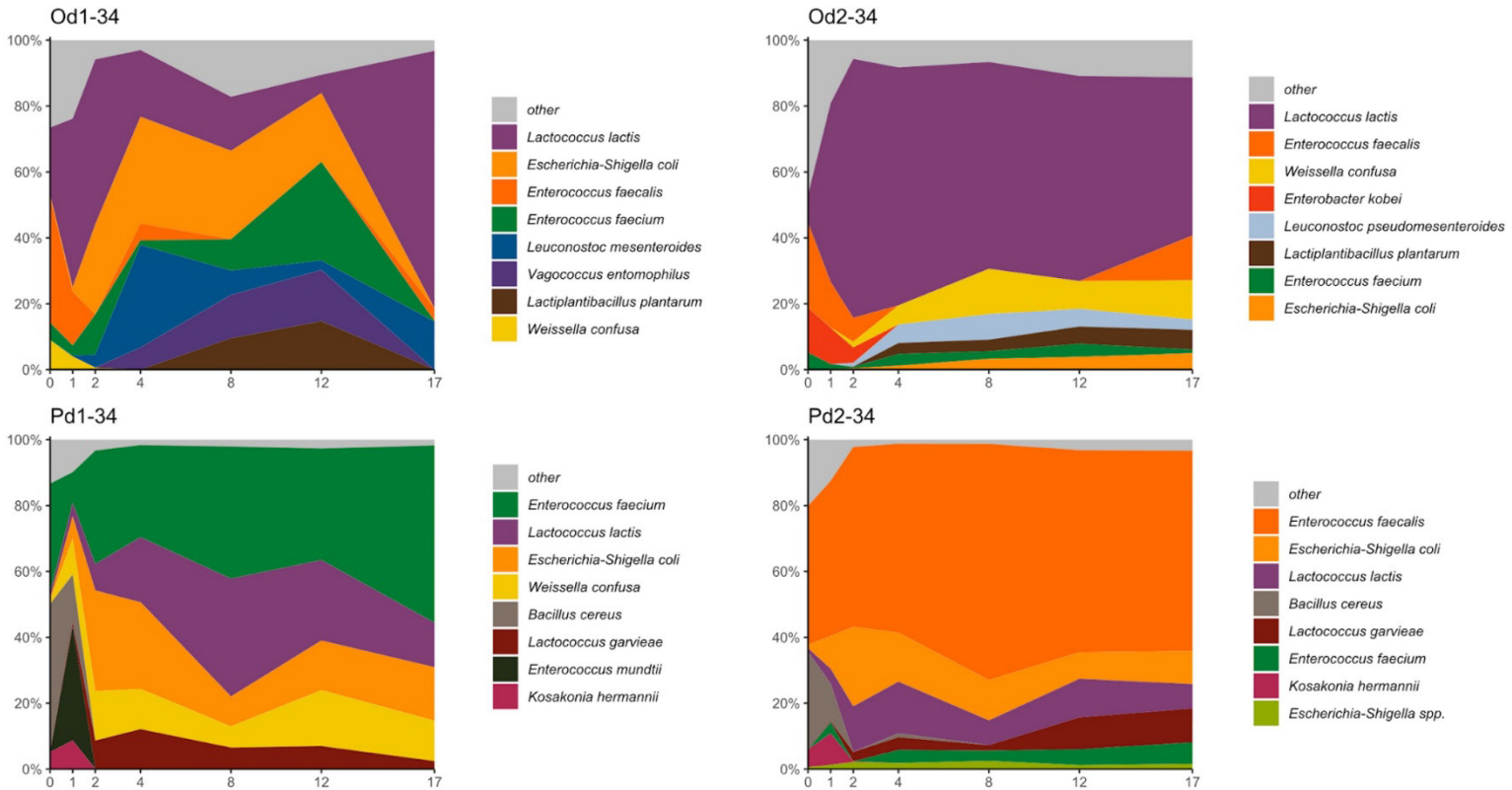

Figure 11. The dynamics in microbial composition according to 16S rRNA NGS from backslopping over 17 cycles at 34 °C. The 0 signifies the macerated floral mass composition together with the microbial biomass from the plant-based drinks, and further numbers the backslopping points. Graphs names indicate Oat drink 1 cultivated at 34 °C (Od1-34), Oat drink 2 cultivated at 34 °C (Od2-34), Pea drink 1 cultivated at 34 °C (Pd1-34), and Pea drink 2 cultivated at 34 °C (Pd2-34). Obtained from Andreson et al., (2024).

Unlike the other 42 °C samples, Od2-42 retained two LAB species—*Lb. johnsonii* and *Lactococcus garvieae* (*Lc. garvieae*)—both of which are known to tolerate temperatures up to 42–45 °C. *Lb. johnsonii* is capable of growing up to 45 °C [150] and *Lc. garvieae* is known to adapt to elevated temperatures and exhibit temperature-responsive gene regulation [151]. Natural consortium backslopping in Od2 at 42 °C showed that thermotolerant LAB within this community were adapted and survived well under high-temperature backslopping, whereas mesophilic LAB such as *Lc. lactis* did not persist. This contrasts with controlled thermophilic fermentations such as Xu et al., (2022), where inoculated thermophiles (*S. thermophilus*, *Lb. delbrueckii* subsp. *bulgaricus*) dominate the community at 42 °C. In Od2-42, the absence of true thermophiles allowed thermotolerant LAB to coexist with opportunistic species such as *E. faecium* and *E. coli*, resulting in a more diverse community than in the other 42 °C samples.

In summary, IMC was not the most reliable method for assessing the stability of the evolved microbial consortia because its results were not fully consistent with those obtained by 16S metabarcoding. The IMC profile suggested that the consortia reached a stable state several backslopping cycles earlier than indicated by the 16S metabarcoding data (Figure 10, Figure 11, Figure 12). This discrepancy is likely attributable to the fact that IMCs measures the total heat generated by microbial population, which primarily reflects overall biomass and metabolic activity rather than the actual changes in community composition [145], [153]. Consequently, IMC is better suited for monitoring the growth of single species, low-complexity microbial consortia, or consortia cultivated in chemically defined media, where community composition is relatively stable and well defined.

Different incubation temperatures contributed to the emergence and dominance of specific bacterial species during the backslopping adaptation process. Notably, *Lc. lactis* and *Leuconostoc* spp. were more prevalent at lower temperatures, whereas *Enterococcus* species were more abundant at higher fermentation temperatures. The pattern may be explained by the creation of optimal growth conditions for each species. Despite the historical use of lactococci in the production of a wide range of dairy-based fermented products, plant-based isolates of these bacteria may offer desirable traits, potentially enhancing the flavor and aroma of traditional dairy products [154]. Moreover, their plant origin suggests potential for incorporation into plant-based matrices, opening new avenues for environmentally sustainable foods. The dominance of *E. faecium* at 42 °C samples can likely be attributed to this species' high competitiveness, supported by bacteriocin production and resilience across a wide range of temperatures and pH levels [155]. However, although *E. faecium* strains may possess beneficial probiotic properties, strains of both *E. faecium* and *E. coli* are not used in the food industry and can also exhibit virulence, raising safety concerns [155], [156].

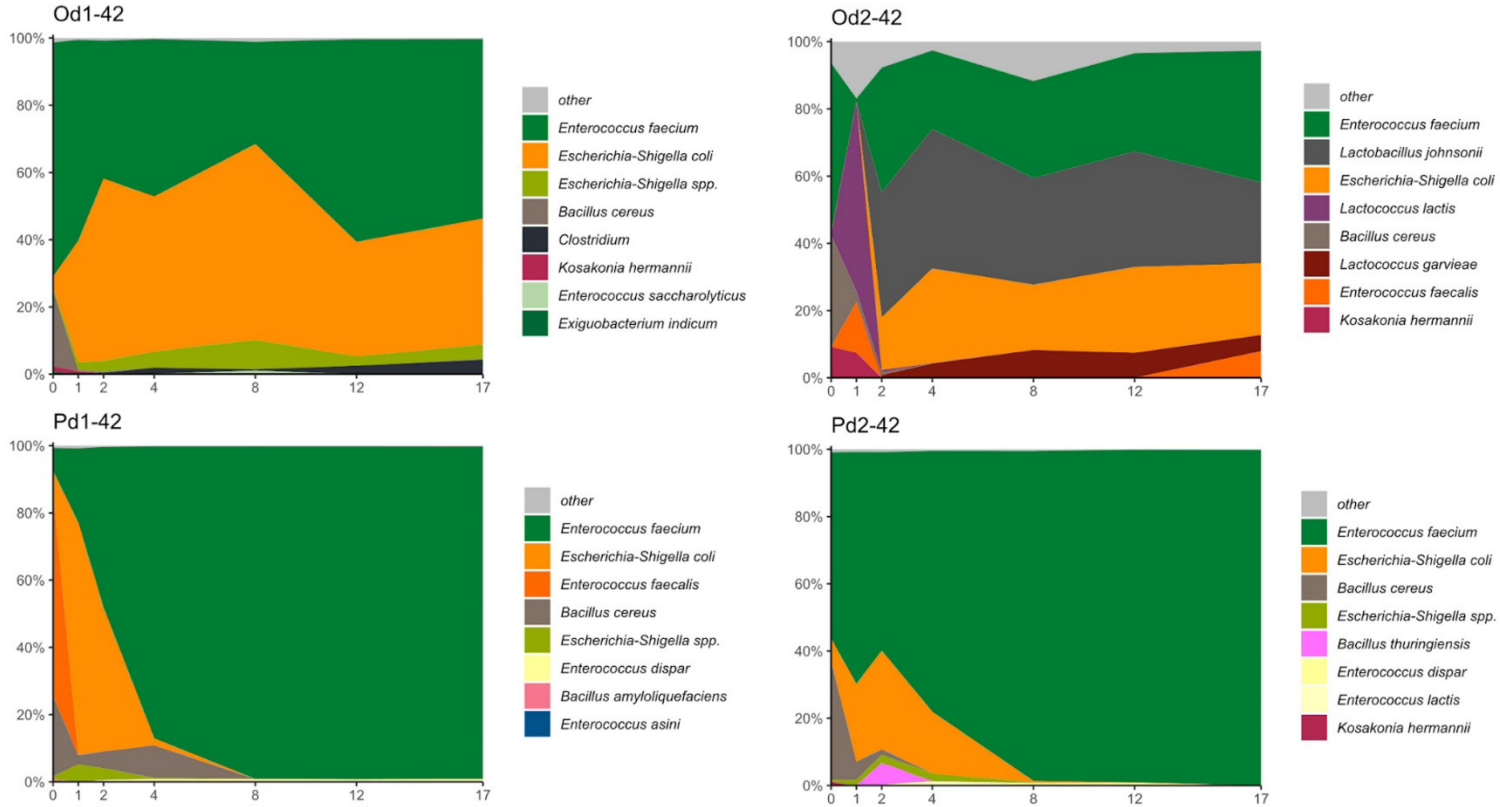

Figure 12. The dynamics in microbial composition according to 16S rRNA NGS from backslopping over 17 cycles at 42 °C. The 0 signifies the macerated floral mass composition together the microbial biomass from the plant-based drinks and further numbers the backslopping points. Graphs names indicates Oat drink 1 cultivated at 42 °C (Od1-42), Oat drink 2 cultivated at 42 °C (Od2-42), Pea drink 1 cultivated at 42 °C (Pd1-42), and Pea drink 2 cultivated at 42 °C (Pd2-42). Obtained from Andreson et al., (2024).

4.4 Relations between microbial composition, sensory attributes and chemical composition (Publication I)

The microbial variability across the commercial kombucha from the same manufacturer was previously described in **section 4.1.1**. Commercial kombuchas differed in culture composition; however, products from the same manufacturer often exhibited similar microbial profiles across different matrices (Figure 2). In Publication I, the chemical and sensorial profiles of the commercial kombuchas were characterized (Table S 1). To deepen the understanding of the biological mechanisms underlying these observations, the relationships between microbial composition, sensory attributes and chemical components were examined using Spearman's rank correlation analysis (Figure 13, Figure 14). However, it is important to consider that information about the fermentation process and its stages, shelf-life of the beverages, any possible attempts to preserve the product, or the time on the shelf was unknown. Additionally, the content of other additives (Table S 1), which could be added to the drinks either before or after fermentation, were unspecified. All these factors are assumed to influence the sensory profile and microbial and chemical composition of the final product. This in turn could cause false positive correlations that are difficult to refute or justify. Nevertheless, the most interesting positive and negative correlations are pointed out in this section, which could still indicate the synergies between different attributes in kombucha.

The negative correlation between acetic acid and *B. coagulans* was shown in Figure 13. It is justified by the fact that *B. coagulans* cannot produce acetic acid [157], [158]. However, it is noteworthy that there is no positive correlation between this bacterium and lactic acid, even though *B. coagulans* is widely used for lactic acid production [157]. As a negative correlation with acetic acid was observed, it is logical to also see a negative correlation between vinegar taste and this bacterium. Nevertheless, a positive correlation between *Bacillus* spp. and lactic acid was shown. On the other hand, *Bacillus* spp. showed a positive correlation with acetic acid, which is unexpected because *Bacillus* spp. are generally not acetic acid producers. However, acetic acid is a key metabolite produced by the vegetative cells of *Bacillus thuringiensis* and stored within the cells as poly-β-hydroxybutyrate, which is then consumed during sporulation [159]. Therefore, in some cases, *Bacillus* spp. can produce small amounts of acetic acid under oxygen-limited conditions, although this acid is not listed as a primary metabolite of *Bacillus* spp. [160].

A strong positive correlation of *K. rhaeticus* and acetic acid was demonstrated. This correlation is not fully consistent with the literature, as *K. europeus* is typically considered the major acetic acid producer. However, Figure 14 does not show any correlation with this bacterium. Nevertheless, Spearman's analysis did not produce entirely contradictory results, because *K. rhaeticus* does produce acetic acid during kombucha fermentation [161]. This analysis also showed a positive correlation with glucose for two *Komagataeibacter* species (*K. intermedius* and *K. rhaeticus*), which is consistent with the literature [162]. Spearman's rank correlation analysis further showed a strong correlation between *K. rhaticus* and tea taste. Although the literature does not explicitly support this direct correlation, it does show that this species influences mouthfeel due to the production of large amounts of bacterial cellulose [163]. Bacterial cellulose in kombucha binds polyphenols, reduces bitterness, and modifies viscosity, and therefore, has an effect on mouthfeel [54].

Lactobacillus spp. showed a positive correlation with lactic acid concentration (Figure 13). This is logical because these bacteria produce lactic acid as their main metabolite [9]. Off-taste intensity correlated positively with *Pseudomonas* spp. (*P. fluorescens*, *P. libanensis*, *P. putida*, *P. tolaasii*), which is consistent with their well-documented role as spoilage organisms that produce ketones, aldehydes, sulfur compounds, and biogenic amines responsible for earthy, rancid, and metallic off-flavors [164].

As expected, vinegar taste showed a strong positive correlation with acetic acid, because this acid is the primary molecule responsible for vinegar flavor (Figure 14). Astringent taste correlated negatively with sucrose concentration and positively with ethanol. The article by Ickes & Cadwallader, (2017) shows that ethanol affects mouthfeel and trigeminal sensations such as warmth, irritation, and dryness. These effects overlap with sensory qualities associated with astringency, so higher ethanol levels can make astringent sensations more noticeable, even at low concentrations.

In conclusion, the analysis demonstrated that key microbial groups showed correlations with chemical and sensory traits that align with their known metabolic roles. These relationships offer insight into how microbial dynamics contribute to flavor development and variability in commercial kombucha.

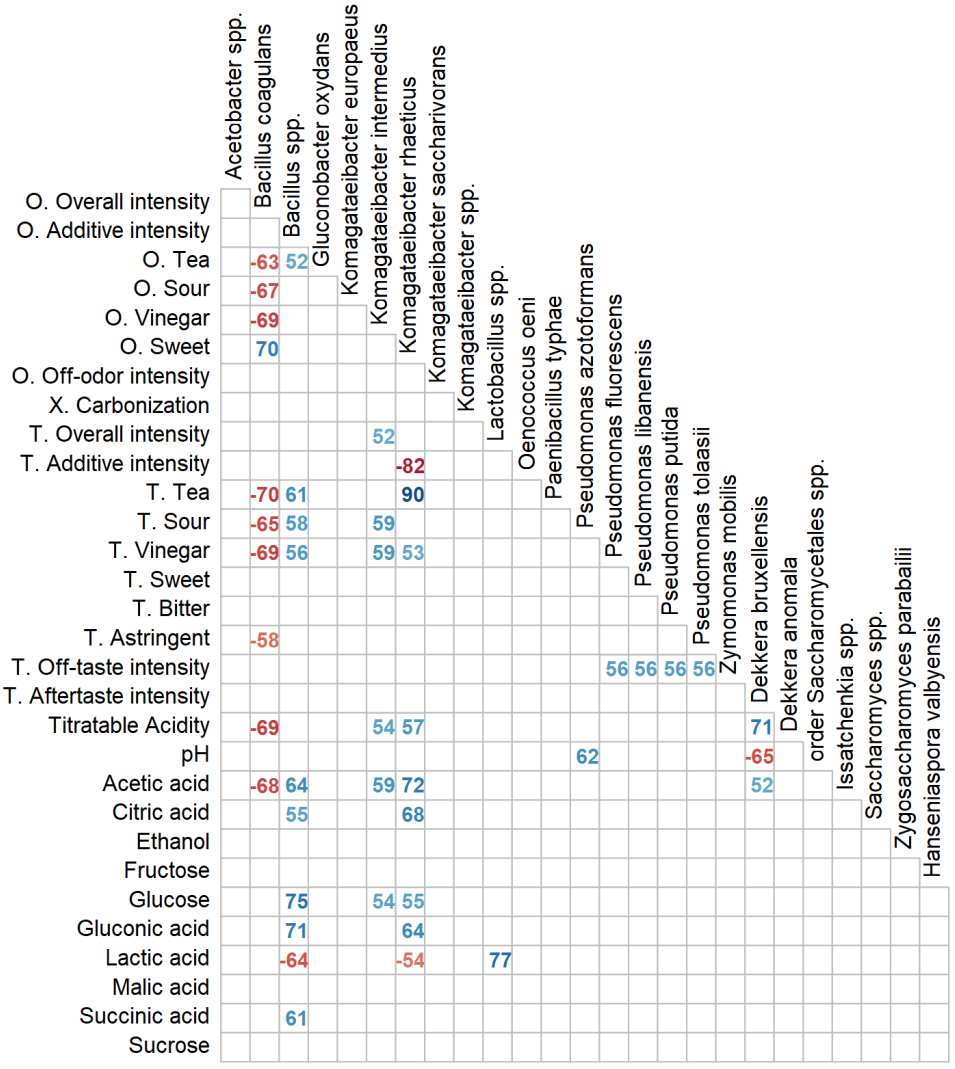

Figure 13. Spearman´s rank correlations (as %) between microbial and chemical composition and sensory attributes; only statistically significant (α = 0.05) coefficients are shown. The letters O. and T. are abbreviations from odor and taste, respectively. Obtained from Andreson et al., (2022).

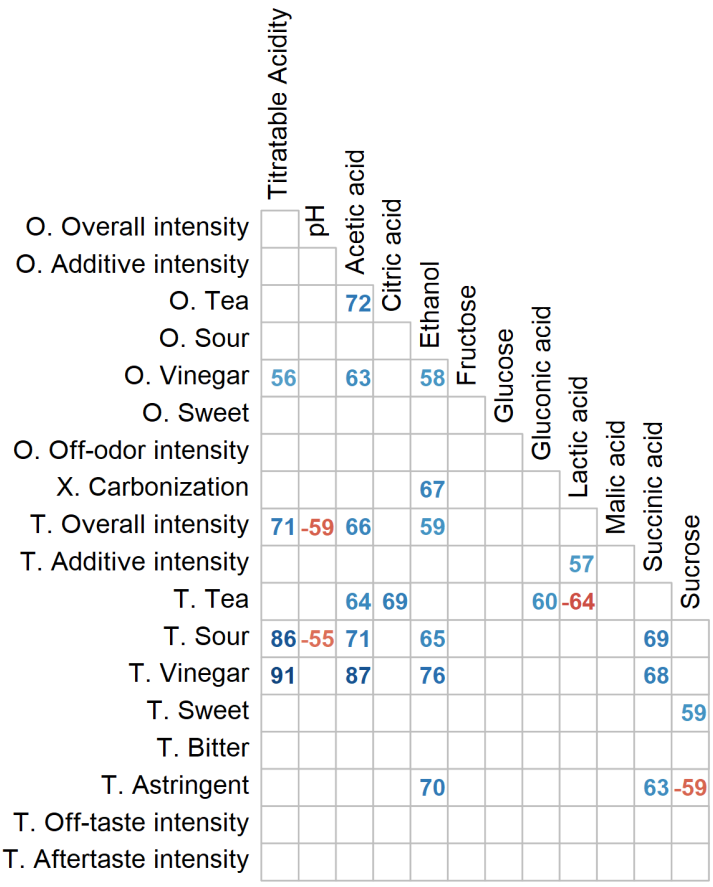

Figure 14. Spearman´s rank correlations (as %) between sensory attributes and chemical composition; only statistically significant (α = 0.05) coefficients are shown. The letters O. and T. are abbreviations from odor and taste, respectively. Obtained from Andreson et al., (2022).

4.5 Strain isolation of novel non-dairy starter culture candidates from fermented and backslopped pea-oat base fermented drinks (Publication III)

To further exploit the potential of the backslopped pea– and oat-based fermented drinks and the evolved microbial consortia, a strain isolation experiment was conducted to obtain plant-adapted bacterial strains for future fermentation applications. Samples for isolation were selected based on their bacterial community profiles determined by 16S rRNA gene sequencing and their favorable sensory characteristics. These data guided the design of selective cultivation conditions to facilitate the isolation of the targeted strains.

The sensory evaluation was performed after each BS cycle by some of the panel members. The evaluators characterized the odor, taste, and texture of the samples. The sensory parameters changed throughout the backslopping process, and the most promising drinks were obtained from the 17th (last) BS cycle in Od1-25, Pd1-34, Pd1-42, and Od2-42 (Table S 2). Od1-25 had a sour cream-like odor, a good sour–sweet balance, and a texture similar to cowmilk-based yogurt. The next sensorially best sample was Pd1-42. It had a sweet and caramelly odor, tasted like cow milk-based yogurt, and had an oat

porridge-like texture. Pd1-34 had a sweet and caramelly odor, a watery texture, and a similar taste to mild unflavored plant-based yogurt. Od2-42 also had a good sour–sweet balance, but the texture was the least similar to real cow milk-based yogurt.

Four above-described samples (Od1-25, Pd1-34, Pd1-42, and Od2-42) from the last backslopping cycle (BS17) were chosen as promising candidates for plant-based starter cultures isolation. Bacterial strain isolation was performed using a standard outplating technique, and colonies were identified by MALDI-TOF MS. To maximize diversity in candidate selection, we used two different growth media, MRS (common for LAB) and M92, and tested all previous incubation temperatures and under both aerobic and anaerobic conditions (Figure S 3).

In this study, a total of 65 isolates were analyzed by MALDI-TOF MS; out of them, eight isolates could not be identified. The most abundantly identified species were *Lc. lactis, L. mesenteroides*, and *E. faecium* (Table 3). Among detected species, some prospective candidates were identified. Thus, *Lc. lactis* are *L. mesenteroides* are mostly used in industrial dairy fermentation as main starter culture contributors (Song et al., 2017). It was shown that *E. faecium* are responsible for antimicrobial activity [167] or expressing probiotic properties [168]. The majority of isolates, such as *Lc. lactis, L. mesenteroides*, and some species of *E. faecium* are generally recognized as safe (GRAS) and may be beneficial to a host's health [168].

The selective consortia differed clearly between samples and media, with each combination showing a distinct dominant species and a characteristic set of additional taxa. Od1-25 on MRS was dominated by *L. mesenteroides*, with smaller contributions from related *Leuconostoc* species (*L. mesenteroides* and *L. citreum*) and substantial unidentified bacteria (Table 3). Pd1-34 on MRS showed a balanced presence of *Lc. lactis*, *E. faecium*, and *W. cibaria*, together with a minor amount of *Lc. garvieae* and many unidentified bacteria. On M92, Pd1-34 shifted to a profile dominated by *Lc. garvieae*, followed by *E. faecalis*, *Lc. lactis*, *E. faecium*, and a group of unidentified bacteria. Od2-42 on MRS mainly consisted of *E. faecium*, with notable amounts of *Lb. johnsonii* and a smaller amount of *E. faecalis*. On M92, Od2-42 contained comparable levels of *E. faecalis* and *E. coli*, along with a moderate presence of *E. faecium* and unidentified bacteria. Only *E. faecium* was isolated from Pd1-42 on M92.

Comparing MALDI-TOF MS results with 16S sequencing data showed that bacterial isolates from Od1-25 on MRS and Pd1-42 on M92 had the same bacterial species across both approaches. These findings suggest that extreme conditions, such as lower or higher fermentation temperatures, which favor a narrower range of species, may be more selective [169]. Greater differences appeared when incubation was performed at an intermediate temperature (approx. 35 °C) on different media (MRS and M92). These results were expected, as certain species preferentially grow on MRS while others thrive on M92 [170]. Nevertheless, all major species identified on both media via agar plates were also detected in 16S rRNA gene sequencing (Table 3). For example, in Pd1-34 incubated at 34 °C on MRS, the dominant species were *E. faecium, Lc. lactis,* and *W. cibaria*, whereas on M92, *Lc. garvieae* predominated. All these species were also confirmed by 16S sequencing. Comparing MALDI-TOF MS and 16S sequencing highlights the complementary strengths of each method: 16S sequencing, though more costly, is not selective and capable of identifying even trace bacterial species within a sample. Conversely, plate incubation combined with MALDI-TOF MS allowed us to isolate and identify preferred species, paving the way for developing non-dairy starter cultures tailored for plant-based products.

Table 3. The species identified according to MALDI-TOF-MS from the last backslopping point (BS17) compared with 16S metabarcoding sequencing results. Obtained from Andreson et al., (2024).

Matrix	Temp, °C	Cultivation media	MALDI-TOF MS			16S	
			Species	Count	%	Species	%
Od1-25	**25**	MRS	*Leuconostoc mesenteroides*	6	30.0	*Leuconostoc mesenteroides*	48.7%
			Leuconostoc pseudomesenteroides	3	15.0	*Leuconostoc pseudomesenteroides*	22.7%
			Leuconostoc citreum	2	10.0	*Lactococcus lactis*	11.4%
			Unidentified	9	45.0	*Vagococcus entomophilus*	6.4%
						Escherichia-Shigella coli	6.4%
						Leuconostoc citreum	2.2%
						Raoultella ornithinolytica	0.8%
						Lactococcus unclassified	0.4%
						Other	1.0%
Pd1-34	34	MRS	*Enterococcus faecium*	2	16.7	*Enterococcus faecium*	53.9%
			Lactococcus lactis	2	16.7	*Escherichia-Shigella coli*	16.2%
			Weissella cibaria	2	16.7	*Lactococcus lactis*	13.5%
			Lactococcus garvieae	1	8.3	*Weissella confusa*	12.3%
			Unidentified	5	41.7	*Lactococcus garvieae*	2.5%
						Escherichia-Shigella unclassified	1.2%
		M92	*Lactococcus garvieae*	4	40.0	*Lactococcus unclassified*	0.1%
			Lactococcus lactis	2	20.0	*Latilactobacillus unclassified*	0.1%
			Enterococcus faecalis	2	20.0	Other	0.3%
			Enterococcus faecium	1	10.0		
			Unidentified	1	10.0		
Od2-42	42	MRS	*Enterococcus faecium*	6	60.0	*Enterococcus faecium*	39.3%
			Lactobacillus johnsonii	3	30.0	*Lactobacillus johnsonii*	24.1%
			Enterococcus faecalis	1	10.0	*Escherichia-Shigella coli*	21.3%
			Unidentified	0	0.0	*Enterococcus faecalis*	7.9%
						Lactococcus garvieae	4.8%
		M92	*Enterococcus faecalis*	3	30.0	*Escherichia-Shigella unclassified*	1.8%
			Escherichia coli	3	30.0	*Latilactobacillus unclassified*	0.4%
			Enterococcus faecium	2	20.0	*Lactobacillus unclassified*	0.2%
			Unidentified	2	20.0	Other	0.3%
Pd1-42	42	M92	*Enterococcus faecium*	5	100.0	*Enterococcus faecium*	98.9%
			Unidentified	0		*Enterococcus dispar*	0.9%
						Enterococcus saccharolyticus	0.1%
						Lactococcus lactis	0.1%
						Other	0.1%

4.6 Whole genome sequencing and annotations of isolated strains – (Publication III and unpublished data)

Exposing of the initial plant inoculum to different fermentation temperatures and plant-based matrices enriched the microbial consortia with strains capable of adapting to each set of conditions. As a result, the potential to isolate unique strains with distinctive technological characteristics increased. The repeated backslopping cycles selected for LAB species that were able to survive, adapt and evolve throughout the fermentation process, indicating their robustness and potential for industrial applications. To accurately identify the isolates and evaluate their functional potential, WGS was performed on several selected strains. In total, 12 genomes were sequenced, representing isolates belonging to the genera *Leuconostoc, Enterococcus, Lactobacillus*, and *Lactococcus*.

The next phase of this research will involve high-throughput *in silico* genome analysis combined with comparative genomics to evaluate the technological properties of the isolates, including their carbohydrate metabolism, proteolytic capacity, and exopolysaccharide production. Within the scope of the present work, the genomic safety of the isolates was assessed by screening for features relevant to food applications, including the absence of acquired antibiotic resistance genes, intact prophage regions, and genes involved in the production of biogenic amines. These analyses were performed to ensure that the isolated strains are suitable candidates for use in food applications.

According to EFSA's guidance for the safety assessment of microorganisms intended for use in food and feed including QPS assessments, novel foods, and starter cultures, [10], microorganisms belonging to taxa with QPS status are presumed to be safe for their intended uses, provided that any applicable qualifications are met. This streamlined safety assessment facilitates their use in food and feed applications. Based on WGS-supported taxonomic identification, five of the isolated LAB strains (2 of *Lc. lactic*, *L. pseudomesenteroides*, *L. mesenteroidis,* and *L. citreum*) belong to species included in the EFSA Qualified Presumption of Safety (QPS) list and are therefore considered suitable for food applications.

For microorganisms that do not have QPS status, the most important genetic traits to evaluate are those related to consumer safety and the potential for horizontal gene transfer. WGS should be used to assess the presence of: (1) antimicrobial resistance genes (ARG), (2) virulence factors, (3) toxin production genes, (4) mobile genetic elements, (5) biogenic amine production, (6) undesirable metabolic pathways. The presence or absence of these key genetic determinants have been characterized for each isolate to support its safety assessment and evaluate its suitability for food applications.

The species belonging to the genera *Enterococcus* and *Weissella*, are not included in the EFSA QPS list (Table 3), therefore, their safety assessment was conducted in accordance with the EFSA guidance list from 2024 [10].

A more comprehensive genomic analysis was conducted in June 2026. As the initial species identification was performed in early 2022 using MALDI-TOF MS, the isolates were classified as *E. faecium* based on the taxonomic databases available at that time, which did not yet distinguish *E. lactis* from *E. faecium*. Reanalysis using WGS and updated reference databases enabled a more accurate taxonomic assignment. Accordingly, isolates VF031, VF005, and VF042 were reclassified as *E. lactis*.

A subsequent review of the EFSA guidance for the characterization and safety assessment of microorganisms intended for use in food and feed applications highlighted

an important distinction. The guidance cites the work of Belloso Daza et al., (2021), who demonstrated that *E. faecium* consists of genetically distinct subpopulations, including a clade that has since been recognized as the separate species *E. lactis*.

The community-associated clade B—containing strains typically colonizing the human and animal gut—has recently been reassigned to *E. lactis* based on WGS analyses. *E. lactis* strains are generally more susceptible to antimicrobials than *E. faecium* and lack hospital-associated genetic markers [171], [172], [173]. In addition, genes involved in adhesion and colonization are often incomplete or show low identity compared with *E. faecium* reference genomes [172], [174].

According to the EFSA guidance manual, average nucleotide identity (ANI) analysis (ANI > 94%) was performed on the WGS data using the type strains *E. faecium* NCTC 7171^{T} and *E. lactis* LMG 25958^{T}. The bioinformatic results showed that VF031 displayed *E. lactis* similarity with an ANI value of 98.94%, VF005 had an ANI value of 98.46%, and VF042 had an ANI value of 98.57%. All previously mentioned isolates ANI values were also calculated against *E. faecium* NCTC 7171^{T}, the ANI values were between 94-95%. Therefore, these isolates can be classified as *E. lactis,* and they should not be pathogenic. Nevertheless, the safety of these isolates should be confirmed through assessment of strain-specific traits, including the absence of virulence factors, acquired antimicrobial resistance genes, and other relevant genetic determinants.

First, the presence of AMR genes in the genomes of isolates outside the QPS list was examined (Table 4). The screening focused on resistance determinants associated with gentamicin, kanamycin, streptomycin, vancomycin, erythromycin, telithromycin, tylosin, ampicillin, fosfomycin, colistin, ciprofloxacin, chloramphenicol, thiamphenicol, clindamycin, and tetracycline. Some isolates carried more AMR genes than others. It is important to note that the detection of an AMR gene in the genome does not automatically indicate that the isolate is hazardous or unsuitable for food or feed applications; rather, it means that the phenotypic resistance level must be verified experimentally. The EFSA guidance manual provides the relevant antibiotic cut-off values for this assessment. However, these experiments were not performed during the current PhD study.

The second aspect evaluated was the presence of virulence factor genes. Isolates VF031, VF005, and VF042 did not contain the virulence markers ptsD, esp, IS16, hylEfm, or orf1481. In contrast, isolates VF036 and VF013 carried the virulence genes hylA and hylB.

Toxin-associated genes were also examined in the genomes. None of the isolates contained histidine decarboxylase encoding gene. Five isolates harbored tyrosine decarboxylase—VF042, VF031, VF005, VF013 and VF036. Although VF042 and VF031 contained tyrosine decarboxylase activity at the annotation level, the genes responsible for producing this enzyme (tdc, tdcB) were not detected.

Mobile genetic elements such as plasmids, transposons, integrative conjugative elements (ICE), and prophages carrying undesirable genes in genomes were also evaluated. VF013 and VF036 contained plasmid sex pheromone cAM373, suggesting that these strains possess a pheromone system capable of attracting pheromone-responsive plasmids from neighboring bacteria, even if they do not necessarily harbor such plasmids at the time of sequencing [175].

VF002 contains a pRiA4b Orf3-like domain-containing protein. It indicates sequence similarity to an Ri-plasmid ORF, but it does not by itself prove that the bacterium carries

a plasmid [176], [177], [178]. Its genomic location and surrounding features must be evaluated to determine whether it is plasmid-borne [176], [177].

VF031 contains plasmid mobilization relaxosome protein MobC. The MobC-like annotation indicates similarity to plasmid mobilization proteins, but it does not confirm that the bacterium carries a plasmid [176], [177]. The surrounding genomic features must be evaluated with VF002 to determine whether the gene is plasmid-derived [176], [177].

VF031 also contains a plasmid replication protein. A plasmid replication protein (Rep protein) is a regulatory initiator that binds the plasmid origin of replication and enables independent plasmid DNA replication [179]. However, the presence of a Rep protein alone does not demonstrate that the bacterium currently carries a plasmid [176], [177]. The genomic context—specifically contig size, GC content, replication modules, and structural features—must be examined before concluding that a plasmid is present in the cell [176], [177].

VF042 also contains a plasmid stabilization system protein. This protein is part of a system that ensures stable inheritance of plasmids during cell division. Its presence strongly suggests plasmid origin, but it does not by itself prove that the bacterium currently carries a plasmid, because stabilization genes can integrate into chromosomes or be misassigned during genome assembly [176], [177]. Therefore, most likely the isolates VF031 and VF042 contain plasmid. However, it requires further analysis of genomic data.

VF042 also contains a transposon Tn552 DNA-invertase bin3-like protein. This annotation indicates similarity to the DNA invertase/recombinase associated with the staphylococcal β-lactamase transposon Tn552 [180]. To our knowledge, no published genome analyses or mobile-element surveys report indicates the complete Tn552 transposon in *E. lactis*. However, serine recombinases and Tn552-like recombination modules are widely distributed among Firmicutes mobile elements, including enterococci, due to horizontal gene transfer [181], [182].

None of the isolates contained ICEs and prophages carrying undesirable genes. However, the undesirable metabolic pathways were present in the isolated species. Four pathways are considered particularly relevant: ethyl carbamate precursor formation, D-lactate overproduction, excessive gas production, and off-flavor formation pathway.

The genes responsible for ethyl carbamate precursor formation in LAB belong to the arginine deiminase pathway (ADI), primarily arcA (arginine deiminase), arcB (ornithine transcarbamylase), and arcC (carbamate kinase) [183]. These enzymes convert arginine into citrulline, carbamyl phosphate, and urea, all of which are established precursors of ethyl carbamate [183]. The arcA gene was detected in VF005, VF031, VF042, VF002, VF036, and VF013. The arcC gene (or its variants) was also present in VF005, VF031, VF042, VF002, VF036, and VF013. None of the isolates contained arcB genes.

D-lactate overproduction–associated genes were also evaluated. The primary gene responsible for D-lactate formation in LAB is d-ldh (also known as ldhD), which encodes D-lactate dehydrogenase. This enzyme converts pyruvate into D-lactate, and its activity determines the amount of D-lactate produced.

Excessive gas production in LAB is primarily caused by the phosphoketolase pathway (xpk/xfp pathway), which generates CO_2 during heterofermentative metabolism [11]. Additional CO_2 can be produced through pyruvate-derived pathways, including the acetolactate synthase/decarboxylase system (alsS, aldC) [30] and by enzymes such as pyruvate formate lyase (pfl) and pyruvate decarboxylase (pdc) [11], [184]. The presence

and expression of these genes determine whether a LAB strain has the ability to produce excessive gas. The alsS was detected in isolates VF005, VF031, VF042, VF002, VF036, and VF013. The pflA and pflB genes were present in VF005, VF013, VF036, VF031, and VF042.

Off-flavor formation in LAB results from multiple metabolic pathways. Diacetyl and acetoin are produced via acetolactate pathway (alsS, aldC, butA) [30]. Acetaldehyde is generated through pyruvate decarboxylation (pdc, adhE) [184]. Acetic acid derives from the pyruvate oxidase and acetate kinase pathways (pox, pta, ackA) [11]. Fatty-acid catabolism (lip, est, fadA, fadB) and sulfur-amino-acid degradation (cysK, metC, mgl) generate rancid and sulfurous notes [185]. The arginine deiminase pathway (arcA, arcB, arcC) contributes bitter and chemical off-flavors [185].

All isolates contained adhE gene or genes and alsS gene. The arcA gene and arcC gene/genes were all of the isolates. Also, all isolates contained cysK gene or genes except VF002. The metC genes and ackA gene were presented in VF013 and VF036. Only VF002 carried the mglA gene. No additional off-flavor-associated genes were identified in the isolates.

To conclude, several undesired genes were identified in the genomes of the isolated species. However, further laboratory experiments are required to determine whether these genes are expressed and whether they result in phenotypic off-flavor formation or other undesirable metabolic outcomes.

Table 4. Genomic indicators relevant to the safety assessment of isolates according to EFSA guidance

Strain	Name	AMR genes	Virulence factors	Toxin production genes – biogenic amine production pathways	Mobile Genetic Elements	Biogenic amine production pathways	Undesirable Metabolic Pathways
VF031	*E. lactis*	*tetR genes (7); vanZ genes (2); ampD gene (1); thiT gene (1);*		*Tyrosine decarboxylase gene (1), tyrosine decarboxylase gene (1),*	*Plasmid sex pheromone cAM373; plasmid replication protein*		*arcA gene (1); arcC gene (1); alsS gene (1); pflA gene (1) and pflB gene (1); adhE gene (1), arcA gene (1); arcC gene (1); cysK gene (1)*
VF036	*E. faecalis*	*tetR gene (1); vanZ genes (2); ampC genes (3); genA gene, genB gene, genR gene; strF genA, strF genB, strF genR; thiT gene (1), thiM gene (1), thiE gene (1), thiD genes (3), thiN gene (1)*	*hylA gene (1), hylB gene (1)*	*Tyrosine decarboxylase gene (1),*	*plasmid sex pheromone cAM373*		*arcA gene (1); arcC gene (4); alsS gene (1); pflA gene (1) and pflB gene (1); adhE genes (3); arcA gene (1); arcC genes (4); cysK genes (2); metC genes (2);*
VF013	*E. faecalis*	*tetR genes (1); vanZ genes (2); ampC (3); genA gene, genB gene, genR gene; strF genA, strF genB, strF genR; thiT gene (1), thiM gene (1), thiE gene (1), thiD genes (3), thiN gene (1)*	*hylA gene (1), hylB gene (1)*	*Tyrosine decarboxylase gene (1),*			*arcA gene (1); arcC gene (4); alsS gene (1); pflA gene (1) and pflB gene (1); adhE genes (3); arcA gene (1); arcC genes (4); cysK genes (2); metC genes (2); ackA gene (1)*
VF005	*E. lactis*	*tetR genes (7); vanZ genes (2); thiT gene (1);*		*Tyrosine decarboxylase gene (1),*			*arcA gene (1); arcC gene (1); alsS gene (1); pflA gene (1) and pflB gene (1); adhE gene (1); arcA gene (1); arcC genes (1); cysK gene (1)*

Strain	Name	AMR genes	Virulence factors	Toxin production genes – biogenic amine production pathways	Mobile Genetic Elements	Biogenic amine production pathways	Undesirable Metabolic Pathways
VF002	*W. cibaria*	*tetR genes (2); vanYB protein (1); camS gene (1); thiM gene (1);*			*plasmid pRiA4b Orf3-like domain-containing protein*		*arcA gene (1); arcC gene (1); alsS gene (1); adhE genes (2); arcA gene (1); arcC genes (1); mglA gene (1)*
VF042	*E. lactis*	*tetR genes (7); vanZ genes (2); thiT gene (1);*		*Tyrosine decarboxylase gene (1), tyrosine decarboxylase gene (1),*		*plasmid stabilization system protein; transposon Tn552 DNA-invertase bin3*	*arcA gene (1); arcC gene (1); alsS gene (1); pflA gene (1) and pflB gene (1); adhE gene (1); arcA gene (1); arcC genes (1); cysK gene (1)*

5 Conclusion

The current research demonstrates that traditionally fermented food and beverages´ microbiota are remarkably stable to small modifications and that such changes do not significantly affect microbial composition at the genus level. The investigation of kombucha showed that the addition of new bacteria to the culture did not change the consortia composition at the genus level, indicating an ecologically developed and stable system. Even challenged by backslopping propagation in new matrices, naturally evolved consortia did not significantly change their microbial composition and added LAB species did not capture dominance in the fermented environment. However, the changes can be observed at the sensorial level. The rapid expansion of the plant-based food sector has increased the demand for novel starter cultures that are well adapted to plant-based matrices and capable of ensuring efficient, controlled fermentation. Consequently, the development of new starter cultures should prioritize the isolation of microorganisms from similar ecological niches, where they are naturally adapted to plant-based environments.

The main conclusions that can be drawn from this work are the following:

- The first article research was the first one that compared several products from different brewers, analyzed the chemical composition and microbiological consortia by metabarcoding.
    - The microbial, chemical, and sensory analyses of commercially available kombuchas demonstrated substantial variability among manufacturers; additionally, the matrix environment and preservation technology influenced the kombucha culture's microbial composition.
    - Five products met the definition of the Kombucha Code of Practice - kombucha is made from tea leaves and fermented with symbiotic consortia of acetic acid bacteria and yeast.
    - The findings also highlight the need for clearer standardization, definition, and legislation regarding kombucha. Several commercial products contained relatively high ethanol concentrations that, in some countries, could classify them as alcoholic beverages. Furthermore, some products lacked viable microorganisms, raising questions about whether they should be marketed as kombucha if microbial viability is considered an essential characteristic of the beverage.
- The second study demonstrated that introducing LAB strains originating from a different ecological environment into an established kombucha consortium had little or no effect on the core microbial composition although it influenced the sensory properties of the fermented beverage. Additionally, repeated backslopping in coffee infusion and orange juice did not substantially change the core kombucha microbiota. While transient fluctuations in microbial composition were observed during the backslopping process, the overall community structure remained stable.
    - In both environments, backslopping did not significantly affect the chemical properties of kombucha. However, relatively high concentrations of ethanol were detected, reaching 23.10 g/L and 10.23 g/L in orange juice and coffee infusion, respectively.

    - The dominant bacterial species differed between the two matrices: *Komagataeibacter intermedius* predominated in orange-juice kombucha, whereas *Komagataeibacter rhaeticus* was dominant in coffee kombucha. The dominant yeast species in both matrices was *Schizosaccharomyces pombe*.
    - These findings demonstrate the stability and resilience of the microbial interactions within self-evolved kombucha consortia. They also highlight the potential of continuous backslopping as a strategy for maintaining stable fermentation performance while improving the quality and sensory characteristics of fermented products.
- The third study integrated the findings from the previous investigations by evaluating the isolation of autochthonous microorganisms from plant biomass and their suitability as starter cultures for plant-based dairy alternatives. Continuous backslopping was employed as an adaptation strategy to promote the formation of stable, self-assembled microbial consortia. The results demonstrate that autochthonous microorganisms represent a promising resource for the development of starter cultures for fermented plant-based products. The study further demonstrated that different plant matrices, such as oat- and pea-based beverages, provide distinct nutritional environments, which consequently support the development of different core microbial communities. Furthermore, backslopping at different fermentation temperatures resulted in distinct dominant microbial populations.
    - The initial microbial composition of the macerated plant mash contained mainly environmental bacteria, with the LAB population being less than 0.1 %. However, after 17 cycles of backslopping, LAB species dominated in all environments, demonstrating the effectiveness of backslopping as a selective adaptation strategy.
    - Eleven LAB strains belonging to the genera *Leuconostoc*, *Lactococcus*, *Lactobacillus*, and *Enterococcus* were successfully isolated and identified via MALDI-TOF MS, with their taxonomic classification subsequently confirmed or reclassified by WGS and genome annotation.

In summary, this thesis demonstrates the potential of autochthonous microorganisms to adapt to plant-based environments and form stable microbial consortia through targeted adaptation using backslopping propagation. The results highlight the potential of backslopping as a practical strategy for selecting robust, plant-adapted starter cultures for fermented plant-based foods.

Furthermore, genomic characterization of the isolated strains has been initiated, but additional analyses in accordance with EFSA guidelines are required to fully establish their safety status. Future research should also evaluate the technological potential of these strains across different plant-based matrices and microbial combinations to develop more efficient and robust starter cultures for fermented plant-based foods.

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Abstract

Diversity and stability of microbial consortia in fermented plant-based products

Fermented foods have been part of the human diet for centuries, providing safer, more digestible products with extended shelf life and desirable sensory quality. Today, they remain a regular component of everyday eating habits, offering additional nutritional benefits, and supporting human health through immune-modulating effects.

Traditional fermented foods are shaped by microorganisms originating from raw materials, equipment, containers, and the surrounding environment, with the best-adapted species gradually becoming dominant during fermentation. Over long periods, this spontaneous selection has formed stable mixed communities of bacteria, yeasts, and molds that underpin products such as yogurt, sourdough, wine, kimchi, and kombucha. However, because spontaneous fermentation is inherently variable and may not consistently meet food safety and quality standards, modern production increasingly relies on defined starter cultures to ensure safe, consistent, and reproducible fermentation.

At the same time, rising global demand for high-protein foods and the environmental impact of animal-based production have intensified interest in sustainable plant-based alternatives. Transferring dairy-processing principles to plant matrices introduces technological and sensory challenges, particularly due to strong background flavors or presence of undesirable components in crops such as oats and peas. Fermentation can help mitigate these limitations, especially when using well-adapted microbial cultures.

While dairy fermentations traditionally depend on lactic acid bacteria from genera such as *Lactococcus*, *Lactobacillus*, *Streptococcus*, and *Leuconostoc*, these dairy-adapted cultures often perform poorly in plant substrates. Therefore, selecting autochthonous plant-associated LAB offers a promising direction for developing high-quality fermented plant-based products, and highlights the need for continued research on microbial growth dynamics, metabolism, and fermentation optimization.

This thesis provides an overview of how microbial consortia behave in different fermented systems and how stable these communities remain under changing conditions. Across the studies, traditional fermented foods and beverages—including kombucha—showed a high degree of resilience, with small modifications having little effect on microbial composition at the genus level. Experiments involving the introduction of new bacteria or the use of backslopping in alternative matrices confirmed that the core community structure remained largely unchanged. These observations highlight the robustness of naturally assembled consortia and emphasize the importance of selecting microorganisms that originate from similar ecological environments when developing new starter cultures.

Further work explored how lactic acid bacteria behave when introduced into kombucha and how backslopping affects microbial dynamics in plant-based substrates.

The final part of the research focused on adapting and isolating autochthonous plant-associated species as potential starter cultures for dairy-alternative applications, demonstrating that repeated backslopping can shift microbial dominance toward LAB even when initial populations are low. Together, these studies form a foundation for developing new plant-based starter cultures and highlight the need for continued genomic characterization and application testing to ensure safety, stability, and technological suitability.

Lühikokkuvõte

Mikroobikoosluste mitmekesisuse ja stabiilsuse uurimine fermenteeritud taimsetes toodetes

Fermenteeritud toidud on olnud osa inimeste toidulauast sajandeid, pakkudes ohutumaid, paremini seeditavaid ning pikema säilivusajaga tooteid, millel on ka soovitud sensoorsed omadused. Tänapäeval kuuluvad fermenteeritud toidud jätkuvalt inimeste igapäeva menüüsse, kuna nende söömine toetab immuunsüsteemi toimimist. Traditsiooniliste fermenteeritud toitude mikroobid pärinevad peamiselt keskkonnast näiteks toorainest. Fermentatsiooni käigus hakkavad domineerima need liigid, mis on antud substraatidega kõige paremini kohanenud. Pika aja jooksul on selline spontaanne valik kujundanud stabiilsed mikroobikooslused bakteritest, pärmidest ja hallitusseentest. Head traditsioonilised toidud on näiteks jogurt, leiva juuretis, vein, kimchi ja kombucha. Spontaanse fermentatsioon ettearvamatuse tõttu, tugineb tänapäevane toidu tootmine peamiselt starter kultuuridel, mis tagavad ühtlase toote kvaliteedi.

Samas on üleilmne nõudlus kõrge valgusisaldusega toitude järele aina kasvanud, et vähendada loomse tootmise keskkonnamõju. Sobivaks alternatiiviks on taimsed valgud, näiteks kaeravalk ja hernevalk. Traditsiooniliste piima töötlemise tehnoloogiliste võtete kasutamine tekitab uuendusliku taimse substraadi töötlemisel väljakutseid. Näiteks kaeral ja hernel on tugevad kõrvalmaitsed. Fermentatsiooniga saab neid sensooreseid kõrvalmõjusid vähendadad kui kasutada hästi kohanenud mikroobikooslusi.

Antud hetkel kasutatakse alternatiivsete piimatoodete tootmisel neid samu piimast eraldatud traditsioonilisi starter kultuure, näiteks *Streptococcus thermophilus* ja *Lactobacillus delbrücki* subsp. *bulgaricus*. Kahjuks ei toimi need kultuurid taimsetes substraatides sageli piisavalt hästi. Seetõttu pakub autohtoonsete, taimset päritolu piimhappebakterite valik paljulubavat suunda kvaliteetsete taimsete fermenteeritud toodete arendamiseks ning rõhutab vajadust jätkuva uurimistöö järele mikroobide kasvudünaamika, metabolismi ja fermentatsiooniprotsessi optimeerimise vallas.

Käesolevas doktoritöös antakse ülevaade sellest, kuidas mikroobsed konsortsiumid erinevates fermenteeritud süsteemides käituvad ning kui stabiilsed need kogukonnad muutuvates tingimustes on. Uuringud näitasid, et kombuchas on väga vastupidavad kultuurid ning väikesed muudatused avaldavad mikroobide koosseisule perekonna tasandil vähest mõju. Katsetused uute bakterite lisamisega või *backslopping*-meetodi rakendamisega alternatiivsetes maatriksites kinnitasid, et põhikooslus jäi suures osas muutumatuks. Need tähelepanekud rõhutavad looduslikult kujunenud konsortsiumide tugevust ning vajadust valida uute starterkultuuride loomiseks mikroorganisme, mis pärinevad sarnastest ökoloogilistest keskkondadest.

Samuti uuriti, kuidas käituvad piimhappebakterid kombuchas ning kuidas mõjutab *backslopping* mikroobide dünaamikat taimsetes substraatides. Uurimuse lõpposa keskendus autohtoonsete taimset päritolu liikide kohanemisele ja potentsiaalsete starterkultuuride isoleerimisele piimavabade toodete jaoks. Töös näidati, et korduv *backslopping* nihutab mikroobse domineerimise LAB-i kasuks isegi siis, kui nende algne osakaal on väga väike. Koos moodustavad need uuringud aluse uute taimsete starterkultuuride väljatöötamiseks ning rõhutavad vajadust jätkata genoomset iseloomustamist ja teostada katseid, et tagada kultuuride ohutus, stabiilsus ja tehnoloogiline sobivus.

Appendix 1

Supplementary materials

Table S 1 Information on the bottles of the studied kombuchas. Obtained from: Anderson et al., (2022)

Producer	Product name	Code	Origin country	Batch number	Best before	Container	Ingredients
Cruz Group's, Inc.	Vigo kombucha original	V1	Poland	L07M	04.12.2021	Colorless glass bottle	spring water, fruit extract, cider vinegar/apple vinegar, fermented herbal tea extract, spices, CO_2, lactic acid, malic acid, stabilizer, glycerol esters of raisin, B-vitamins: niacin, B6, B12, biotin
Helde Pruulikoda OÜ	Helde kombucha valge	H1	Estonia	88	03.10.2020	Dark glass bottle	filtered water, white tea (*Camellia sinensis*)*, rooibos*, fennel*, rose hip*, black pepper*, cane sugar, kombucha culture
Helde Pruulikoda OÜ	Helde kombucha roheline	H2	Estonia	99	25.02.2021	Dark glass bottle	filtered water, green tea (*Camellia sinensis*)*, lemongrass*, apple*, spearmint*, cane sugar*, orange peels*, kombucha culture
Helde Pruulikoda OÜ	Helde kombucha punane	H3	Estonia	96	13.02.2021	Dark glass bottle	filtered water, hibiscus tea*, orange blossoms*, ginger*, cane sugar*, kombucha culture
Gut Feeling OÜ	Mjuk kombucha klassikaline	M1	Estonia	253	23.01.2021	Dark glass bottle	water, raw cane sugar*, green tea*, kombucha culture*
Gut Feeling OÜ	Mjuk kombucha humal	M2	Estonia	247	15.12.2020	Dark glass bottle	water, raw cane sugar*, green tea*, kombucha culture*, hops
Gut Feeling OÜ	Mjuk kombucha ingver	M	Estonia	251	10.01.2021	Dark glass bottle	water, raw cane sugar*, green tea*, kombucha culture*, ginger root*
Gut Feeling OÜ	Mjuk kombucha kurkum	M4	Estonia	251	10.01.2021	Dark glass bottle	water, raw cane sugar*, green tea*, kombucha culture*, turmeric root*
Gut Feeling OÜ	Mjuk kombucha münt	M5	Estonia	249	25.10.2020	Dark glass bottle	water, raw cane sugar*, green tea*, kombucha culture*, mint*

Producer	Product name	Code	Origin country	Batch number	Best before	Container	Ingredients
Säsi Pruulikoda OÜ	Säsi kombucha salvei	S1	Estonia	66	13.10.2020	Colorless glass bottle	filtered water, raw cane sugar*, black tea*, kombucha culture
Säsi Pruulikoda OÜ	Säsi kombucha klassikaline	S2	Estonia	209	23.01.2021	Colorless glass bottle	filtered water, raw cane sugar*, green tea*, sage 0.2%, kombucha culture
Tee Veel OÜ	Kuma kombucha jasmiin	K2	Estonia	345	06.02.2021	Dark glass bottle	water, raw cane sugar*, jasmine superior tea*, kombucha culture
Tee Veel OÜ	Kuma kombucha klassikaline	K1	Estonia	343	14.12.2020	Dark glass bottle	water, raw cane sugar*, black tea*, kombucha culture
GUTsy Captain Company	Captain kombucha raspberry	C2	Portugal	L01715A2	04.07.2021	Dark plastic bottle	water filtered by reverse osmosis, organic cane sugar (transformed during fermentation), kombucha culture, organic stevia tea extract (water, organic stevia leaves), organic green tea, natural raspberry flavor 0.14%, organic extract (carrot, apple, and blackcurrant), "*Bacillus coagulants*"
GUTsy Captain Company	Captain kombucha pineapple & peach	C3	Portugal	L02344A2 2-1	05.09.2021	Dark plastic bottle	water filtered by reverse osmosis, organic cane sugar (transformed during fermentation), kombucha culture, organic stevia tea extract (water, organic stevia leaves), organic green tea, natural pineapple flavor 0.15%, natural peach flavor 0.002%, "*Bacillus coagulants*"
GUTsy Captain Company	Captain kombucha original	C1	Portugal	L03A087-1	11.04.2021	Dark plastic bottle	water filtered by reverse osmosis, organic cane sugar (transformed during fermentation), kombucha culture, organic stevia tea extract (water, organic stevia leaves), organic green tea, organic green tea extract 0.0001%, "*Bacillus coagulants*"

* Organic ingredient

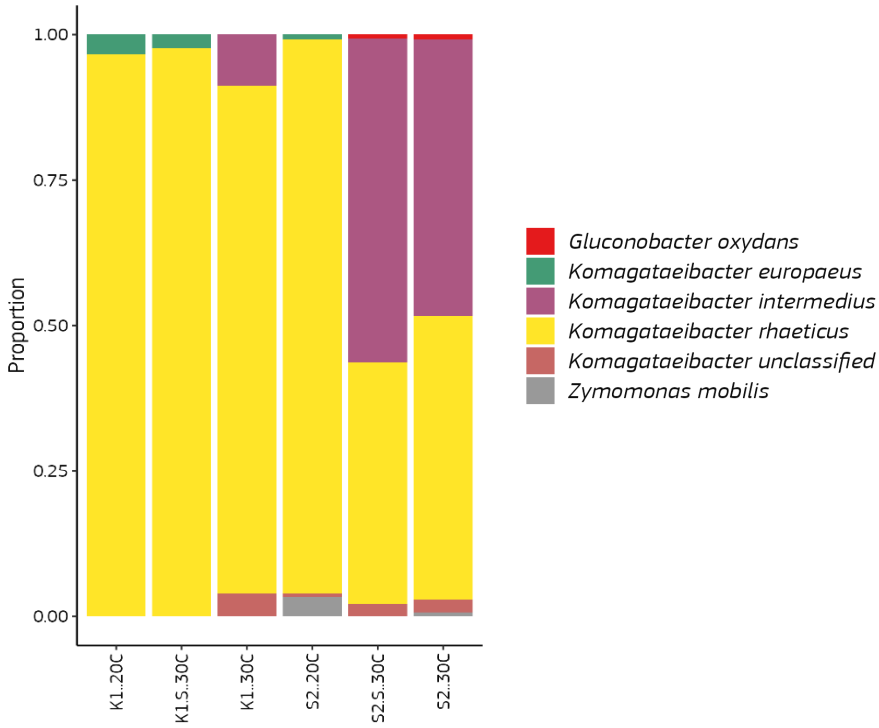

Figure S 1 Bacterial composition of K1 and S2 kombucha cultures cultivated at 20 °Cand 30 °. According to 16S metabarcoding, graphed as relative abundances. The abbreviation S stands for SCOBY. The proportion threshold was set to 0.01. Unpublished data.

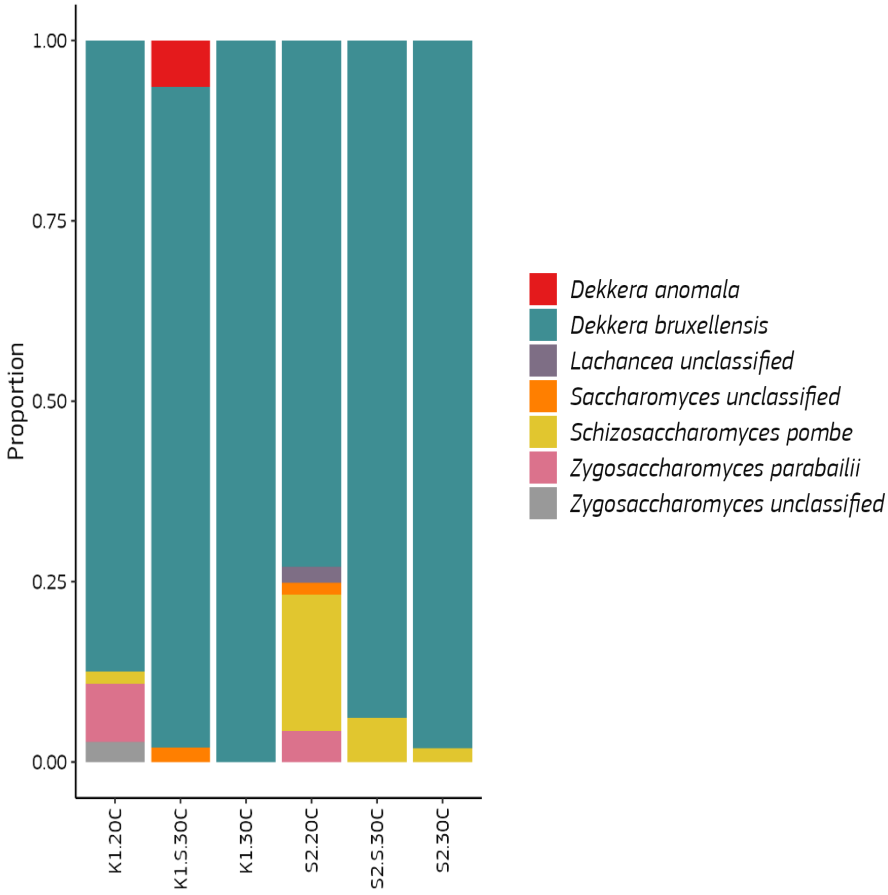

Figure S 2 Yeast composition of K1 and S2 kombucha cultures cultivated at 20 °Cand 30 °. According to ITS2, graphed as relative abundances. The abbreviation S stands for SCOBY and K for kombucha liquid. The proportion threshold was set to 0.01. Unpublished data.

Table S 2 Sensory characteristics and pH measurements from backslopping point 17. Highlighted and bold font represent the sample descriptions

	25 °C								34 °C								42 °C							
	Oat 1		Oat 2		Pea 1		Pea 2		Oat 1		Oat 2		Pea 1		Pea 2		Oat 1		Oat 2		Pea 1		Pea 2	
	Sensorics	pH		pH		pH		pH		pH		pH		pH		pH		pH		pH		pH		pH
Bs 17	**Odor - like sour cream. Taste - good sour-sweet balance, carbonated. Texture - similar to yoghurt.**	**4.76**	Odor - sour. Taste - very sour, slightly bitter, carbonated. Texture - watery.	4.62	Odor - sweet. Taste - salty. Texture - watery.	4.82	Odor - chemicaly and smelled like trash. Taste - did not taste. Texture - watery.	4.86	Odor - intense unpleasant old smell. Taste - intense unpleasant. Texture - watery.	4.68	Odor - sour. Taste - very sour. Texture - watery.	4.38	**Odor - sweet, caramelly. Taste - like mild unflavoured yoghurty. Texture - watery.**	**4.98**	Odor - peany, grass notes. Taste - unpleasant. Texture - watery.	4.96	Odor - like sour cream. Taste - old unpleasant intense sour cream. Texture - watery.	4.96	**Odor - sweet, caramelly. Taste - tasted like yoghurty. Texture - good, like oat porridge.**	**4.52**	**Odor - oat meal, good. Taste- good sour-sweet balance, like oat drink. Texture - watery.**	**4.93**	Odor - bready, sour, decayed wood. Taste - interesting in the wrong sense. Texture - watery.	5.07

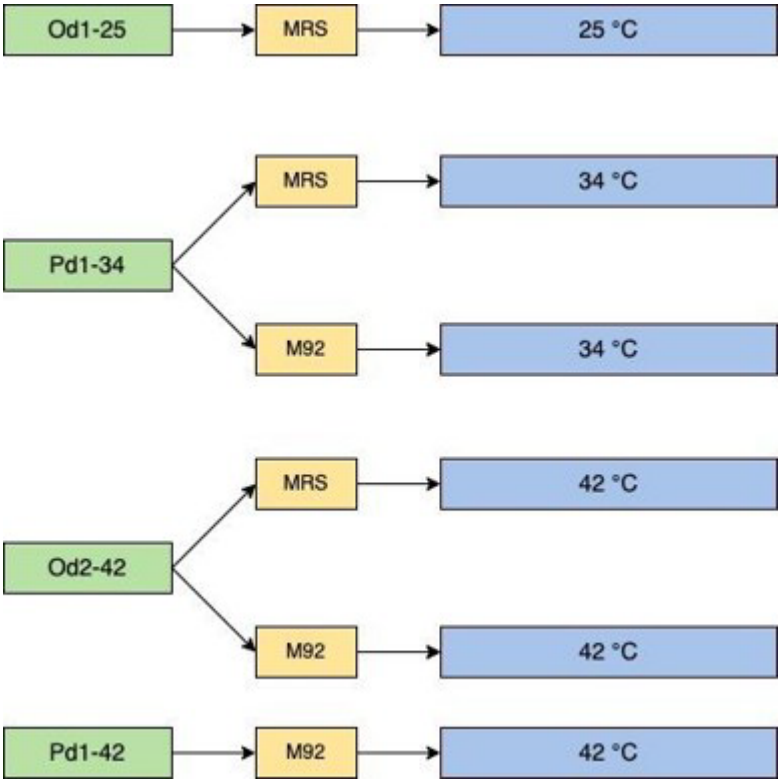

Figure S 3 The process of strain isolation. The backslopping endpoint (BS 17) used for species isolation is indicated in the green box. Green box names indicate: oat drink 1 cultivated at 25 °C (Od1-25), oat drink 2 cultivated at 42 °C (Od2-42), pea drink 1 cultivated at 34 °C (Pd1-34), and pea drink 1 cultivated at 42 °C (Pd1-42). The yellow boxes show the selected agar media for isolating colonies. The blue boxes show the incubation temperature of agar plates.

Appendix 2

Publication I

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Characterisation of chemical, microbial and sensory profiles of commercial kombuchas

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ABSTRACT

The kombucha market is a fast-growing segment in the functional beverage category. The selection of kombuchas on the market varies between the traditional and flavoured kombuchas. Our research aimed to characterise the chemical, microbial, and sensory profiles of the commercial kombuchas. We analysed 16 kombuchas from 6 producers. The dominant metabolites were acetate, lactate, and ethanol, the last of which might put some kombuchas into the alcoholic beverage section in some countries. The metagenomic analyses demonstrated that LAB dominates in green tea, and AAB in black tea kombuchas. The main bacterial species were *Komagataeibacter rhaeticus* and *Lactobacillus ssp*, and yeast species *Dekkera anomala* and *Dekkera bruxellensis*. The sweet and sour balance correlated with acid concentrations. The free sorting task showed that commercial kombuchas clustered into three main categories "fruity and artificial flavour", herbal and tea notes", and "classical notes". Our research results showed the necessity of the definition of kombucha.

1. Introduction

Kombucha is a tea beverage fermented by symbiotic consortium of bacteria and yeast (Dufresne and Farnworth, 2000; Jayabalan et al., 2014). Up to now, the consortia of kombucha and kombucha biofilm in different environmental conditions have been investigated (Arıkan et al., 2020; Bharathiraja et al., 2016; Coton et al., 2017; Marsh et al., 2014). However, there is a lack of information about the chemical, microbial and sensory properties of commercial kombuchas.

In 2018, the kombucha market value was 1.8 billion USD, and by 2025, the expected value will be over 5 billion USD (Kim and Adhikari, 2020). The market is distributed into traditional and flavoured kombucha, where the last segment leads the market. The original brewing medium in flavoured kombucha has been replaced, e.g. by rooibos tea, coffee, juices, or some flavours have been added such as fruits and herbs (Kim and Adhikari, 2020). These untraditional components affect the sensory and microbial profiles of the kombucha causing huge diversity among composition and properties of commercial kombuchas (Dufresne and Farnworth, 2000; Jayabalan et al., 2014; Marsh et al., 2014; Reva et al., 2015).

Traditional kombucha is derived from black or green tea leaves by addition of 5–10% of sugar and inoculum of starter culture that consists of the previous batch of kombucha liquid and SCOBY (Symbiotic Culture of Bacteria and Yeast). The initial geographical origin, environmental conditions, and the growth medium of SCOBY determine the diversity of species in kombucha liquid and SCOBY (Marsh et al., 2014; May et al., 2019).

The dominant bacterial species in the kombucha consortium belong to acetic acid bacteria (AAB), mainly to the *Komagataeibacter* genus (earlier named as *Gluconacetobacter* and before as *Acetobacter* (Chakravorty et al., 2016; Marsh et al., 2014; Yamada et al., 2012)), such as *Komagataeibacter intermedius* (Reva et al., 2015), *Komagataeibacter xylinus* (De Filippis et al., 2018; Reva et al., 2015), *Komagataeibacter rhaeticus* (Semjonovs et al., 2017), *Komagataeibacter saccharivorans* (De Filippis et al., 2018; Reva et al., 2015) and *Komagataeibacter kombuchae* (Reva et al., 2015). Also, species from the genera of lactic acid bacteria (LAB) such as *Lactobacillus*, *Leuconostoc*, and *Bifidobacterium* have been identified in kombucha (Villarreal-Soto et al., 2018). The dominant

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yeast species in the kombucha belong to genera *Zygosaccharomyces (Zygosaccharomyces bailii)*, *Saccharomycodes* (*Saccharomycodes ludwigii*), *Candida*, *Torulaspora*, *Pichia*, *Brettanomyces/Dekkera (Brettanomyces bruxellensis*), *Schizosaccharomyces (Schizosaccharomyces pombe)* and *Saccharomyces (Saccharomyces cerevisiae)* (Coton et al., 2017; Dufresne and Farnworth, 2000; Jayabalan et al., 2014; Marsh et al., 2014; Reva et al., 2015).

The bacteria and yeasts in kombucha cooperate and compete. It leads to the cascades of reactions where different chemical components are formed, such as acetic acid, gluconic acid, glucuronic acid, and ethanol (May et al., 2019). All named components dynamically affect the sensory profile and microbial composition in the kombucha consortium (Dufresne and Farnworth, 2000; Jayabalan et al., 2014; Marsh et al., 2014; Reva et al., 2015). Although some information about the microbial and chemical composition of commercial kombuchas is available, the sophisticated sensory study that characterises kombucha properties has not been implemented (Kim and Adhikari, 2020). Therefore, current research aims to identify the microbial composition by using novel metagenomics methods for consortia analysis, which influences chemical and sensory characteristics of commercial kombuchas.

2. Materials and methods

2.1. Collection of kombuchas

The commercial kombuchas were obtained from the Estonian market in September–October 2020. Sixteen different kombuchas in two biological replicates were acquired for the metagenomic, sensory and chemical analyses. The kombuchas were from several countries and six brands were represented. The coding of kombuchas can be found in Supplementary Table A.1.

2.2. Determination of pH and titratable acidity

Five millilitres of the sample were suspended in 50 mL of distilled water to measure pH and titratable acidity (TA). The Food and Beverage Analyzer (Mettler-Toledo International Inc., Columbus, OH, USA) was used for pH and TA measurements. The acidity was determined through titration to pH 7 using 0.1 N of NaOH and expressed as 0.1 N of NaOH per 1 mL of kombucha. All measurements were performed in replicates.

2.3. Determination of metabolic products in kombucha

The liquid was filtered by using Millipore Millex-LG filters 13 mm Philic PTFE 0.2 μm Non-sterile (Germany). The Waters 2695 HPLC system (Waters Corporation, Milford, MA, USA) was used to analyse metabolic products (organic acids, sugars, and ethanol) in the kombuchas. The HPX-87H column (BioRad Hercules, CA) was used, at 35 °C and the system was eluted isocratically with 0.005 M of H_2SO_4 at 0.6 mL/min. The Waters 2487 dual absorbance detector (Waters Corporation, Milford, MA, USA) and the Waters 2414 refractive index detector (Waters Corporation, Milford, MA, USA) were used for detection and quantification of analytes. The data analysis was performed with Empower software (Waters Corporation, Milford, MA, USA).

2.4. Sensory analysis

Sensory analysis was conducted in the Center of Food and Fermentation Technologies (TFTAK, Tallinn, Estonia) by an expert sensory panel accordingly with ISO 8586:2012. The panel consisted of eight trained assessors (seven females and one male), who were between 25 and 48 years old (average age 34). Panellists were subjected to previous training for kombucha sensory analysis. The evaluation was carried out with two different descriptive analysis methods. Quantitative Descriptive Analysis (QDA) was conducted to compare the sensory profile of different kombuchas. Free sorting was used to map kombuchas based on similarities perceived. Assessors evaluated samples in isolated booths in a sensory room which was in accordance with ISO 8589:2007. Planning and conducting sensory tests followed the ISO 6658:2017. Kombucha samples were stored refrigerated at 4 °C and were served at room temperature (22 °C). All samples were served in transparent 40 mL plastic cups that were coded with three-digit numbers. Palate cleansing was encouraged during evaluations with unsalted water crackers (Pladis LTD, London, UK) and available spring water (Eden Springs Estonia OÜ, Tallinn, Estonia). All sensory data was collected by using RedJade (RedJade Sensory Solutions LLC, Martinez, CA, USA).

QDA was performed as individual evaluations replicated in two sessions. A session lasted about 40 to 50 min. Assessors evaluated intensity of all attributes on a 10-point scale with word anchors (0 = "none", 1 = "very weak", 5 = "moderate", 9 = "very strong"). The initial attribute list was developed based on literature (Gramza-Michalowska et al., 2016; Neffe-Skocińska et al., 2017; Yavari et al., 2017) and was refined in panel discussions. The final attribute list is shown in Supplementary Table A.3. Four modalities were assessed in total: appearance, odour, texture, and taste. The additional comments of modality were possible to write in a voluntary text box.

To avoid loss of carbonization that can affect sensory results (texture), samples were served in groups consisting of 3–4 samples and each group was evaluated immediately after opening the bottle. Samples were grouped based on their flavouring characteristics to prevent the convergence effect where a very distinctive sample may reduce the perceived differences between other samples (Hollowood, 2018). The order of the groups was the same for all panellists, but the sample order within the group was balanced by Williams' Latin square design to avoid the effect of presentation (Macfie et al., 1989). There were two-minute breaks between each group assessment to prevent sensory fatigue. In addition, the first sample in both sessions was a reference kombucha that helped panellists to get ready for the evaluation and to compare it with the upcoming samples. Values on the scale for the reference sample were previously agreed by consensus.

The free sorting task was given in a session lasting about 15 min. All samples were presented simultaneously in a randomized order. Assessors were asked to sort each sample into a group and to arrange as many groups as necessary. Every formed group was characterised based on their differences. Data was cleaned similarly to Pétel et al. (2017) research, where synonyms were assembled, and descriptors used by less than 10% of participants were removed. Since there were 8 participants in total, then the 10% was rounded to one participant.

2.5. Amplicon-based metagenomic analysis

2.5.1. DNA extraction

A bottle with commercial kombucha was roiled and opened carefully in aseptic conditions. 100 mL of the kombucha beverage was centrifuged at 3950 ×*g* for 20 min at 4 °C. Then, the pellet was washed with 500 μL of sterile and cold 1 × PBS (*Phosphate-buffered saline*, BIO-RAD, CA, USA), centrifuged at 10,000 ×*g* for 10 min at 4 °C. For the gDNA extraction, the pellet was resuspended in 200 μL of 1 × PBS and added into the ZR BashingBead™ Lysis Tube (Zymo Research, Irvine, CA, USA). The gDNA isolation was performed by applying Quick-DNA™ Fungal/Bacterial Miniprep kit (Zymo Research) according to the instruction manual. Extracted gDNA was quantified by a Qubit™ 4 Fluorometer (Thermo Fisher Scientific, Waltham, MA, USA) using dsDNA BR Assay Kit (Thermo Fisher Scientific).

2.5.2. Amplicon sequencing

25 ng of extracted microbial gDNA was subjected to the amplicon library preparation. For the study of the bacterial composition, the V4 region of the 16S ribosomal RNA (rRNA) gene (Caporaso et al., 2011) was sequenced as described in Kazantseva et al. (2021) by Next Generation Sequencing (NGS) technology.

For the determination of fungi, the 5.8S-ITS2-LSU region of the rRNA

gene was sequenced using forward 5.8S-Fun 5′-**AACTTTYRRCAAYGGATCWCT**-3′ and reverse ITS4-Fun 5′-**AGCCTCCGCTTATTGATATGCTTAART**-3′ primers (Biomers, Germany); (Taylor et al., 2016). The library preparation and sequencing were carried out as mentioned before (Kazantseva et al., 2021) with some modifications. In the amplification stage of the library preparation, 25 cycles, annealing temperature of 58 °C and elongation time of 45 s were applied. The normalised library pool was sequenced on iSeq100 Sequencing System (Illumina, San Diego, CA, USA) using iSeq 100 i1 Reagent and 300 cycles single-end protocol.

2.6. Bioinformatic analysis

The sequencing data of the V4 region of the 16S rRNA gene were analysed by an open-source BION-meta package (Espinosa-Gongora et al., 2016; McDonald et al., 2016; www.box.com/bion, Danish Genome Institute, Denmark). Raw sequences (paired-end reads 2 × 150 bp) were cleaned at both ends applying a 99.5% minimum quality threshold for at least 18 of 20 bases for 5′-end and 28 of 30 bases for 3′-end, joined and contigs shorter than 150 bp were removed. Created sequences were cleaned from chimaeras and clustered by 95% oligonucleotide similarity (k-mer length of 8 bp, step size 2 bp). Obtained consensus reads were aligned to the SILVA reference 16S rRNA database (v123) using a word length of 8 and a similarity cut-off of 90%. The bacterial designation was analysed at different taxonomic levels down to species if applicable.

The sequencing data of the 5.8S-ITS2-LSU region of the rRNA genes were processed and analysed using custom pipeline of the Quantitative Insights Into Microbial Ecology [QIIME2, version 2019.10, (Bolyen et al., 2019)]. All following procedures in this section were conducted in the QIIME2 environment plug-ins. Demultiplexed single-end reads from iSeq100 (1 × 300 bp) were trimmed to remove primers and poor quality bases and then denoised with DADA2 (Callahan et al., 2016, R package, https://github.com/benjjneb/dada2). Taxonomies were assigned with a q2-feature-classifier plug-in and classify-sklearn (Pedregosa et al., 2011) method using the fungal ITS classifiers trained on UNITE reference database [version 2018.11.18 ("UNITE Community (2019) UNITE QIIME release for Fungi 2.," n.d.)].

2.7. Quantitative real-time PCR

To measure the bacterial and the fungal-specific gDNA concentration of the commercial kombucha, a quantitative real-time PCR (qPCR) was carried out using qTOWER^{3}G thermal cycler (Analytik Jena, Jena, Germany) by implementing a qPCRsoft 4.0 software. Standard curves for the quantification were performed applying DNA standards from Femto Bacterial or Fungal Quantification kits (Zymo Research, Irvine, CA, USA). Specific primer pairs 515F/806R (forward 5′-GTGYCAGCMGCCGCGGTAA-3′ and reverse 5′-GGACTACNVGGGTWTCTAAT-3′; Biomers, Germany) for bacterial V4 region of the 16S rRNA gene (Caporaso et al., 2011), and UNF1/UNF2 (forward 5′-GCATCGATGAAGAACGCAGC-3′ and reverse 5′-TTGATATGCTTAAGTTCAGCGG-3′) for fungal ITS2 region of the ribosomal gene (Fiedorová et al., 2019) were used. Each reaction in a total volume of 10 μL included 5× HOT FIREPol® SolisGreen qPCR Mix (Solis BioDyne, Tartu, Estonia), 5 pmol of each primer and 1 μL of extracted gDNA sample or commercial standard. The reaction conditions were as follows: initial denaturation at 95 °C for 12 min; 42 cycles of denaturation at 95 °C for 30 s, annealing at 55 °C for 30 s and elongation at 72 °C for 30 s; final elongation at 72 °C for 7 min. To determine the specificity of amplification, an analysis of the product melting curve was performed after the last cycle of the amplification. All samples and standards were analysed in duplicates.

Based on the quantification data of the qPCR (Supplementary Table A.1) and according to the taxonomic proportional abundances of the sequencing results, the content of detected bacterial and yeasts species of each investigated kombucha was calculated. The number of the amplified PCR products (16S rRNA or ITS2 rRNA gene copies) per volume was calculated based on the equation: Copy number $= \frac{N_A \times m \times D}{AS \times M}$, where N_A is the Avogadro number 6.02 × 10^{23} $\left(\frac{\text{copies}}{\text{mol}}\right)$, m is the measured mass of DNA (g), D – dilution coefficient, AS is the amplicon size (bp) and M is the dsDNA molar mass 660 $\left(\frac{g}{\text{mol bp}}\right)$ (Duewer et al., 2018). To estimate the cell numbers, the average number of 16S rRNA genes in bacteria was considered as 4.2 (Větrovský and Baldrian, 2013), and 150 copies for ITS2 (Kobayashi, 2011).

2.8. Statistical analysis

Sensory results were statistically analysed and visualised with R version 4.1.0 (R Foundation for Statistical Computing, Vienna, Austria). For quantitative descriptive analysis, results were interpreted using principal component analysis (PCA), analysis of variance (ANOVA), and variable distributions presented as violin plots. Free sorting results were statistically analysed by multiple correspondence analysis (MCA) from R package "FactoMineR" 2.4. Dissimilarities between microbial consortia were assessed using non-metric multidimensional scaling (NMDS) function from R package "vegan" 2.5-7.

3. Results

3.1. Chemical composition of commercial kombuchas

All commercial kombuchas were evaluated for acidity and amounts of metabolites. The acidity was estimated by pH and TA measurements. The pH values of all products stayed below 4, and the average TA value was 3.1 ± 0.3 mL of 0.1 N NaOH (Supplementary Fig. A.1). Commercial kombucha K2 had the highest pH with the value of 3.7 ± 0.3, and the product H3 pH value was 2.8 ± 0.3, which was the lowest detected. This kombucha TA value was also the highest with the value of 6.0 ± 0.3 mL of 0.1 N NaOH. The pH values of fermented beverages of manufacturers H, M, and S changed between different products. However, the beverages of producer C showed rather equal results, the pH fluctuation was 0.1 units. The kombucha C3 showed the lowest TA value (0.9 mL of 0.1 N NaOH). The TA values also varied between different kombuchas of the same manufacturer and were negatively correlated to pH (Pearson's r – 0.56).

The concentration of acetic acid in analysed beverages was relatively high (Fig. 1A). Kombuchas H1–H3 showed elevated concentrations in the range of 3.54–6.40 g/L. The product V1 showed the lowest level of acetic acid (0.35 ± 0.002 g/L). In contrast, only C1, C3, M1, M2, M3, M5, S1, S2, and V1 beverages contained lactic acid. The highest concentration of lactic acid was in kombucha M5 and the lowest in C1 that corresponds to 3.01 ± 0.17 g/L and 0.21 ± 0.02 g/L, respectively.

Additionally, the commercial kombuchas contained residues of citric acid, malic acid, and succinic acid (Fig. 1B). The concentrations of these acids were 10-folds lower than that of acetic and lactic acid. Products H1 and M2 had the highest concentration of citric acid—0.36 and 0.34 g/L, and C1 had the lowest at 0.07 g/L. Malbaša et al., 2011 measured the citric acid concentration accounted for 2.5% of the total acidity, and Jayabalan et al., 2007 citric acid concentrations varied between 0.03 and 0.11 g/L. Our results showed this acid content in studied kombucha was insignificant, what was confirmed by the literature.

All products with except of the kombucha S1 contained gluconic acid (Fig. 1B). Manufacturer H beverages showed the highest concentrations of gluconic acid, other products contained relatively low amounts of this acid. Only four kombuchas contained glucuronic acid: C1, C2, C3 and S2, where the acid concentrations were up to 0.33 ± 0.02 g/L (data not shown).

The average concentration of detected ethanol was 7.35 ± 0.23 g/L. The highest concentration was in kombucha H1 at 14.77 ± 0.77 g/L,

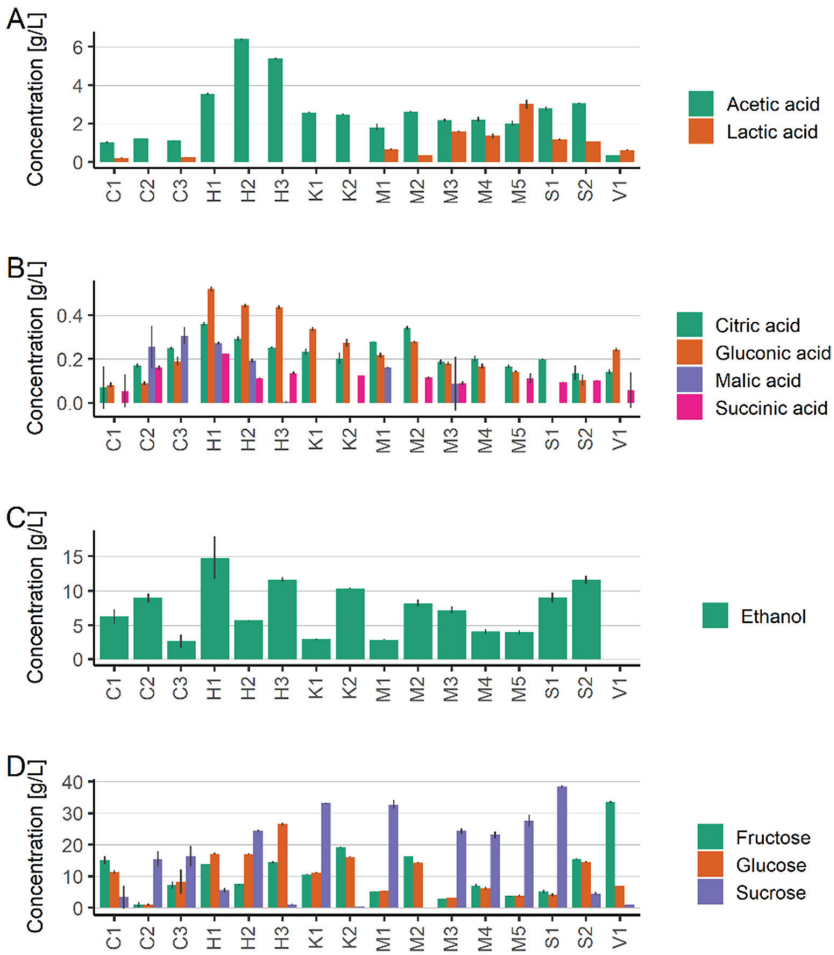

Fig. 1. Organic acids, ethanol, and sugar profiles of commercial kombuchas are shown as mean with standard deviations (n = 2). Metabolite profiles presented in acetic and lactic acids profiles (A); citric, gluconic, malic and succinic acids profiles (B); ethanol profiles (C); fructose, glucose, and sucrose profiles (D). The coding of kombuchas is explained in Supplementary Table A.1.

and the lowest in C3 with a value of 2.66 ± 0.75 g/L (Fig. 1C). The beverage V1 did not contain ethanol.

All products included sucrose; the concentrations varied between 0.21 ± 0.01 g/L to 38.49 ± 0.30 g/L (Fig. 1D). The highest sucrose concentration occurred in kombucha S1 and the lowest in M2. Also, relatively low concentrations of sucrose were in kombuchas V1 and H3 1.13 ± 0.01 g/L and 1.19 ± 0.03 g/L, respectively. Additionally, these two commercial kombuchas showed the highest concentrations of the monosaccharides' glucose and fructose.

3.2. Microbial composition of commercial kombuchas

Microbial gDNA were isolated from 15 out of 16 commercial kombuchas (Supplementary Table A.2). Repeated extraction of microbial DNA from V1 product failed because of the absence of any viable microbes which was confirmed by additional out-plating analysis (data not shown). The common universal origin of kombuchas from one starter culture among definite manufacturers was confirmed by NMDS clustering analysis (Supplementary Fig. A.2). Kombuchas from manufacturers S, M, and H formed distinct groups, but K1–K2 and C1–C3 did not, which is confirmed by their difference in bacterial composition (Fig. 2). However, K1 showed similarities with the H cluster but K2 could not be clustered.

The metagenomic 16S amplicon NGS results revealed that the most abundant genera among the kombuchas consortia refer to *Komagataeibacter*, *Lactobacillus* and *Bacillus* (Fig. 2A) that belong to the potential probiotic strains such as *Lactobacillus* and *Oenococcus*, and bacterium *Bacillus coagulans*. Also, some products contained *Acetobacter*, *Gluconobacter*, *Pseudomonas* and *Zymomonas* species.

The dominant species in kombuchas H1–H3 were related to *Komagataeibacter* and *Bacillus*, while *Lactobacillus* prevailed in M1–M5 beverages. The highest proportion of bacteria in S1and S2 beverages belonged to *Zymomonas mobilis*, but K1 and K2 kombuchas included mainly *Komagataeibacter* and *Gluconobacter* species. The most diverse bacterial composition across the one manufacturer was in C1–C3 products. Here, the common bacterial pattern was not detected, thus all three tested kombuchas had totally different major bacteria – *Pseudomonas putida* in C1, *Bacillus coagulans* in C2, and *Gluconobacter oxydans* in C3 beverage. Inclusively, the distinctive pattern in the bacterial composition of commercial kombuchas was shown - LAB dominated in green tea

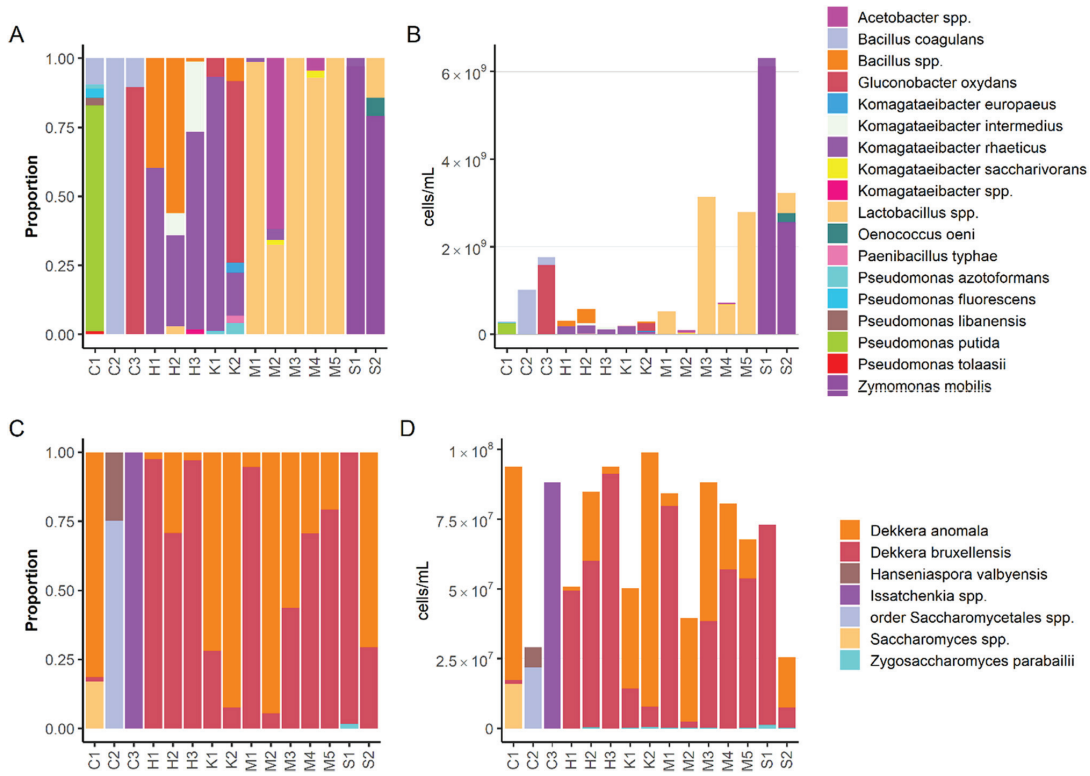

Fig. 2. Microbial composition of kombuchas according to 16S NGS for bacteria (A, B) and ITS2 NGS for yeasts (C, D) graphed as relative (A, C) and normalised abundances (B, D). The coding of kombuchas can be found in Supplementary Table A.1.

and AAB in black tea beverages.

In general, most kombuchas had 2–3 dominant bacteria in their composition. However, beverages H2, H3, and M2 contained mixture of four dominant bacterial cultures, and K2 and C1 were even richer. Products H2, H3 and M2 consisted mainly of common bacterial species, while the richness of C1 was due to the presence of various *Pseudomonas* species along with *B. coagulans*. The diversity of K2 drink was also caused by additional atypical bacteria for kombuchas like *Pseudomonas azotoformans* and *Paenibacillus typhae*.

The proportion of different bacterial species in fermented beverage is a valuable parameter to characterise and compare commercial products. The results of normalised absolute data based on bacteria-specific qPCR are represented on Fig. 2B. The products of S and M manufacturers have the highest number of bacterial cells in their composition, up to 6.4×10^9 cells/mL. At the same time, manufacturers H and K have a lower bacterial load in their drinks (~5.0×10^8 cells/mL), while C manufacturer shows intermediate values (~0.5×10^8–1.8×10^9 cells/mL). We also detected the difference in the bacterial load of the manufacturers M and C kombuchas, which points to the fact that diversity is not always connected with the abundance of bacteria. The prevalent bacterial genera for S and M were *Zymomonas* and *Lactobacillus*, correspondently, while the C manufacturers' drinks were rich in *B. coagulans* and *G. oxydans* species.

The diversity of yeasts in analysed kombuchas detected by metagenomic ITS2 amplicon NGS was more modest. All identified yeasts belonged to order *Saccharomycetales*, mainly to genus *Dekkera/Brettanomyces* (Fig. 2C). Kombuchas H, K, M and S had different proportions of *Dekkera anomala* and *Dekkera bruxellensis* in their composition. The S1 beverage along with dominant *D. bruxellensis* contained low portion of *Zygosaccharomyces parabailii.* Similarly to the bacterial composition, C group products stayed apart and were more unique regarding the yeasts presented. Moreover, they differed even inside the manufacturing group. Together with *Dekkera* species, C1 product contained *Saccharomyces* spp. Despite detected *Dekkera* genus, C2 included *Hanseniaspora valbyensis* and other *Saccharomycetales spp*, while C3 was dominantly represented by *Issatchenkia* spp.

For absolute quantification, we evaluated the fungal cell number based on DNA concentration measured by ITS2-specific qPCR and considering the average *Saccharomycetales* amplicon size. This normalisation did not affect the pattern of the cell amount so drastically as in the case of bacteria (Fig. 2). So, the pattern of yeasts determined by ITS2 sequencing remained more uniformed and without manufacturers distinctness. To compare, the difference in cell numbers between different kombuchas was only fourfold: the lowest count of fungal cells we found in beverages S2 and C2 (~2.5–3×10^7 cells/mL), and the highest number in H3, K2 and C1 (~1.0×10^8 cells/mL).

3.3. Sensory analysis

According to the ANOVA, all sensory attributes were significant. The kombuchas were mostly distinguishable by their sweet odour and taste based on the QDA results presented as a PCA plot (Fig. 3). In general, kombuchas from the same manufactures were sensorially similar. Overall additives intensities varied in taste and odour due to the different content of flavours. Tea, sourness, and vinegar notes are rather characteristic attributes for kombuchas, which was also noticeable in various products such as H1-H3. Products C1–C3 and V1 were perceived as the sweetest. Each manufacturer's (C, H, K, M) own products were

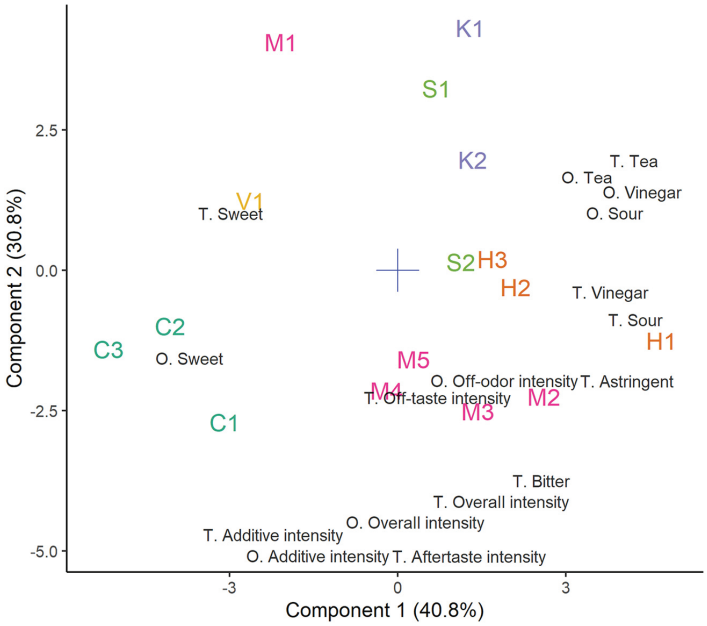

Fig. 3. Principal component analysis (PCA) of quantitative sensory analysis of kombuchas. Abbreviations: O – odour; T – taste. The coding of kombuchas is explained in Supplementary Table A.1.

clustered together on the plot. Product M1 was the only one that differed from other M kombuchas.

The sensory results were in concurrence with the chemical analyses (Fig. 4), which indicate good sensory panel stability. However, the sourness and sweetness may be influenced by other properties, e.g. ingredients used. In general, products with higher TA were perceived as sourer in taste (Fig. 4A). This also corresponds to the sensory results that beverages C1–C3 and V1 were the sweetest and the least sour. The kombuchas with higher total sugar content were mostly perceived as having a sweeter taste (Fig. 4B). However, based on the chemical analyses, the beverages C1–C3 had lower sugar content compared to others. It could be explained with the unbalanced taste profile between sourness and sweetness or the most intense additional flavours. According to the comments from the sensory panel, all C beverages were perceived as fruity and floral, that in turn may be sensorially associated with sweeter nuances.

Free sorting task produced 36 different descriptors, that were categorized into 10 groups: herbal (6 assessors), classical kombucha (5), fruity (4), artificial flavour (4), tea (4), vinegar (3), sweet (3), sweet and sour (2), berry (2), off-flavour (2). Based on the sorting task, the commercial kombuchas can be grouped into three clusters (Fig. 5). The first cluster includes kombuchas with “fruity” and “artificial flavours”,

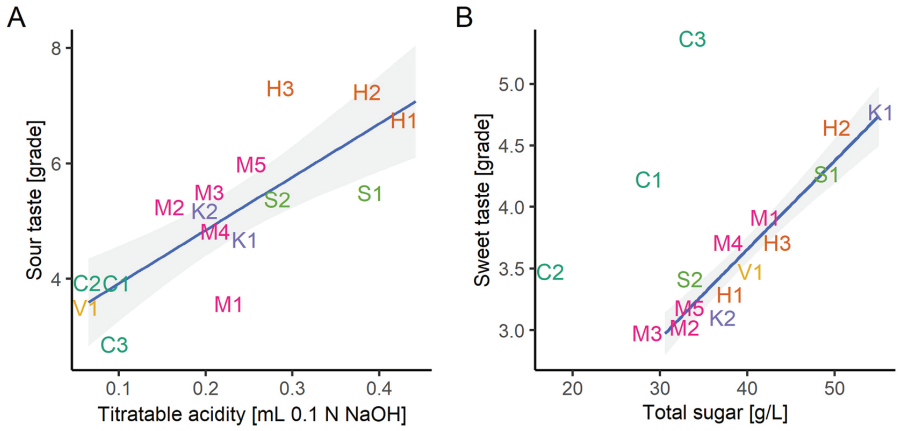

Fig. 4. Average sensory results in ascending order compared with the trendline for chemical analysis average results. A represents comparison for sour taste (sensory analysis) and TA (chemical analysis); B represents comparison for sweet taste (sensory analysis) and total sugar (chemical analysis). The blue lines are linear fit, and the grey areas show their confidence intervals. The coding of kombuchas can be found in Supplementary Table A.1. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

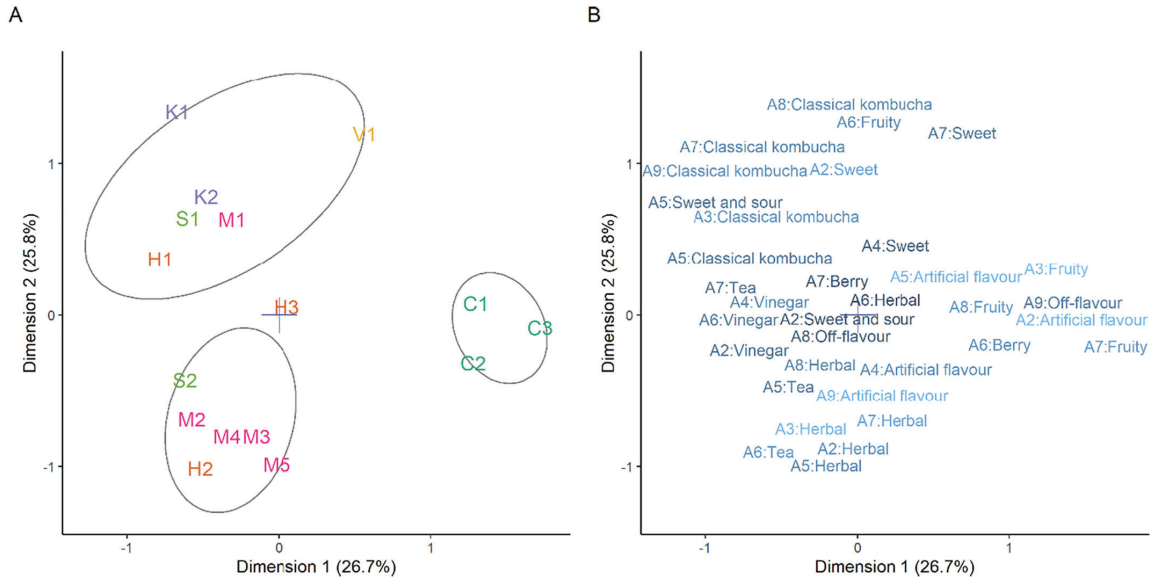

Fig. 5. Multiple correspondence analysis (MCA) for free sorting task, where A demonstrates clustering of different samples and B characterises the associating descriptors. Colour of variable categories is according to cos^2; the lighter, the better this category is represented. Ellipses are drawn at 80% confidence level for three groups identified with hierarchical clustering. The coding of kombuchas is explained in Supplementary Table A.1.

mostly from manufacturer C. As the QDA confirmed, these kombuchas were distinctive by their additive intensity, highest perceived sweetness, and lower sourness. The second cluster (M2–M5, H2, S2) can be described as kombuchas with "herbal" and "tea" notes. These products had herbal ingredients like sage, hop, ginger, turmeric, mint. However, some kombuchas among that group had "vinegar" nuances. The third cluster (K1, K2, S1, M1, H1, V1) was often described as "classical kombuchas" with some "sweet" nuances. All unflavoured versions of kombuchas (coded with 1) and all kombuchas produced by K were included into this group. Another interesting tendency was that all kombuchas produced by H were clustered differently. Kombucha H1 was made with white tea and was grouped into classical kombucha cluster, whereas H2 was made with green tea and grouped into the herbal cluster. H3 (based on red tea) was the only sample that was not clustered into any group as it had similarities to both previously mentioned clusters.

3.4. Finding relations between microbial composition, sensory attributes, and chemical components

The abundance of bacteria and yeast cells, sensory attributes, and chemical composition were compared using Spearman's rank correlation analysis (Supplementary Figs. A.3, A.4). This revealed positive correlations between acetic acid concentration and *Komagataeibacter rhaeticus*, *Komagataeibacter intermedius*, and *Bacillus* spp., and a negative correlation with *Bacillus coagulans*. As expected, vinegar taste had a strong positive correlation with acetic acid. Astringent taste correlated negatively with sucrose concentration and positively with ethanol. The off-taste intensity correlated positively with *Pseudomonas* spp., while *Lactobacillus* spp. showed positive relation to lactic acid concentration.

We must emphasize that we had no information about the fermentation process and its stages, any possible attempts to preserve the product, the time on the shelf, or the content of other additives (Supplementary Table A.1), which could be mixed into the drinks either before or after fermentation. All these factors are expected to strongly influence the sensory profile and microbial and chemical composition of the final product. This in turn could cause spurious correlations that are difficult to refute or justify. For all these reasons, Supplementary Figs. A.3 and A.4 should be interpreted with caution.

4. Discussion

Few publications connected with kombucha manufacturing have explored the marketing (Kim and Adhikari, 2020), the SCOBY composition (Harrison and Curtin, 2021), the diversity of the microbial community and its dynamics on an industrial scale (Coton et al., 2017). To the best of our knowledge, this study is the first that identifies the microbial composition of commercially available kombuchas by using metagenomic methods for consortia analysis and characterises their chemical and sensory profiles.

All studied kombuchas differed by their microbial composition, chemical parameters, and thereby sensory characteristics. The presence of AAB and LAB in kombucha and their production of organic acids determine the level of pH in the beverage. According to the Food and Drug Administration (FDA) Food Code model, the pH of commercial kombuchas must be in the range of $2.5 \leq pH < 4$ (Kim and Adhikari, 2020; Nummer, 2013). Low pH values are required to decrease the contamination risk. Also, the pH must be $\geq$2.5 to prevent damages caused by drinking acidic drink (Greenwalt et al., 2000; Kim and Adhikari, 2020; Nummer, 2013). The kombuchas in this study were in the required range (pH 2.8–3.7) and therefore according to this criterion were safe to consume.

All beverages contained acetic acid produced primarily by the AAB. This acid has been shown to inhibit the growth of 10 of the 14 pathogenic bacteria such as *Escherichia coli* and *Salmonella enteritis* (Sreeramulu et al., 2000). Our results also confirmed AAB dominance in market kombuchas with a high concentration of acetic acid and lack of pathogenic strains.

Another organic acid frequently found in kombuchas is lactic acid (Coton et al., 2017; Dufresne and Farnworth, 2000; Reiss, 1994) that is produced by LAB after fermentation from added sucrose (Dufresne and Farnworth, 2000; Reiss, 1994). It was detected in ten studied

commercial kombuchas at relatively high concentrations. The metagenomic analysis revealed the appearance of some LAB species, such as *Lactobacillus* and *Oenococcus*. LAB species were dominant in the kombuchas from manufacturer M and, correspondingly, the concentration of lactic acid was also the highest.

Others distinctive acids in kombucha are gluconic and glucuronic acid, whose central producers are AAB (Chen and Liu, 2000; Dufresne and Farnworth, 2000; Jayabalan et al., 2014; May et al., 2019). Our results showed that the amount of these acids was insignificant. Controversially, these concentrations vary in the literature from 2.3 g/L (Jayabalan et al., 2007) to 7.36 g/L (Chakravorty et al., 2016). Moreover, the metabolic pathways of gluconic and glucuronic acids showed that these acids could be metabolised to other chemical components, such as ascorbate, amino sugars, cofactors, and inositol ("KEGG COMPOUND C00191," n.d.).

The chemical analysis demonstrated significantly elevated concentrations of ethanol. AABs use ethanol to produce acetic acid (De Filippis et al., 2018; Jayabalan et al., 2014; Matsushita et al., 2016; Nguyen et al., 2015; Yavari et al., 2017). In some cases, high concentrations of ethanol can decrease the population of LAB due to toxicity (May et al., 2019). This could happen in the case of H and C kombucha products, as the increased ethanol concentration was accompanied by a low number of bacterial cells. Moreover, the level of acetic acid for manufacturer H was high, which was confirmed both by the chemical analysis results as well as the highest sourness in taste. However, not only yeasts have the capability to produce ethanol. It was shown that *Z. mobilis* possesses this feature (Cao et al., 2019; Doelle et al., 1993; Marsh et al., 2013; Weir, 2016), which could be the reason of the heightened level of ethanol in the products of manufacturer S. Additionally, the incorrect storage temperature of products could cause overproduction of ethanol. Only kombucha V1 from the investigated kombuchas did not contain ethanol. Also, it was very distinct from other beverages and did not have any residues of microbial DNA or alive microbes, which might indicate that it was somehow processed (Kim and Adhikari, 2020; Nummer, 2013). The accepted concept of kombucha is that it should be a "living organism". Therefore, the sample V1 should not be categorized as kombucha but might be named as a carbonated soft drink. Sensory results also confirmed that V1 was the most different sample.

In our work, the sensorial free sorting task and QDA showed that the commercial kombuchas can be grouped into three clusters. The "fruity and artificial flavour" cluster included products C1–C3 that distinguished by the higher sweetness, additive intensity, and lower sourness. It could be associated with the processed product, where the bacteria are added after fermentation to preserve the title of kombucha (Kim and Adhikari, 2020). It can also be confirmed by the detection of bacteria that are atypical in kombucha, and the information on the bottle label (*Bacillus coagulans* as an ingredient). Market kombuchas M2–M5, H2, and S2 had "herbal" and "tea" notes. All these products were made from green tea, contained *Lactobacillus* species, and some herbal additives. Coton et al., 2017 also showed that LAB dominated in green tea kombuchas. The commercial kombuchas K1, K2, S1, M1, H1, and V1 were clustered as "classical kombuchas" without distinctive non-characteristic attributes. Samples K1, K2, S1, M1 and H1 contained *Komagataeibacter rhaeticus* and yeast *Dekkera* species, and all of them except M1 were made of black tea. The same observation was made by Villarreal-Soto et al., 2020 and Coton et al., 2017, who demonstrated the same *K. rhaeticus* dominance in the black tea beverage.

According to the sensory analysis, the products of manufacturer H demonstrated the most "vinegar" notes in odour and taste. It can be caused by the presence of *K. rhaeticus* and other species of the *Komagataeibacter* genus, due to their ability to withstand high acetic acid and ethanol content (Gaggìa et al., 2019; Nguyen et al., 2015; Semjonovs et al., 2017). Interestingly, the bacterial cell numbers of H2 kombucha were higher than in the case of H1 and H3, while the yeast cell numbers were the medium ones. These fluctuations in bacterial and fungal cell numbers could be explained by dynamic and symbiotic processes in kombuchas (Jayabalan et al., 2014; Reva et al., 2015; Villarreal-Soto et al., 2020). Furthermore, Kombucha H2 had the highest concentration of acetic acid and the lowest concentration of ethanol from all H manufacturer products. Additionally, the relative population of the H2 yeasts was decreased probably due to the increase of *Komagataeibacteria* species amount. These findings may indicate that the kombuchas of the same producer H tended to be in different fermentation phases (Coton et al., 2017; Jayabalan et al., 2014; Villarreal-Soto et al., 2018). Moreover, all three kombuchas were produced with different substrates (H1 – white tea, H2 – green tea, H3 – hibiscus), which might also affect the consortia composition (Villarreal-Soto et al., 2018).

The absence of a commonly recognised kombucha definition and uncertain technological characteristics allow high diversity in market kombuchas. This permits the appearance of a beverage with a kombucha label but omits its possible beneficial qualities, as in the case of several drinks we studied. The FDA is elaborating to set the variance limits for kombucha safety plan (Nummer, 2013), and the Kombucha Code of Practice has stated the product standards and safety requirements ("Kombucha Code of Practice – Kombucha Brewers International," n.d.). The Kombucha Code of Practice has defined that kombucha is made from tea leaves and fermented with symbiotic consortia of bacteria and yeasts. Despite that, no official definition of kombucha has been declared yet by European Food Safety Authority or FDA.

5. Conclusion

In this paper, we analysed 16 kombuchas from 6 manufacturers. Their chemical and microbiological composition differed between production companies and inside the same manufacturer. The prevailing acid in the majority of kombuchas was acetic acid, however, lactic acid dominated in one beverage. Due to the fact the ethanol concentrations in analysed kombuchas were relatively high (2.66 to 14.77 g/L), some of the commercial kombuchas depending on the local legislation could be considered as alcoholic beverages. Based on the sensory analysis, the commercial kombuchas were sorted into three clusters "fruity and artificial flavour", "herbal and tea notes", and "classical". The analyses of the kombucha consortia showed that LAB is dominant in green tea and AAB in black tea kombuchas. Also, sensory and metagenomic analyses confirmed that the substrate and microbial composition of starter culture are the main influencers of the final product properties.

We were the first ones who compared several products from different brewers, analysed the chemical composition and microbiological consortia by metagenomic amplicon NGS, and established the connections with sensory profiles. Further research should investigate the substrate effect on temporal microbial composition in more detail. Definite and accepted industry standards have to be set to ensure product quality in the fast-growing market of kombuchas.

CRediT authorship contribution statement

Maret Andreson, Rain Kuldjärv, Mary-Liis Kütt: Conceptualization. Maret Andreson, Jekaterina Kazantseva: Data curation. Maret Andreson, Esther Malv, Aleksei Kaleda: Formal analysis. Rain Kuldjärv: Funding acquisition. Maret Andreson, Rain Kuldjärv, Esther Malv, Jekaterina Kazantseva, Helen Vaikma: Investigation, Methodology. Rain Kuldjärv, Maret Andreson: Project administration, Resources. Aleksei Kaleda: Software, Mary-Liis Kütt, Raivo Vilu: Supervision. Maret Andreson, Rain Kuldjärv, Jekaterina Kazantseva, Aleksei Kaleda: Validation. Maret Andreson, Esther Malv, Jekaterina Kazantseva, Helen Vaikma: writing-original draft. Maret Andreson, Jekaterina Kazantseva, Esther Malv, Helen Vaikma, Rain Kuldjärv, Mary-Liis Kütt, Aleksei Kaleda, Raivo Vilu: writing-review and editing.

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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.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ijfoodmicro.2022.109715.

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Appendix 3

Publication II

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Article

Evaluation of Microbial Dynamics of Kombucha Consortia upon Continuous Backslopping in Coffee and Orange Juice

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Abstract: The kombucha market is diverse, and competitors constantly test new components and flavours to satisfy customers' expectations. Replacing the original brewing base, adding flavours, or using "backslopping" influence the composition of the symbiotic starter culture of bacteria and yeast (SCOBY). Yet, deep characterisation of microbial and chemical changes in kombucha consortia in coffee and orange juice during backslopping has not been implemented. This study aimed to develop new kombucha beverages in less-conventional matrices and characterise their microbiota. We studied the chemical properties and microbial growth dynamics of lactic-acid-bacteria-tailored (LAB-tailored) kombucha culture by 16S rRNA next-generation sequencing in coffee and orange juice during a backslopping process that spanned five cycles, each lasting two to four days. The backslopping changed the culture composition and accelerated the fermentation. This study gives an overview of the pros and cons of backslopping technology for the production of kombucha-based beverages. Based on research conducted using two different media, this work provides valuable information regarding the aspects to consider when using the backslopping method to produce novel kombucha drinks, as well as identifying the main drawbacks that need to be addressed.

Keywords: kombucha; backslopping; lactic acid bacteria; metagenomic analysis; fermented beverage; coffee; orange juice

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1. Introduction

Kombucha is the common name for an ancient tea-based beverage fermented with a symbiotic community of acetic acid bacteria (AAB) and yeast. The symbiotic culture of bacteria and yeast in kombucha is named SCOBY. A variety of kombucha species have been identified so far. The most commonly detected bacterial species belong to the genera *Komagataeibacter* and *Gluconobacter* [1,2]. However, genera of lactic acid bacteria (LAB) *Lactobacillus* and *Oenococcus* have also been found in kombucha [2,3]. The identified yeasts generally belong to the genera *Saccharomyces*, *Dekkera/Brettanomyces*, *Zygosaccharomyces*, *Pichia*, *Torulaspora*, *Candida* and *Schizosaccharomyces* [2,4]. The kombucha fermentation process occurs spontaneously and changes rapidly due to the cooperative and competitive processes required for nutrient symbiosis of bacteria and yeast [5]. Each cultivation leads to the formation of a new SCOBY layer that can be used to start new fermentation [6]. The addition of a small portion of the previous batch of fermented food or drink to refresh or start a new fermentation stage is named "backslopping" (BS) [7]. This method is well established by homebrewers to renew the kombucha culture and has been used for centuries.

Traditional kombucha is made of black or green tea, and usually has 5–10% of added sugar. The kombucha culture has been used to ferment different juices, such as

pomegranate, red grape, sour cherry, and apple juice [8]. Sun et al. [9] used kombucha culture to ferment wheatgrass juice to accelerate the increase of antioxidant liberation, such as those in phenolic compounds and several flavonoids. Nizioł-łukaszewska et al. [10] investigated green coffee bean fermentation with a kombucha culture. They detected that the fermented beverage had biologically active compounds which were potential candidates for antioxidants. Additionally, Tu et al. [11]) used kombucha culture to ferment a by-product originating from the production of tofu and soy protein isolates. The authors showed an enhancement of antioxidant activity and greater antimicrobial activity of fermented soy whey. Another part of kombucha studies has focused on the dynamical changes of culture during one growth cycle in different fermentation times [6,12]. Savary et al. [13] created a kombucha culture from the AAB and yeast species previously selected by Coton et al. [4] and monitored the microbial, chemical and physical changes during one batch cycle [4,13].

The origin and composition of kombucha culture and growth matrix and the number of backslopping cycles influence the chemical and microbial properties of the final product [5,14]. Due to the growing popularity of commercial kombuchas, producers are finding ways to introduce new flavours and nuances for this traditional drink. One of the ways is to modify the SCOBY by introducing new bacterial cultures that affect the sensory properties of the drink. Another option is to replace the classical growth environment, usually black or green tea, with a new one. However, it is important to maintain the constant quality of every batch of the final product and obtain stable and reproducible results, which could be possible with the application of backslopping technology. An examination of the effects of SCOBY modification and a profound description of chemical and microbial changes in kombucha community in different novel growth media during backslopping has not been implemented yet. Therefore, we wanted to characterise modified kombucha fermentation in new environments using backslopping methodology with continuous chemical and microbiological monitoring throughout. Based on the research in two different media, orange juice and coffee infusion, in this work we provide valuable information regarding the aspects to consider for this type of kombucha production and what are the main drawbacks to be solved.

2. Materials and methods

2.1. Creation of Kombucha Culture

The initial kombucha consortium, originating from a commercial Estonian kombucha brewery, was re-inoculated into a 10% sucrose black-tea mixture. The fermentation continued for seven days at 30 °C, after which the lactic acid bacteria (LAB) species were added to the initial kombucha culture. The LAB strains were *Levilactobacillus brevis*, *Lactiplantibacillus plantarum*, *Pediococcus pentosaceus* and *Companilactobacillus paralimentarius*, and they were obtained from the TFTAK's (Center of Food and Fermentation Technologies, Tallinn, Estonia) culture collection. The strains were originally isolated from sourdough [15]. The LAB species were pre-cultured in a de Man, Rogosa, Sharpe (MRS) broth (Lab M, Neogen Company, London, UK) at 30 °C for 12 h. Based on the optical density (OD), some strain biomass was concentrated, so that all strains had the same initial OD value before introduction into the kombucha. All strains were added in equal amounts at the concentration rate of 1% (1×10^7 cells/mL). The kombucha fermentation supplemented with LAB species continued for two weeks.

2.2. Kombucha Cultivation in Coffee and Orange Juice

The experimental plan of the study is shown in Figure 1. The coffee infusion was made using ground coffee beans (100% Arabic) purchased from a local market and boiled in tap water. The coffee concentration in the infusion was 51 g/L, supplemented with 10% of sucrose. The coffee infusion was brewed for 3 min and then filtered through a paper coffee filter into a beaker and cooled down to 25 °C. The pasteurised orange juice was purchased from a local market.

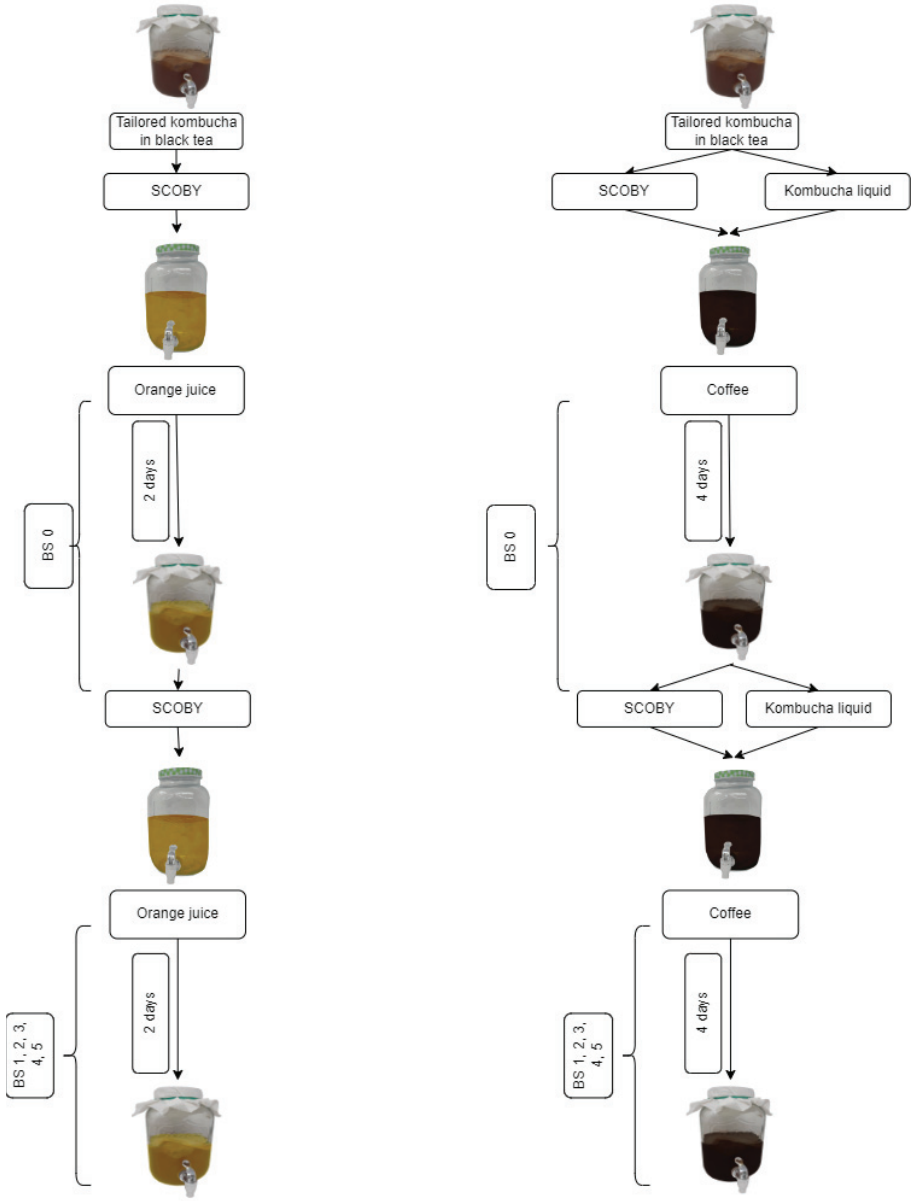

Figure 1. The schematic figure of the backslopping (BS) experiment workflow. The samples for pH, TA, organic acid, and sugar measurements were collected and analysed, and sensory analysis was conducted every day throughout the experiment. Samples for metagenomic analyses were collected and analysed at the end of each backslopping cycle.

The LAB-tailored kombucha culture was inoculated in orange juice and coffee infusion. Kombucha SCOBY was divided into eight equal sectors, and one sector was added to the fresh matrix (either in orange juice or in coffee). As the initial pH of the orange juice was 4, a piece of SCOBY was added to the orange juice without pH adjustment. In the case of the coffee environment, at first, the pH was adjusted to 4.5 with the initial batch of kombucha liquid, and thereupon, a piece of SCOBY was added. The backslopping

experiment continued for five backslopping cycles. The duration of a single cycle varied between the environments: two days for the orange-juice kombucha and four days for the coffee kombucha.

2.3. Determination of pH and Titratable Acidity

Five millilitres of kombucha sample were suspended in 50 mL of distilled water to measure pH and titratable acidity (TA). For the pH and TA analyses, the Food and Beverage Analyzer (Mettler-Toledo International Inc., Columbus, OH, USA) was used. The acidity was determined through titration to pH 7 using 0.1 N of NaOH and expressed as 0.1 N of NaOH per 1 mL of kombucha. All measurements were performed in sets of two replicate measurements.

2.4. Determination of Metabolic Products in Kombucha

13 mm Philic PTFE 0.2 µm Non-sterile Millex-LG filters (Millipore, Darmstadt, Germany) were used to filter the kombucha samples. A Waters 2695 HPLC system (Waters Corporation, Milford, MA, USA) was used to analyse the metabolic products (organic acids, sugars, and ethanol) of the kombuchas. An HPX-87H column (BIO-RAD Hercules, Hercules, CA, USA) was used, and the system was eluted isocratically with 0.005 M of H_2SO_4 at 0.6 mL/min at 35 °C. A Waters 2487 dual absorbance detector (Waters Corporation, Milford, MA, USA) and a Waters 2414 refractive index detector (Waters Corporation, Milford, MA, USA) were used for the detection and quantification of analytes. Empower software 3 (Build 3471 FR5 SR4, Waters Corporation, Milford, MA, USA) was used to perform the data analysis.

2.5. Microbial Quantitative Metagenomic analysis

2.5.1. Sample Preparation

For the microbial analysis of the original, laboratory-modified by lactic acid bacteria (LAB-tailored), and backslopping kombucha, 40 mL of liquid and 0.75 mL of SCOBY samples were collected aseptically. During the backslopping, the samples were taken according to the experimental plan (Figure 1)—at the starting point from tailored kombucha (BS 0.0) and at each end of the backslopping cycle from orange juice (BS 0.2, 1.2, 2.2, 3.2, 4.2, 5.2) and coffee kombucha (BS 0.4, 1.4, 2.4, 3.4, 4.4, 5.4). In the case of the orange-juice kombucha, only the sample of SCOBY was taken. The liquid sample was prepared as described in Andreson et al. [2], with additional centrifugation at the beginning at 700× *g* for 5 min at 4 °C. The supernatant was poured into a new tube and centrifuged at 3950× *g* for 15 min at 4 °C. The SCOBY sample was cut into small pieces with a sterile scalpel and then washed with 750 µL of sterile and cold 1 × PBS (Phosphate-buffered saline, BIO-RAD, Hercules, CA, USA). The sample was centrifuged at 10,000× *g* for 10 min at 4 °C, and the pellet was subjected to the gDNA extraction. The gDNA isolation of each type of kombucha sample was performed and assessed as in Andreson et al. [2].

2.5.2. Next-Generation Amplicon Sequencing

To determine the bacterial and fungal composition of studied kombuchas, the v4 region of the 16S ribosomal RNA (rRNA) gene [16] and the ITS2 region between the 5.8S and LSU rRNA genes [17] were used for sequencing library preparation. For this, 25 ng of extracted microbial gDNA was taken for the amplification stage, and dual indices were used for final libraries and carried out as described in Andreson et al. [2] and Kazantseva et al. [18]. The next-generation sequencing (NGS) was performed using the iSeq100 platform (Illumina, San Diego, CA, USA) with iSeq 100 i1 Reagent v2 and applied as 2 × 150 cycles paired-end protocol for 16S and 300 cycles single-end protocol for ITS2 region. The corresponding bioinformatic data analyses were performed using an open-source BION-meta package [19,20] for bacterial identification and by the Quantitative Insights Into Microbial Ecology [QIIME2, version 2019.10, [21]] for fungal identification, as described in Andreson et al. [2].

2.5.3. Data Quantification

For absolute quantification of the bacterial and yeast species detected by the 16S and ITS rRNA amplicon sequencing in the studied kombuchas, a quantitative real-time PCR (qPCR) on a qTOWER3G thermal cycler (Analytik Jena, Jena, Germany) using calibration curve methodology was performed, as mentioned before Andreson et al. [2]. All experiments were performed in triplicate. Standard primers of amplifying the v4 region of the 16S rRNA gene [16] for bacteria, and the ITS2 region of the ribosomal gene [22] for fungi were used. For cell number calculations, the average genome size of the detected microbial species was taken as 4 Mbp for bacteria and 14 Mbp for yeasts.

To estimate the cell number from qPCR data, the following equation was used:

$$Cell\ number = \frac{m \times N_A}{GS \times M},$$

where m is the mass of DNA (g) measured by qPCR, N_A is the Avogadro constant 6.02×10^{23} L/mol, GS is the mean genome size in bp, and M is the molar mass of 1 bp of dsDNA equal to 660 g/mol.

2.6. Data Visualisation

Titratable acid, pH, organic acids, sugars, ethanol, and ITS and 16S amplicon data were visualised using packages "ggplot2" 3.3.6 and "scales" 1.2.0 in R version 4.0.2 22 June 2020 (R Foundation for Statistical Computing, Vienna, Austria).

3. Results

3.1. LAB-Tailored Kombucha Culture

To modify the original kombucha culture after 7 days of its propagation in the black-tea environment, four lactic-acid bacteria (LAB) species were added to the initial culture. After two weeks of fermentation, the LAB-tailored kombucha was sensorially different from the original drink. While the initial kombucha had a strong acidic taste, the LAB-tailored kombucha was less sour (Tables S1 and S2). The sourness was softer and similar to the taste of lactic acid. However, the pH and titratable acidity did not differ significantly between the initial and LAB-tailored fermented drinks.

The bacterial and yeast composition of liquid and SCOBY from the initial and tailored kombucha were determined by 16S or ITS2 metabarcoding, accordingly (Figure 2). In the initial kombucha, the predominant bacterial species was *Komagataeibacter rhaeticus*, whilst *K. europaeus* and *K. intermedius* were identified in smaller proportions. In the LAB-tailored kombucha, *K. rhaeticus* prevailed. While LAB species were detected at the beginning, they were not found later in tailored kombucha by 16S NGS. Additionally, the yeast composition of tailored kombucha differed from the initial drink. The dominant yeast species in the initial kombucha was *Dekkera bruxellensis*, but the tailored kombucha had more than one dominant yeast species. Prevalent yeast species belonged to the genus *Zygosaccharomyces*.

As we could not identify added LAB by 16S metabarcoding in tailored kombucha, we performed qPCR with strain-specific primers at five sampling points during a four-month period (Table S3). Despite the fact that the measured LAB amount was relatively high at the first sampling point, with values between 10^4 to 10^5 cells per millilitre of kombucha, the abundance of LAB species decreased 1–3 log in the second sampling point and stayed below the detection threshold until the end of the fourth month. Still, modified LAB-tailored and propagated kombucha culture was used further in backslopping experiments.

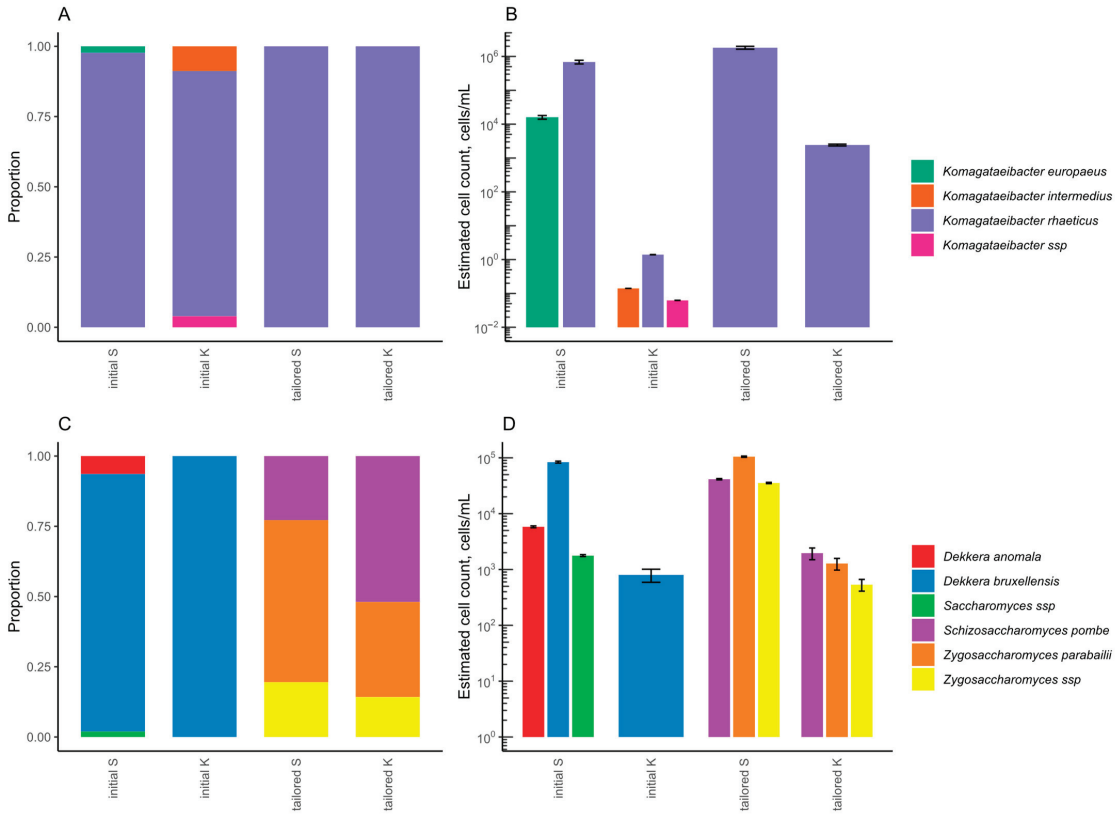

Figure 2. Microbial composition of initial and tailored kombucha at the end of the backslopping process according to 16S NGS for bacteria (**A**,**B**) and ITS2 NGS for yeast (**C**,**D**), graphed as relative (**A**,**C**) and normalised abundances (**B**,**D**). The abbreviation S stands for SCOBY, and K for kombucha liquid.

3.2. Orange-Juice Kombucha Backslopping

3.2.1. Chemical Composition and Sensory Evaluation

In the preliminary optimisation study, the fermentation of tailored kombucha in orange juice was monitored over a period of five days. It included measurements of pH, titratable acidity, and sensory characterisation (Table S4). The kombucha fermented in orange juice was sensorially unacceptable after more than two days of fermentation. The fermented beverage was becoming rotten and developing a strong acetic taste. Thus, a single backslopping cycle duration for orange juice was chosen, specifically, two days. The whole experiment continued for five backslopping cycles.

The orange-juice kombucha was evaluated for acidity and metabolites during the whole backslopping process. The pH of natural orange juice was 3.99 $\pm$ 0.02 (Figure 3). The highest TA values were detected at the fourth and fifth backslopping cycles, with values of 8.05 $\pm$ 0.04 g/L and 7.94 $\pm$ 0.06 g/L, respectively. A trend showed that after the end of each backslopping cycle, the TA increased even more than in the previous cycle.

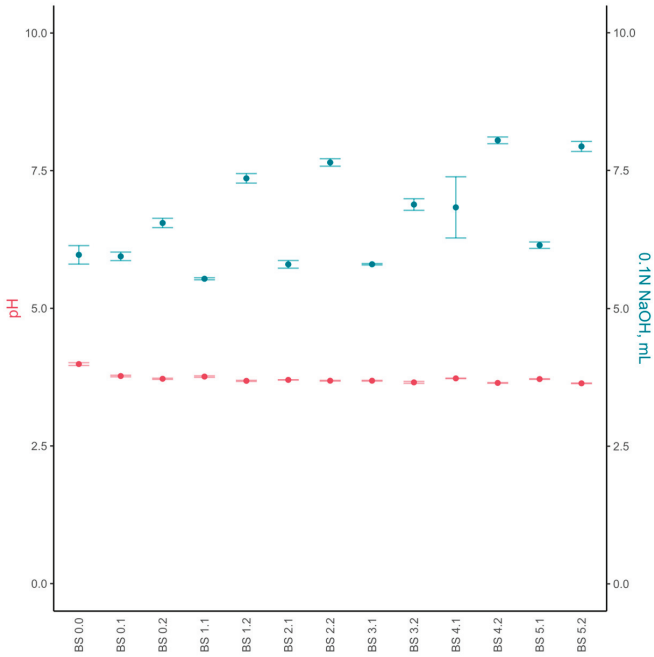

Figure 3. The pH (red dots) and titratable acidity (blue dots) values of orange-juice kombucha during backslopping cycles. Titratable acidity units are mL of 0.1 N NaOH. The abbreviation BS stands for backslopping. The first number shows the backslopping cycle and the second number refers to the day of the backslopping cycle.

Metabolic analysis showed that despite citric acid being a dominating acid throughout the experiment, its concentration showed minor changes in range of 8.3 to 9.1 g/L (Figure 4A). Acetic acid showed the greatest increase, increasing from 0.04 ± 0.00 to 3.08 ± 0.02 g/L throughout the backslopping cycles (Figure 4A). Ethanol concentration increased throughout the backslopping cycles (Figure 4C). The highest concentration occurred at the end of fermentation, with a value of 23.10 ± 0.04 g/L. The highest sucrose content was detected at the beginning of backslopping, with a value of 40.97 ± 0.09 g/L (Figure 4D). The fructose and glucose concentrations were one-half of the sucrose concentration in the beginning of the fermentation. However, a switch in the consumption of sugars happened between backslopping points 1.2 and 3.1. After that shift, the sucrose concentration was lower than the fructose and glucose concentrations. At the end of the fifth cycle, the sucrose was almost depleted, and some fructose and glucose were left. Organic acid, sugars, and ethanol profiles demonstrated that the kombucha fermentation accelerated during the backslopping, which indicates fastened fermentation after each backslopping cycle (Figure 4E).

By the end of the first cycle, the sensory evaluation showed that the kombucha culture changed orange juice's taste towards the sour and carbonated it. The fermented orange-juice kombucha had a chemical taste and bitter notes by the end of the second and third cycles, respectively. By the fourth and fifth backslopping cycles, the fermented orange juice was very sour and bitter and had profound chemical notes (Table S5).

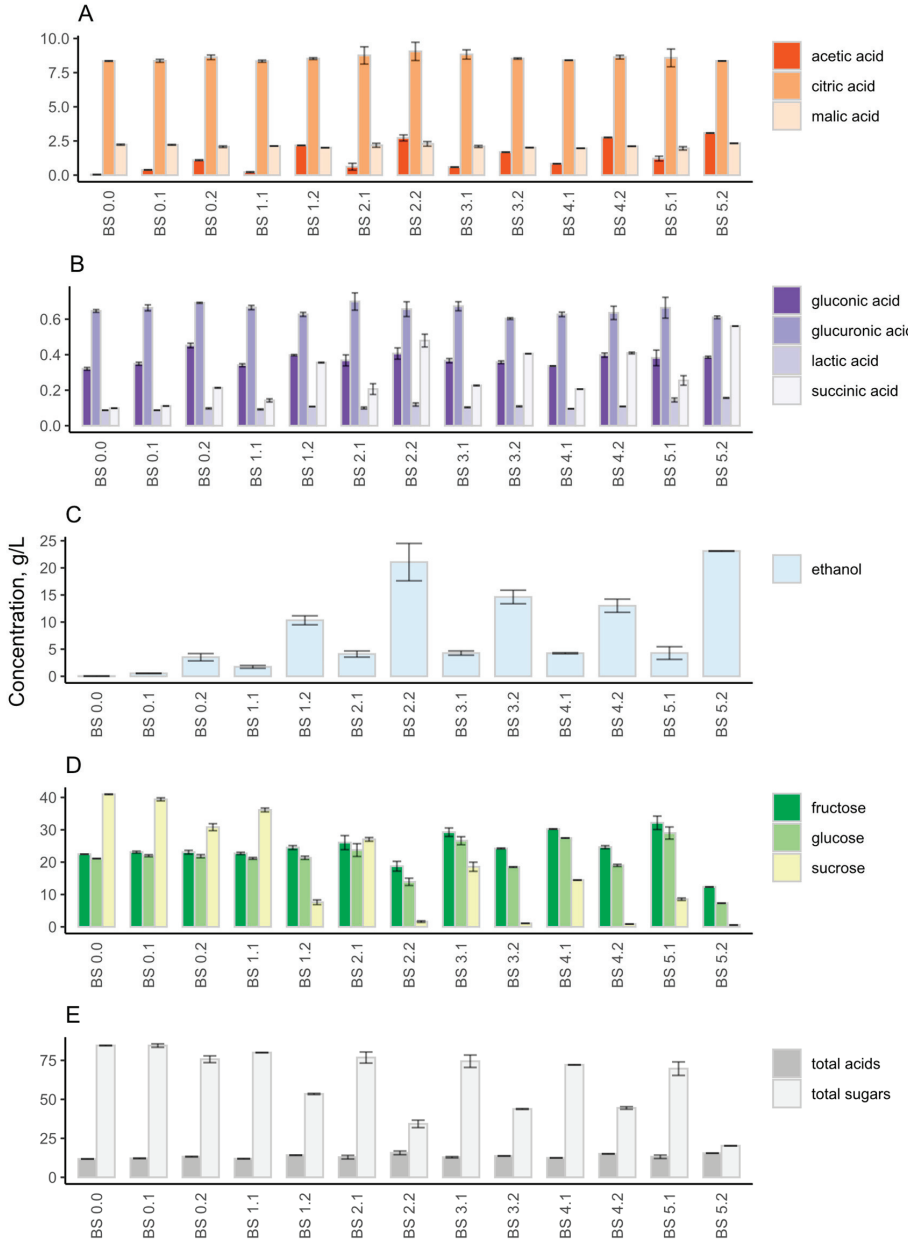

Figure 4. Organic acids, sugars, and ethanol concentrations (g/L) of orange-juice kombucha during backslopping. Concentrations of acetic, citric, and malic acid (**A**); gluconic, glucuronic, lactic, and succinic acid (**B**); ethanol (**C**); fructose, glucose, and sucrose (**D**); and total acids and sugars (**E**) are indicated. The abbreviation BS stands for backslopping. The first number shows the backslopping cycle and the second number states the day of the backslopping cycle.

3.2.2. Microbial Composition

The microbial composition of every backslopping cycle was detected solely from SCOBY, as it was the transferring component applied to the next kombucha batch during the

backslopping process. The changes in bacterial proportion revealed by 16S metabarcoding and the enumerated data normalised by bacteria-specific qPCR during the backslopping cycle are shown in Figure 5A,B, respectively. The BS 0 cycle signifies the adaptation period of the microorganisms, with the switch between black tea (BS 0.0) and orange juice (BS 0.2) matrices.

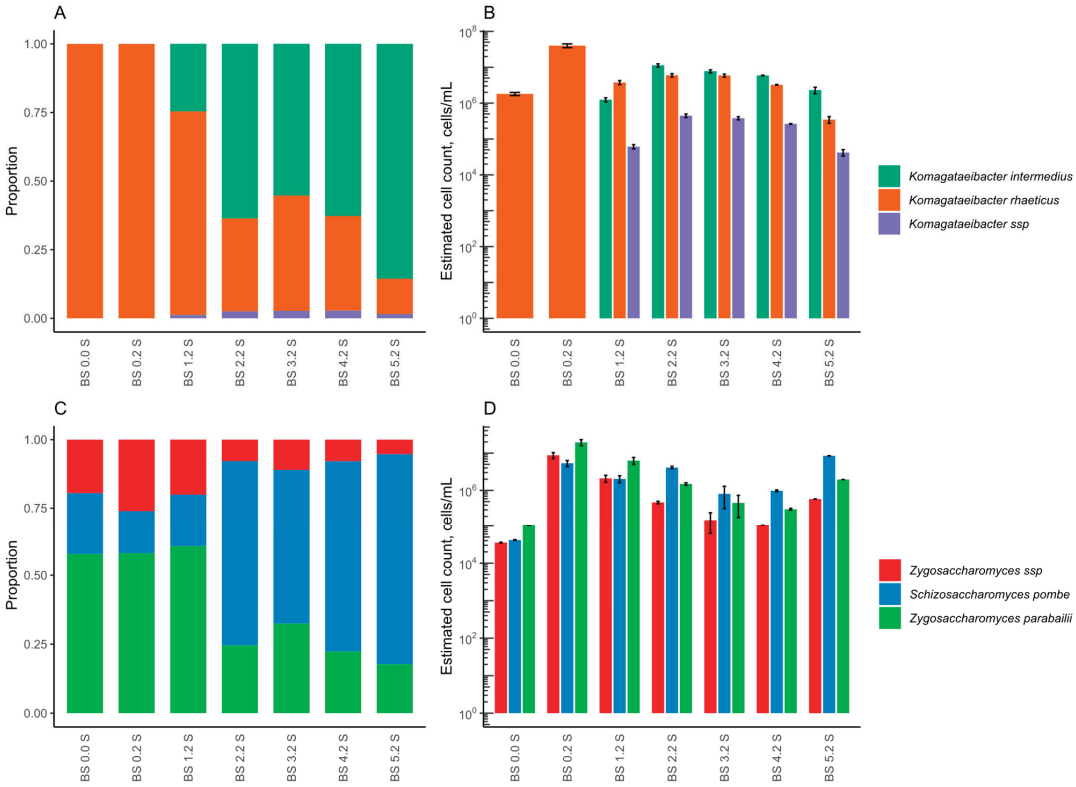

Figure 5. Microbial composition during orange juice backslopping cycles according to 16S NGS for bacteria (**A**,**B**) and ITS2 NGS for yeast (**C**,**D**), graphed as relative (**A**,**C**) and normalised abundances (**B**,**D**). The abbreviation BS stands for backslopping. The first number shows the backslopping cycle and the second number states the day of the backslopping cycle.

The results showed that two main bacterial species—*Komagataeibacter rhaeticus* and *Komagataeibacter intermedius*—were detected throughout the experiment. The *K. rhaeticus* was the only bacteria detected at the point of BS 0.0 where the lowest numbers of bacterial cells were $1.81 \pm 0.18 \times 10^6$ cells/mL. *K. rhaeticus* prevailed until the point of BS 0.2, when the drink contained the highest number of cells ($4.00 \pm 0.49 \times 10^7$ cells/mL). From the second backslopping cycle (BS 2.2) until the end (BS 5.2), the *K. intermedius* started to dominate over others. The *K. intermedius* cell count was increased to $1.12 \pm 0.12 \times 10^7$ cells/mL in BS 2.2. At the end of the fifth cycle (BS 5.2), the *K. intermedius* proportion was raised to 0.86, while *K. rhaeticus* was diminished to 0.13, with a lower total cell number of $2.69 \pm 0.55 \times 10^6$ cells/mL. To some extent, 16S NGS revealed species only at the genus level, so from BS 1.2 to BS 5.2 the proportions of 0.01–0.03 species were identified as members of the genus *Komagataeibacter*. At the end of the experiment on the fermented orange juice, *K. intermedius* was found to be the dominant species. Even though the content of *K. intermedius* increased significantly during the last cycle, the overall number of bacteria showed a decreasing tendency after the point of BS 2.2 and stayed especially

low ($2.69 \pm 0.55 \times 10^6$ cells/mL) at the end of the cycles compared to the beginning of the backslopping (downtrend 2.4 log).

The yeasts community was detected by ITS2 metabarcoding along with the cell numbers determined by ITS-specific qPCR in analysed time points, as presented in Figure 5C,D, respectively. The results showed that two genera were detected throughout the experiment. In the beginning, at the point of BS 0.0, the dominant species was *Zygosaccharomyces parabailii*, with a proportion of 0.58, followed by *Schizosaccharomyces pombe* and *Zygosaccharomyces* spp., with proportions of 0.23 and 0.20, respectively. Similarly to the bacterial cell load, the lowest yeast cell count was detected at the time point of BS 0.0, $1.81 \pm 0.05 \times 10^5$ cells/mL. In the same way, the highest total yeast cell count was measured at the point of BS 0.2, $3.35 \pm 0.62 \times 10^7$ cells/mL, while species abundances did not change remarkably, with dominating *Z. parabailii* followed by the *Zygosaccharomyces* spp. and *S. pombe*. From BS 2.2 until the end (BS 5.2), the *S. pombe* started to dominate with a proportion of 0.68 and had a cell count of $4.10 \pm 0.31 \times 10^6$ cells/mL. A minor decrease of dominance occurred at the point of BS 3.2, but in BS 4.2 and BS 5.2, the *S. pombe* content was increased to 0.70 and 0.77, respectively. Therefore, the *S. pombe* dominated at the end of the backslopping cycles. Although, at the end of backslopping, the yeast cell load increased to 1.10×10^7 cells/mL, it is still lower than in the beginning.

3.3. Coffee Kombucha Backslopping

3.3.1. Chemical Composition and Sensory Evaluation

The preliminary optimisation study was also conducted in a coffee environment (Table S6). The fermentation of tailored kombucha was monitored over a period of five days, with measurements taken for pH, titratable acidity, and sensory characteristics. As the sensory properties of fermented drinks became unpleasant after the fourth day, the duration of one backslopping cycle for coffee was limited to four days. The whole experiment continued for five backslopping cycles.

The chemical composition and acidity of kombucha during its backslopping in the coffee infusion were evaluated by analogy to orange juice. The pH of coffee infusion was 5.69 ± 0.04, which was adjusted to a pH of around 4.7–4.9 (Figure 6). The lowest pH obtained during the backslopping process for coffee kombucha was 4.02 ± 0.01. As in the case of orange-juice kombucha, the TA values demonstrated similar changes in acidity during the backslopping cycles. Similar to the orange juice, the later backslopping cycles in coffee ended faster.

Metabolic analysis showed that acetic acid increased from 0.32 ± 0.01 to 4.86 ± 0.02 g/L throughout the backslopping cycles (Figure 7A). The acetic acid concentration rise was higher than corresponding rise in the orange juice. The ethanol concentration increased to 8.58 ± 0.28 g/L at the end of the first cycle but decreased to 5.91 ± 0.48 g/L after the second cycle (Figure 7C). The second and third cycles of backslopping had similar ethanol concentrations. At the end of the fifth backslopping cycle, the concentration of ethanol was increased to 10.23 ± 0.32 g/L. This concentration was two times lower than that in the orange-juice kombucha. The highest sucrose concentration occurred at the beginning of backslopping, with a value of 107.22 ± 0.01 g/L (Figure 7D), while fructose and glucose were not detected. The sucrose concentration decreased during the backslopping cycles, and the final concentration was 49.06 ± 0.04 g/L. The fructose and glucose amounts increased throughout the backslopping cycles. Coffee kombucha had minor changes in the sugar concentration compared to the orange-juice kombucha, in which the balance of sugars changed at the end of the backslopping cycles. The coffee kombucha organic acid, sugars and ethanol profiles showed the same trends as those of the orange-juice kombucha regarding the acceleration of the fermentation rate during the backslopping (Figure 7E).

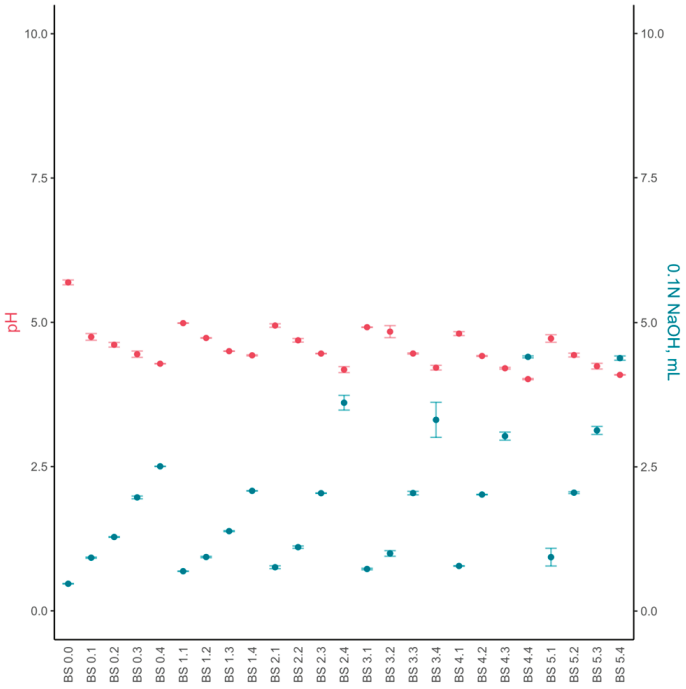

Figure 6. The pH (red dots) and titratable acidity (blue dots) values of coffee kombucha during backslopping cycles. Titratable acidity units are mL of 0.1 N NaOH. The abbreviation BS stands for backslopping. The first number shows the backslopping cycle and the second number states the day of the backslopping cycle.

The sensory analysis showed that by the fourth day of fermentation, the kombucha culture imparted a sweet and sour taste to the coffee. Additionally, by the end of the first cycle, the coffee kombucha had developed a bitter taste, and by the end of the third cycle, the beverage had acquired carbonisation and a strong bitter taste. In the fourth and fifth backslopping cycles, the coffee kombucha exhibited intense sourness, bitterness, and carbonation (Table S7).

3.3.2. Microbial Composition

In the coffee kombucha, the microbes were detected from kombucha liquid and SCOBY because both parts were transferred during the backslopping. The changes in bacterial composition during the backslopping cycles in the coffee kombucha detected by metagenomic 16S amplicon NGS and enumerated by bacteria-specific qPCR data are shown in Figure 8A,B, respectively. As in the orange-juice kombucha, the sequencing results revealed that the coffee kombucha contained the same two major bacterial species during the backslopping. *Komagataeibacter rhaeticus* occurred only from point BS 0.0 to BS 1.4 in both parts of the kombucha, and it dominated in the kombucha liquid at all backslopping points (BS 0.0 to BS 5.4). However, the bacterial cell load in the kombucha liquid was relatively low throughout the experiment. On the other hand, the bacterial cell count was quite stable during the whole experiment, reaching, at the endpoint, a value of $1.32 \pm 0.07 \times 10^5$ cells/mL, which is comparable to that of the backslopping point BS 0.4.

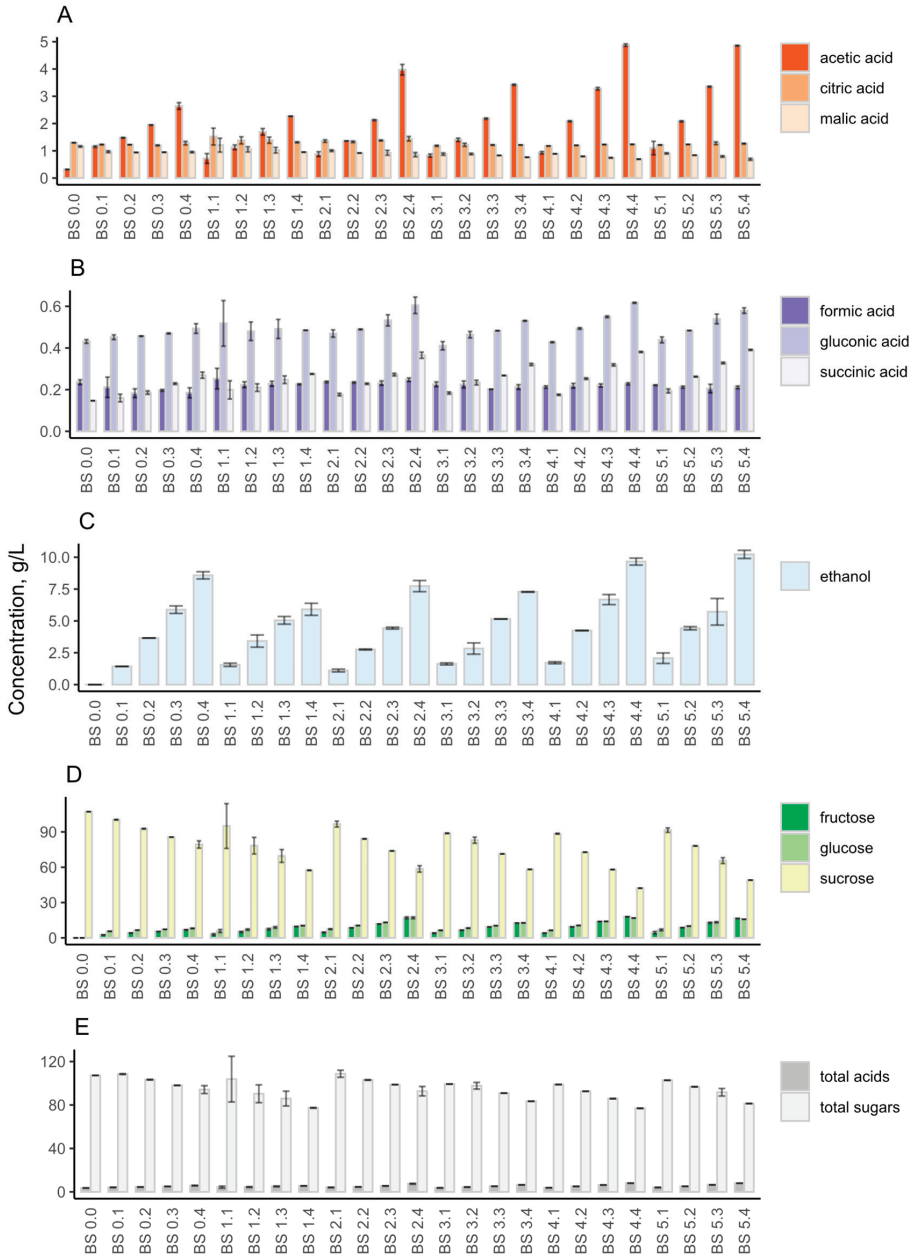

Figure 7. Organic acids, sugars, and ethanol concentrations (g/L) of coffee kombucha during backslopping. Concentration of acetic, citric, and malic acid (**A**); formic, gluconic, and succinic acid (**B**); ethanol (**C**); fructose, glucose, and sucrose (**D**) and total acids and sugars (**E**) are indicated. The abbreviation BS stands for backslopping. The first number shows the backslopping cycle and the second number states the day of the backslopping cycle.

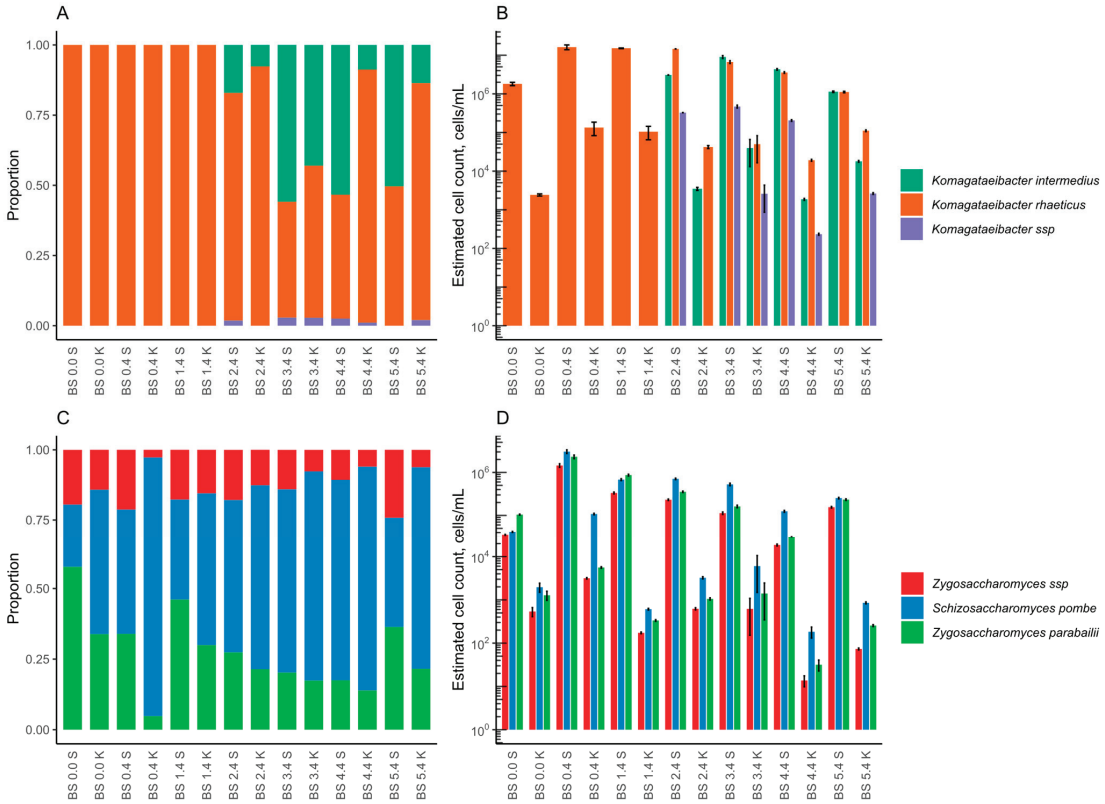

Figure 8. Microbial composition during coffee backslopping cycles according to 16S NGS for bacteria (**A**,**B**) and ITS2 NGS for yeast (**C**,**D**), graphed as relative (**A**,**C**) and normalised abundances (**B**,**D**). The abbreviation BS stands for backslopping. The first number shows the backslopping cycle and the second number states the day of the backslopping cycle.

Compared to the SCOBY, the *K. rhaeticus* was not the dominating species at the points of BS 3.4 and BS 4.4. Additionally, the detected bacterial load in SCOBY was twofold higher than in the kombucha liquid. The highest number of bacterial cells in SCOBY was detected for the *K. rhaeticus* at the point of BS 0.4 ($1.62 \pm 0.23 \times 10^7$ cells/mL) and for the *K. intermedius* at the point of BS 3.4 ($9.03 \pm 0.77 \times 10^6$ cells/mL). From the last-mentioned point (BS 3.4), the proportions of both species were approaching equality. By the end of the experiment (BS 5.4), the content levels of the *K. rhaeticus* and *K. intermedius* in the SCOBY were in a ratio of 50:50. Simultaneously, the total count of bacterial cells in SCOBY at the backslopping endpoint (BS 5.4) was $2.25 \pm 0.10 \times 10^6$ cells/mL, which was approximately sevenfold lower than in the beginning. This result is quite equal to the value measured in the tea medium (BS 0.0). In general, the coffee backslopping showed stabilizing effects on the bacterial composition and content.

The yeast community was detected by metagenomic ITS2 amplicon NGS and is presented with the fungal cell number determined by ITS-specific qPCR in Figure 8C,D, respectively. The results showed that the coffee kombucha contained the same two yeast genera as the orange-juice kombucha. The SCOBY and kombucha liquid had different dominant yeast species in the beginning of the experiment (BS 0.0—black tea). The *Schizosaccharomyces pombe* prevailed in the kombucha liquid, and *Zygosaccharomyces parabailii* in SCOBY. However, the *S. pombe* was more prevalent than was the *Z. parabailii* in the SCOBY at the starting point of the backslopping (BS 0.4—coffee). Throughout the experiment, the *S. pombe* was

dominant in the kombucha liquid. Still the total yeast cell count in the kombucha liquid was two- to threefold lower than in the SCOBY, with the lowest value in the liquid at the point of BS 4.4 ($2.29 \pm 0.65 \times 10^2$ cells/mL). At the same time, the SCOBY was a little bit more dynamic. However, the *S. pombe* was beginning to dominate in the SCOBY, but was still less than at the same point in the kombucha liquid. Generally, the yeast population in coffee kombucha liquid and SCOBY decreased during the backslopping experiment, reaching to the values of $6.42 \pm 0.26 \times 10^5$ cells/mL and $1.18 \pm 0.06 \times 10^3$ cells/mL, respectively.

4. Discussion

Previous research on kombucha has primarily focused on studying its health benefits. Some studies in which kombucha culture was introduced in different environments, such as pomegranate juice [23], grape juice [24], and others [8], accented the description of its antioxidant properties. SCOBY composition is variable depending on its origin and fermentation conditions. Its consortia are mainly enriched by acetic acid bacteria (AAB) and yeasts. This study aimed to develop and characterise new beverages based on modified LAB SCOBY fermentation in non-traditional growth environments by backslopping technology. The focus was on describing the chemical and microbial changes during backslopping cycles that were supported by sensory evaluation.

The first stage of the new kombucha product development was to modify the initial kombucha culture with several LAB species. *Levilactobacillus brevis*, *Lactiplantibacillus plantarum*, *Pediococcus pentosaceus* and *Companilactobacillus paralimentarius* are strains that were isolated from plants [15], and therefore are more capable of adapting to the kombucha environment than are the classical milk-origin LABs. After introducing the LABs to kombucha fermentation, we expected changes in the SCOBY composition, with new probiotic cultures added to it, and the taste modified.

Despite the evident effects on sensory properties, the added LAB did not show measurable viability in kombucha. We could not detect them after serial backslopping cycles even by strain-specific primers by qPCR. Probably, these strains could not compete for substrates due to the microbial richness in the kombucha. However, the composition of original microbial consortia changed after LAB tailoring, shifting from three species (*K. intermedius*, *K. rhaeticus*, and *K. europeaus*) in the initial composition, to the one for the LAB-tailored kombucha. Despite the fact that we detected lactic acid throughout the entire fermentation process, it was not dominating the organic acid. In their study, Nguyen et al. [25,26] supplemented kombucha with LAB strains and demonstrated a significant increase in glucuronic acid and antioxidant activity However, they did not measure the abundance of the lactic acid bacteria after kombucha fermentation. Our data also proved that, although the tailored kombucha had a low abundance of LAB strains, it contained a higher amount of gluconic acid than did the initial kombucha (Figure S1). Thus, we can assume that the added LAB influenced kombucha properties positively.

In this study, we observed dynamic changes in microbial composition during the backslopping cycles. In both environments, initially detected bacterial species disappeared through several backslopping cycles, but they were again detected at the end of the experiment. Thus, it showed fluctuation in species' behaviour and their dependence on the environment. Moreover, the dominant species were different for various matrices. In the initial tea kombucha, the prevailing bacterium was *K. rhaeticus*, but in the orange juice, it was *K. intermedius*; whereas, in the coffee kombucha, the *K. rhaeticus* retained its dominance only by the end of the backslopping. From the perspective of yeasts, the LAB addition totally changed the yeast composition of kombucha. In the initial kombucha, the primary yeast was *Dekkera bruxellensis*, but the *Zygosccharomyces* species dominated in the LAB-tailored kombucha. The yeast composition in the orange juice and coffee did not change back to the initial kombucha composition, as had happened with bacteria. These changes demonstrate that the microorganisms which are detectable in the initial kombucha do not disappear, but are still present in very small amounts and are metabolically active, waiting for the environment to become favourable to increase their populations. Thus,

backslopping plays an important role in the balance of the microbial consortia, supporting or reducing the growth of definite microorganisms, depending on the environment.

Although, in both environments, backslopping did not significantly affect the chemical properties of kombucha, the results showed relatively high concentrations of ethanol during the analysed process. The ethanol concentrations reached 23.10 g/L and 10.23 g/L in orange juice and coffee environments, respectively. Chen and Liu [27] also showed that the ethanol concentration in kombucha increased with time and reached 5.5 g/L by the end of fermentation. Another study by Chakravorty et al. [12] determined the highest concentration of ethanol to be on day 7, with a value of 0.28 g/L. Besides, Tan et al. [28] showed that ethanol concentration reached 70.7 g/L in juice fermented with yeast. It is known that different species have different levels of ethanol tolerance [3,29]. Thus, we assume that the elevated concentrations of ethanol in the coffee and orange-juice kombuchas affected the viability of bacteria and yeast. The lowest bacterial cell counts were detected when the ethanol concentration was the highest in both orange juice and coffee. However, the yeast cell counts were not the lowest in orange juice at that point compared to that in coffee.

One additional trend observed in coffee kombucha was that an increase in ethanol levels contributed to a higher concentration of acetic acid. This observation aligns with the classical kombucha metabolism, in which the yeast produces ethanol, which is then converted to acetic and other acids by AAB [5,6]. However, this tendency was not as apparent in orange-juice kombucha. Metagenomic results indicated that the content of *S. pombe* was initially high, slightly decreased, and by the end of the backslopping process, the yeast content was even higher than at the beginning of the experiment. This may be attributed to the yeast species adapting to the orange juice environment. Additionally, it appears that the ethanol concentration in both orange juice and coffee kombucha reached levels deemed unacceptable, preventing the AAB from converting it to acetic acid. The *Komagataeibacter* species exhibited tolerance to the elevated ethanol environment and adapted accordingly, whereas the supplemented LAB species did not.

Despite the fact that the orange-juice kombucha acquired a carbonated orange lemonade-like taste after two days of fermentation, prolonging the backslopping cycles led to changes in its sensory characteristics, rendering the taste sensorially unacceptable. On the other hand, coffee kombucha exhibited a pleasant balance of sweet and sour flavours and developed some carbonation after four days of fermentation. However, an excessive number of backslopping cycles caused a deterioration in its taste. In general, the backslopping experiments conducted in both environments demonstrated accelerated fermentation and a shortened backslopping cycle. Therefore, when it comes to production purposes, the application of backslopping technology for nontraditional kombucha matrices should be evaluated individually for each specific environment and condition.

Our sensory evaluation highlighted the importance of thorough monitoring of all chemical and microbial changes as a critical aspect of developing a new beverage. The study revealed that each backslopping cycle reduced the time required for bacterial adaptation, resulting in an accelerated fermentation process and earlier attainment of acceptable taste and odour. These changes must be taken into account to achieve a stable product with consistent sensory and chemical qualities during production. Moreover, meticulous monitoring of the ethanol concentration is necessary to market the beverage as a soft drink [30]. In terms of enhancing the health benefits of kombucha, it is desirable to enrich it with additional beneficial microbes, such as LAB, which positively impact the beverage's chemical properties, sensory characteristics, and stability [31]. It is possible to find a SCOBY that already contains LAB [2] or select LAB strains that can adapt to the existing SCOBY culture.

5. Conclusions

The development of novel fermented drinks with unique flavours and properties poses a significant challenge. The SCOBY used in kombucha production is a complex

and wild culture that dynamically adapts to new conditions and environment. This adaptability makes it difficult to achieve consistent batch-to-batch quality and control in the industrial manufacturing of kombucha. In our study, we examined the changes in the chemical and microbiological compositions of kombucha tailored by LAB cultures in orange juice and coffee, using the backslopping technology. Although the added lactic acid bacteria were not detected in the kombucha, the LAB-tailored kombucha exhibited improved sensory parameters and a modified microbial composition compared to the initial beverage. The transfer of the kombucha culture to the new matrices changed the original microbial community, and subsequent backslopping cycles further modified the consortia composition, thereby accelerating the fermentation rate.

There are various industrial technologies available for producing new kombucha-based beverages. The first option is to utilise the classical kombucha liquid and SCOBY and introduce them into the new environment. This approach requires more initial kombucha volume but tends to result in a more stable product quality. The second option is to employ the backslopping technology, which is easier to implement, but requires diligent and continuous monitoring of the microbial consortia and metabolites changes. Our study elucidated the advantages and disadvantages of different technologies for producing new kombucha-based beverages. Depending on the production capacity and available options, kombucha brewers can select the most optimal technology and adapt it to their specific goals.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/foods12193545/s1, Table S1: pH, TA, and sensory characteristics of the initial kombucha in black tea; Table S2: pH, TA and sensory characteristics of the LAB-tailored kombucha in black tea; Table S3: The lactic acid bacteria species abundance as measured by qPCR in LAB-tailored kombucha; Table S4: pH, TA and sensory characteristics of the orange juice kombucha in the preliminary optimisation study; Table S5: Description of sensory characteristics during the orange-juice kombucha backslopping (BS). The sensory testing was conducted by three assessors; Table: S6: pH, TA and sensory characteristics of the coffee kombucha in the preliminary optimisation study; Table S7: Description of sensory characteristics during the coffee kombucha backslopping. The sensory testing was conducted by three assessors; Figure S1: Gluconic acid concentrations in initial and LAB-tailored kombucha.

Author Contributions: Conceptualization, M.A.; Methodology, M.A., R.K., E.M. and J.K.; Software, R.P.; Validation, M.A., R.K. and J.K.; Formal Analysis, M.A., E.M. and R.P.; Investigation, M.A., R.K., E.M. and J.K.; Resources, R.K. and M.A.; Data Curation, M.A.; Writing—Original Draft Preparation, M.A.; Writing—Review and Editing, M.A., J.K., E.M., R.K., R.P. and M.-L.K.; Visualization, R.P.; Supervision, M.-L.K. and J.K.; Project Administration, R.K. and M.A.; Funding Acquisition, R.K. All authors have read and agreed to the published version of the manuscript.

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Data Availability Statement: The data used to support the findings of this study can be made available by the corresponding author upon request.

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Conflicts of Interest: The authors declare no conflict of interest.

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Appendix 4

Publication III

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Article

Isolation and Identification of Novel Non-Dairy Starter Culture Candidates from Plant Matrix Using Backslopping Propagation

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Abstract: The majority of non-dairy starter cultures on the market are originally isolated from milk and therefore do not provide the most optimal fermentation for plant matrices. Developing plant-derived starter cultures is essential for creating high-quality, tasty dairy alternatives. This study aims to isolate and characterize bacterial strains with the potential to be used as non-dairy starters from plant sources via backslopping evolution. A natural consortium of macerated plants was inoculated into two oat and two pea commercial drinks and backslopped for seventeen cycles to evolve the bacterial consortium at 25 °C, 34 °C, and 42 °C. The results showed that the initial natural consortium contained less than 1% lactic acid bacteria, and after the seventeenth cycle, lactic acid bacteria dominated in all investigated consortia. Oat Od1-25 and Od2-42 and pea Pd1-34 and Pd1-42 samples were selected for strain isolation based on amplicon-based metagenetic analysis of 16S rRNA gene sequencing and sensory properties. The strain isolation was performed using an out-plating technique, and colonies were identified by MALDI-TOF mass spectrometry. Altogether, eleven lactic acid bacteria species of plant origin were obtained. The strains belonged to the *Leuconostoc*, *Enterococcus*, *Lactobacillus*, and *Lactococcus* genera.

Keywords: plant-based food; backslopping technique; lactic acid bacteria; metagenetic analysis; non-dairy starter cultures; MALDI-TOF

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1. Introduction

The increasing global population has led to rising demand for high-protein foods. Research has shown that it is rather difficult to use traditional dairy and meat products to feed the rising population from an ecological perspective [1]. The higher demand for animal-based protein will increase the conversion of forests, wetlands, and natural grasslands into agricultural lands because of the need to produce more animal feed [2]. That, in the end, will increase the production of greenhouse gas emissions and decrease biodiversity and other important ecosystem services [3].

Current consumption trends emphasize the importance of conserving natural resources and protecting global ecosystems. Therefore, developing sustainable and health-supporting plant-based dairy alternatives has become indispensable. A key component in this effort is selecting suitable native crops for production to help reduce the carbon footprint [3]. Among Scandinavian crops, oats and peas are some of the most commonly used in plant-based dairy alternatives. However, certain challenges arise in translating dairy product manufacturing processes to dairy-alternative production [3]. For example, these Scandinavian crops have a strong background flavor, often perceived as an astringent by some consumers [4]. Fermentation offers a promising approach to reduce these sensory issues.

Artisan food fermentation uses well-investigated starter cultures that have a long historical background. The group of lactic acid bacteria (LAB) plays a central role in that process [5–7]. These organisms are a group of bacteria that produce lactic acid by utilizing carbohydrates. Also, LAB produce acetic acid, ethanol, aroma compounds, bacteriocins, exopolysaccharides, and several enzymes, which are important to ensure the end-product shelf-life [8], microbial safety, texture, and pleasant sensory profile [7]. The main species used in food fermentation are from the genera *Lactococcus*, *Lactobacillus*, *Streptococcus*, *Leuconostoc*, *Pediococcus*, *Enterococcus*, *Oenococcus*, *Bifidobacterium*, and *Weisella* [9].

Traditional yoghurt is made through the fermentation of cow's milk by using LAB-based starter cultures. Conventional yoghurt starter cultures are *Lactobacillus delbrueckii* subsp. *bulgaricus* and *Streptococcus thermophilus*; additionally, in fermented probiotic cow's milk production, *Lactobacillus casei*, *Lactobacillus acidophilus*, *Lactobacillus rhamnosus*, *Lactobacillus johnsonii*, *Bifidobacterium lactis*, *Bifidobacterium bifidum*, *Bifidobacterium brevis*, are used [7]. Rapid acidification causes the pH to drop to 4.5, which in turn results in changes in the casein structure, through which the gelation of yoghurt appears [8]. Due to this process, the yoghurt obtains a good creamy texture.

Most non-dairy starter cultures on the market are isolated from the native microbiota of traditional dairy products. Additionally, fermented end products may contain many additives such as protein extracts, inulin, thickeners, and emulsifiers [8,10]. All these additives impact the fermentation process by providing new substrates for microbes, potentially altering the usual metabolic pathway and leading to the production of unpleasant aroma compounds. Despite being non-dairy, these alternatives are fermented using the same dairy-based starter cultures [11]. Product development has found that these dairy cultures are not proficient in creating appealing aroma, taste and texture profiles, nor reducing undesirable flavors from raw materials in fermented plant-based products [8]. One option to solve this issue is to select autochthonous cultures that originated from plants. The most common plant-associated LAB species are *Lactobacillus plantarum*, *Leuconostoc mesenteroides*, *Leuconostoc citreum, and Weissella cibaria* [12,13]. However, there are also representatives from *Enterococcus* and *Streptococcus* species [13].

Previous studies have shown that autochthonous species have more potential in non-dairy product formulation than allochthonous species [12,14,15]. Thus, Di Cagno et al., 2009 [16] showed that tomato juice fermented with autochthonous species had a shorter lag phase, higher total antioxidant activity during storage, and a positive odor effect due to the synthesized volatile compounds than allochthonous species. Therefore, isolation and investigation of autochthonous species could be a turning point in non-dairy product production. Furthermore, Kütt and colleagues demonstrated that in the final stages of fermentation, the indigenous species *L. plantarum* from mixed cultures of dairy and non-dairy sources started to dominate the consortium in an oat-based matrix [11]. This could be because *L. plantarum* originates from botanical sources and is well-adapted to plant-based environments [17]. The main goal of this research was to isolate novel non-dairy starter culture candidates adapted through backslopping in oat- and pea-based drinks, and characterize the chemical, microbial, and growth changes during the backslopping procedure coupled with sensory descriptive analysis. In our study, the autochthonous species were not only identified but also evolved through backslopping cycles to find the strongest starter candidates for plant-based dairy alternatives development. Additionally, the current study investigates the time and temperature effects to find the most suitable lactic acid species, indicating their repetition robustness and possible scalability.

2. Materials and Methods

2.1. Collection and Preparation of Floral Mash for Culture Isolation

The oat florets were collected from local South Estonia farmers. The pea pods and plant leaves (horseradish, blackcurrant) were collected from a garden located in southwest Estonia. The collected plant materials were preserved under +4 °C conditions. Then, the collected floral materials were blended with tap water and crushed in a Vorwerk

Thermomix® TM6® blender cooker (Thermomix; Vorwerk & Co., Wuppertal, Germany). The ratio between solid material and tap water was 2:5. Then, the solid parts of the floral mash were extracted through muslin cloth and the liquid part was used for inoculation.

2.2. Backslopping Procedure

The experimental plan of the study is shown in Figure 1. The backslopping (Bs) was conducted at 25 °C, 34 °C, and 42 °C. A total of 1% floral mash was inoculated into plant drinks [oat drink 1 (Od1), oat drink 2 (Od2), pea drink 1 (Pd1), pea drink 2 (Pd2)]. The pasteurized plant drinks were purchased from the local grocery. The list of ingredients is presented in Table S1. Samples cultivated at 25 °C were reinoculated every 48 h, and samples fermented at 34 °C and 42 °C were reinoculated every 16 h. Three 1 mL aliquots were taken after every Bs point for further analyses.

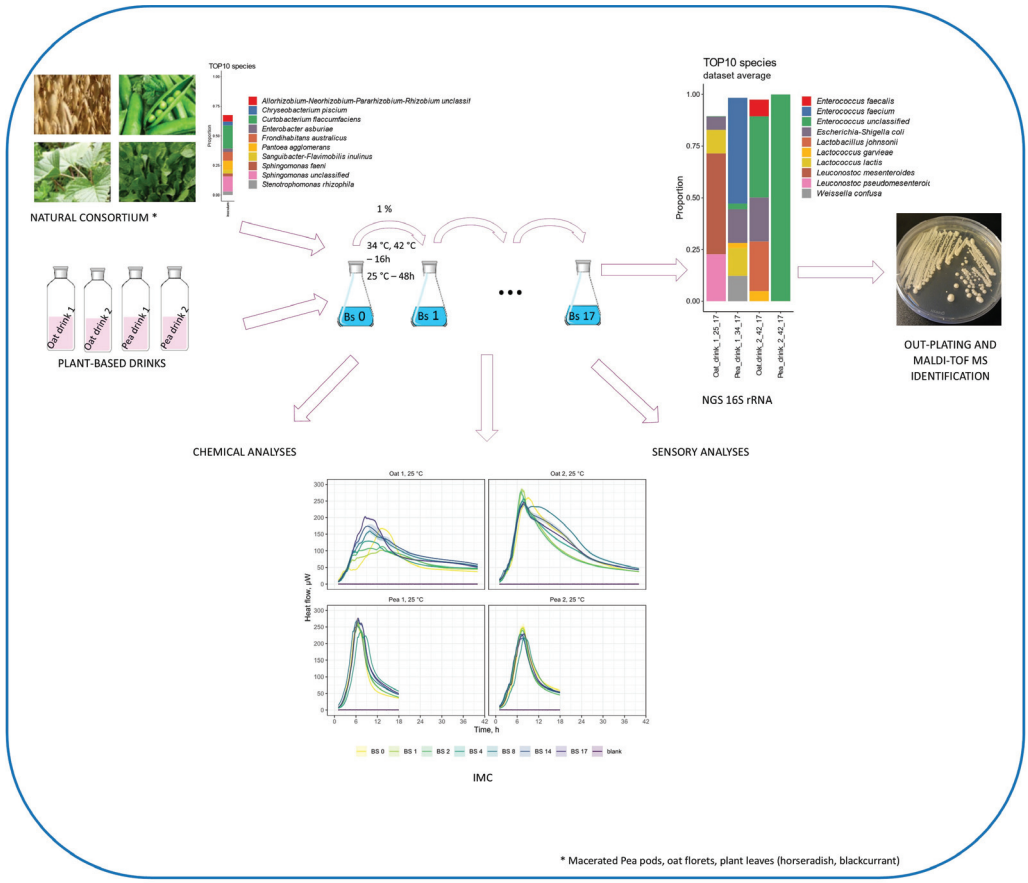

Figure 1. The floral mash was added to the oat and pea drinks and backslopped for 17 cycles. During the experiment, the sensory parameters were evaluated, and the growth of bacteria was monitored by isothermal microcalorimetry TAM III and TAM IV. The microbial composition was detected with next-generation sequencing (NGS). Four samples from backslopping were selected for strain isolation based on the 16S rRNA sequencing and aroma characteristics. The isolated strains were detected with MALDI-TOF.

2.3. Microbial Cell Isolation, PMAxx Treatment, and Genomic DNA Extraction

Microbial cells from fermented oat and pea drink samples were isolated aseptically. For that, 10 mL of each sample was diluted in 40 mL of sterile 0.85% NaCl solution, vortexed thoroughly, and centrifuged at 300× *g* for 10 min at 4 °C (Hettich ROTANTA 460R, fixed-angle rotator). If the supernatant contained bigger floating particles or aggregates after the first centrifugation step (some pea drink 1 samples), the supernatant was filtered through folded (pleated) filter paper. To pellet the microbial cells, the supernatant was transferred to a new 50 mL tube and centrifuged at 5000× *g* for 15 min at 4 °C (Hettich ROTANTA 460 R, fixed-angle rotator). The pellet was washed in 1 mL of sterile 0.85% NaCl solution and then transferred to a 2 mL tube.

For determination of the total bacterial community (viable and non-viable bacteria), the cell suspension was centrifuged at 10,000× *g* for 10 min at 4 °C (Thermo Scientific MicroCL 21R Centrifuge, Thermo Scientific™, Waltham, MA, USA), the supernatant was aspirated, and the pellet containing the microbial cells was stored at −20 °C until gDNA extraction. For the discrimination of viable bacterial consortia, the cell suspension was centrifuged at 5000× *g* for 10 min at 4 °C (Thermo Scientific MicroCL 21R Centrifuge), the supernatant was aspirated, the pellet was resuspended in 400 µL sterile 0.85% NaCl solution, and PMAxx treatment was performed as published by Ref. [18]. After PMAxx treatment, cells were pelleted as described above and stored at −20 °C until gDNA extraction.

For gDNA extraction, cell pellets were resuspended in 250 µL 1 × PBS and subjected to gDNA extraction using the Quick-DNA Fungal/Bacterial Miniprep Kit (Zymo Research, USA) according to the manufacturer's instructions. The concentrations of the extracted DNAs were quantified by a Qubit™ 3 Fluorometer (Thermo Fisher Scientific, Waltham, MA, USA) using the Qubit dsDNA BR Assay Kit (Thermo Fisher Scientific).

2.4. 16S rRNA Gene Amplicon Sequencing and Data Processing

Amplicon libraries targeting the V4 region of the 16S rRNA gene by the primer pair 515F/806R were prepared according to Illumina's dual indexing protocol as published in Ref. [19]. Multiplexed and normalized libraries were sequenced with the MiSeq Sequencing System (Illumina, San Diego, CA, USA) using the MiSeq V2 Reagent Kit and 300 cycles of the paired-end sequencing protocol. DNA sequence data were analyzed as published before in Refs. [19–21] using the open-source BION-meta program (https://github.com/nielsl/mcdonald-et-al, accessed on 21 December 2024, Danish Genome Institute, Copenhagen, Denmark) according to the author's instructions.

2.5. Isothermal Microcalorimetry Study

The 48-channel isothermal microcalorimetry (IMC) TAM IV (TA Instruments, New Castle, DE, USA) and 24-channel IMC TAM III (TA Instruments, New Castle, DE, USA) were used to determine the growth of consortia during the backslopping experiment. The IMC protocol was published before by Refs. [22,23].

The experiment was conducted at 25 °C, 34 °C, and 42 °C. The ampoules were filled with pasteurized plant drinks, and 1% inoculum was added under aseptic conditions. Samples cultivated at 34 °C and 42 °C were fermented for 16 h, and fermentation at 25 °C lasted 48 h. After baseline collection, the filled ampoules were inserted in the IMC for 30 min. Ampoules were kept in an equilibration position for 15 min and then lowered into a measuring position. After lowering the samples, it took an additional 45 min to stabilize the signal. Data were not collected during the first hour. The data were acquired by TAM Assistant Software V2.0.105 (TA Instruments, New Castle, De, USA) and analyzed in Microsoft Excel 2021 (Microsoft Corporation, Redmond, WA, USA).

2.6. Determination of Metabolic Products in Plant Matrices

The determination of metabolic products in plant matrices was performed as published by Ref. [11]. For free amino acid identification, fermented plant drinks were centrifuged at 14,000× *g* for 20 min at room temperature (Hettich ROTANTA 460 R, fixed-angle rotator).

The supernatant was filtered through a 3 kDa molecular weight cut-off filter (Amicon® Ultra-0.5, Merck KGaA, Darmstadt, Germany) and diluted with 2 parts ultrapure water before analysis. Prior to injection, free amino acids were derivatized with AccQ•Fluor Reagent (Waters Corp., Milford, MA, USA) according to the manufacturer's procedure. Analysis of free amino acids was performed on an ultra-performance liquid chromatography (UPLC) system (Acquity UPLC; Waters Corp, Milford, MA, USA), including a binary solvent manager, a sample manager, and a photodiode array (PDA) detector (Waters Corp, Milford, MA, USA), controlled by Waters Empower™ 3.0 software (Build 3471, Waters Corp., Milford, MA, USA). Separations were performed on a Waters Acquity UPLC AccQ•Tag Ultra Column (2.1 × 100 mm, 1.7 µm particle size) operated at +55 °C. The injection volume was 1.5 µL, the amino acids were eluted at a flow rate of 0.3 mL/min, and absorbance was recorded at 260 nm. The running time was 25 min. Empower software (Waters Corp., Milford, MA, USA) was used for data processing.

The organic acids of fermented plant drinks were measured by a Waters 2695 HPLC system (Waters Corporation, Milford, MA, USA). For that, 13-mm Philic PTFE 0.2 µm Non-sterile Millex-LG filters (Millipore, Darmstadt, Germany) were used to filter the plant drink samples. An HPX-87H column (BIO-RAD Hercules, Hercules, CA, USA) was used, and the system was eluted isocratically with 0.005 M of H2SO4 at 0.6 mL/min at 35 °C. A Waters 2487 dual absorbance detector (Waters Corporation, Milford, MA, USA) and a Waters 2414 refractive index detector (Waters Corporation, Milford, MA, USA) were used for the detection and quantification of analytes. Empower software 3 (Build 3471 FR5 SR4, Waters Corporation, Milford, MA, USA) was used to perform the data analysis.

2.7. Strain Isolation

The isolation was carried out from the last backslopping cycle of selected fermented drinks on agar plates according to Figure 2. For that, MRS (De Man–Rogosa–Sharp) agar (NEOGEN Culture Media) was used and incubated either at 25 °C, 34 °C, or 42 °C. Additionally, trypticase soy yeast extract medium (M92) was used at 34 °C and 42 °C. All plates contained cycloheximide in concentration at 50 mg/L to inhibit the growth of yeast species and 10 g/L sucrose (Table S2). After incubation, based on colony formation, five to twenty colonies from each agar plate were picked and sent to MALDI-TOF MS identification.

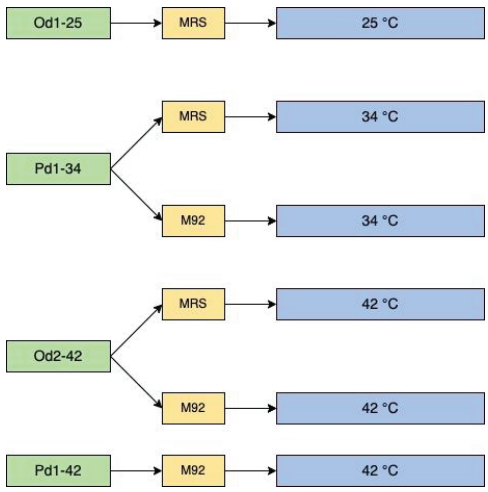

Figure 2. The process of strain isolation. The backslopping endpoint (Bs 17) used for species isolation is indicated in the green box. Green box names indicate: oat drink 1 cultivated at 25 °C (Od1-25), oat drink 2 cultivated at 42 °C (Od2-42), pea drink 1 cultivated at 34 °C (Pd1-34), and pea drink 1 cultivated at 42 °C (Pd1-42). The yellow boxes show the selected agar media for isolating colonies. The blue boxes show the incubation temperature of agar plates.

2.8. MALDI-TOF Mass Spectroscopy

MALDI-TOF-MS (Bruker MALDI Biotyper, Billerica, MA, USA) was used to identify isolates at the species level. The analysis was conducted at Tallinn University of Technology in the Department of Chemistry and Biotechnology. Fresh colony biomass was spread on the two spots of the target plate and covered with 1 µL of formic acid (70%), followed by matrix solution (HCCA, Bruker Daltonics, Billerica, MA, USA). Each series of MALDI measurements was preceded by a calibration step with the bacterial test standard (BTS; Bruker Daltonics, Billerica, MA, USA) to validate the run. Identifications were obtained by comparing the mass spectra to the Bruker MSP database using the Bruker Compass software (https://www.bruker.com/en/products-and-solutions/mass-spectrometry/ms-software/biopharma-compass.html, Bruker Daltonics, Billerica, MA, USA) at the default settings. The identification scores equivalent to or higher than 2 were considered for the identification on the species level.

2.9. Data Visualization and Statistical Analysis

The 16S and HPLC graphs were generated using R studio version 4.3.3 (29 February 2024 ucrt, R Foundation for Statistical Computing, Vienna, Austria) and the tidyverse package suite version 2.0.0. Within tidyverse, ggplot2 3.5.0 was used for data visualization, dplyr 1.1.4 was used for data manipulations, and readxl 1.4.3 was used for reading Excel files. For quantitative descriptive analysis, free amino acids analysis was interpreted using principal component analysis (PCA) and visualized with R studio version 4.3.1 (16 June 2023 ucrt, R Foundation for Statistical Computing, Vienna, Austria). The libraries used to create PCA graph were readxl (v 1.4.1), ggbiplot (v 0.55), factoextra (v 1.0.7), and FactoMineR (v 2.9).

3. Results and Discussion

3.1. The Initial Consortium

To obtain bacterial strains with enhanced adaptability to plant-based environments, we isolated bacteria from supporting plant materials, including oat florets, pea pods, and leaves from horseradish and blackcurrant. Two types of commercial plant-based beverages—oat and pea—were selected as matrices for evolutionary adaptation due to the relevance of these crops to the Nordic market and their potential for future applications. To enrich the final consortia with the most adaptable cultures, we applied backslopping technology. The microbial compositions before and after backslopping cycles were analyzed using 16S metabarcoding. Both standard and PMAxx-modified 16S rRNA NGS were employed to distinguish between total and viable microbial consortia (Figure 3) [18]. The initial microbial composition was assessed following maceration of the plant material.

The composition of total and viable mash microbiota turned out to be similar and diverse, where the majority of consortia were represented by regular plant inhabitants. The largest proportion of the initial inoculum was formed by *Curtobacterium flaccumfaciens*, followed by *Sphingomonas* spp (Figure 3). *C. flaccumfaciens* is a well-known bean pathogen [24]. *Sphingomonas* spp. are plant tissue inhabitants that can control plant pathogens and promote plant growth [25]. *Neorhizobium galegae* and *Pantoea ananatis* were determined in the PMA-treated sample but not in the sample prepared by the standard method. Before enrichment by backslopping, the lactic acid bacteria (LAB) population was less than 0.1%. The PMA-treated sample showed that *Enterococcus* spp. were dominant, with *Enterococcus faecium* prevailing in the standard sample. Also, *Lactobacillus* spp., *Lactobacillus delbrueckii*, and *Ligilactobacillus aviarius* were detected among viable bacteria but not in the standard sample. Thus, the initial consortia contained many viable environmental bacteria, and the LAB species proportion was particularly low, with *Enterococcus* spp. dominance.

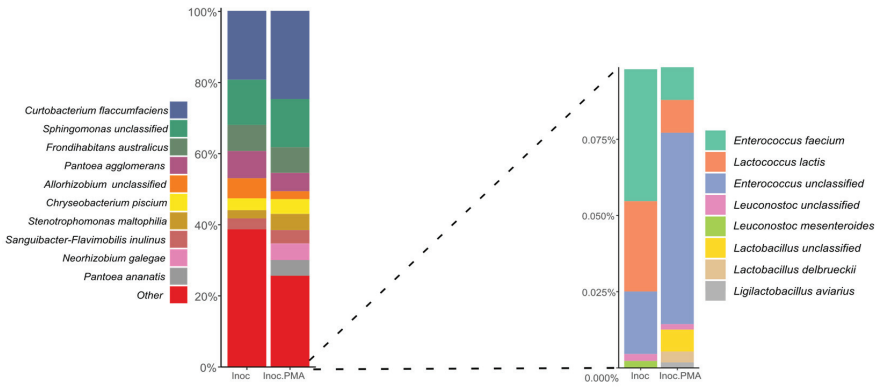

Figure 3. Bacterial composition of the inoculum. Standard DNA sequencing methodology (Inoc) and DNA sequencing methodology modified with PMAxx treatment (Inoc.PMA) for detection of viable bacteria species. Less than 0.1% of the detected species were LAB, and they are enlarged on the right side of this figure.

3.2. The Variation in Consortia Composition During Backslopping

Next, we performed the laboratory evolution of initial plant bacterial consortia in oat and pea drinks by backslopping propagation for 17 cycles. In order to diversify and stabilize the adapted microbiota, the backslopping was carried out under different temperature regimes (25 °C, 34 °C, and 42 °C). A temperature of 25 °C was selected to obtain LAB that produce diacetyl, the main aroma component that gives dairy products a milky and creamy aroma [26]; additionally, 34 °C was the optimum chosen to enrich the microbiota with strains of mesophilic bacteria, and 42 °C was chosen to isolate thermophilic species [27]. Even though backslopping is a commonly used inoculation procedure based on the addition of a small part of previously fermented product into a fresh similar environment [28], this technology is not often used for bacterial strain evolution and novel complex starter culture generation.

The dynamic changes in the proportion of viable bacteria during backslopping cycles at different temperatures were monitored by 16S rRNA metabarcoding (Figures 4–6), starting from the microbial composition of the macerated floral mass combined with the microbial biomass from the plant-based drinks (zero time-point), with subsequent numbers indicating each backslopping (Bs) cycle.

The fluctuations in microbiota composition were affected by both the fermentation temperature and the specific formulation of the plant-based drink. Even similar matrices from different commercial beverages promoted the growth of various bacteria species. Thus, during fermentation and backslopping at 25 °C, *L. lactis* initially dominated in oat drink 1 at 25 °C (Od1-25), but from the 8th Bs cycle onward, *L. pseudomesenteroides* and *L. mesenteroides* began to prevail, with the latter becoming dominant by the end of the experiment. Oat drink 2 at 25 °C (Od2-25) displayed greater initial diversity than oat drink 1, though *L. lactis* was slightly more abundant. By the middle of the experiment (8th Bs cycle), *L. pseudomesenteroides* and *L. citreum* started to prevail, with *L. pseudomesenteroides* ultimately overtaking the consortia.

The pea samples were less diverse than the oat samples, showing minimal changes in bacterial composition. *L. lactis* dominated throughout the experiment in both pea drink 1 (Pd1-25) and pea drink 2 (Pd2-25) samples at 25 °C. The only notable difference appeared in *E. faecalis* abundance, which showed a slight increase in Pd1 in the 8th Bs cycle, while in Pd2, this increase occurred earlier in the 4th Bs cycle. By the 17th Bs stage, the bacterial microbiota had stabilized, with *L. lactis* continuing to dominate in the pea matrices, while more complex consortia developed in the oat drinks.

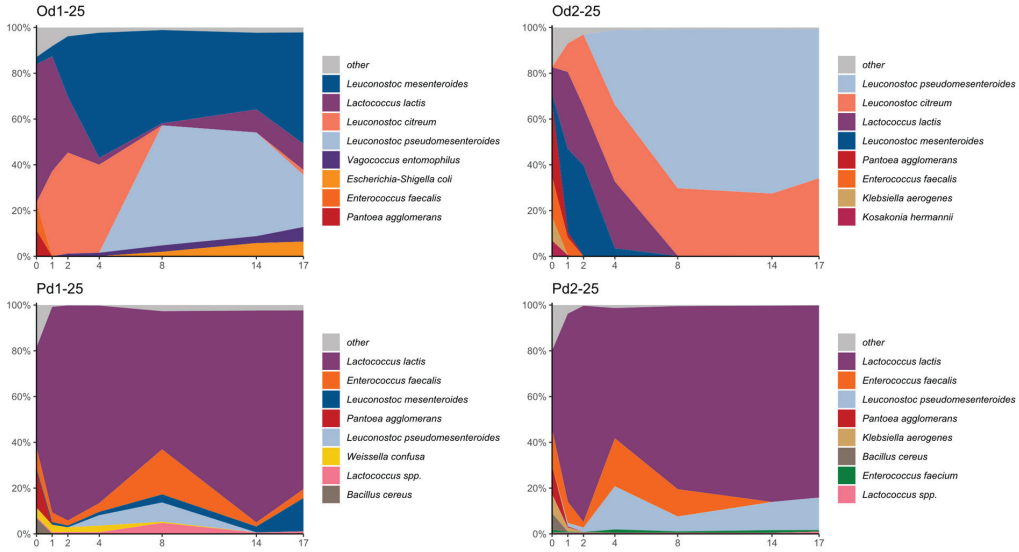

Figure 4. The dynamics in the microbial composition according to 16S rRNA NGS from backslopping over 17 cycles at 25 °C. The 0 signifies the macerated floral mass composition with the microbial biomass from the plant-based drinks, and further numbers indicate the backslopping points. Graphs names indicate: oat drink 1 cultivated at 25 °C (Od1-25), oat drink 2 cultivated at 25 °C (Od2-25), pea drink 1 cultivated at 25 °C (Pd1-25), and pea drink 2 cultivated at 25 °C (Pd2-25).

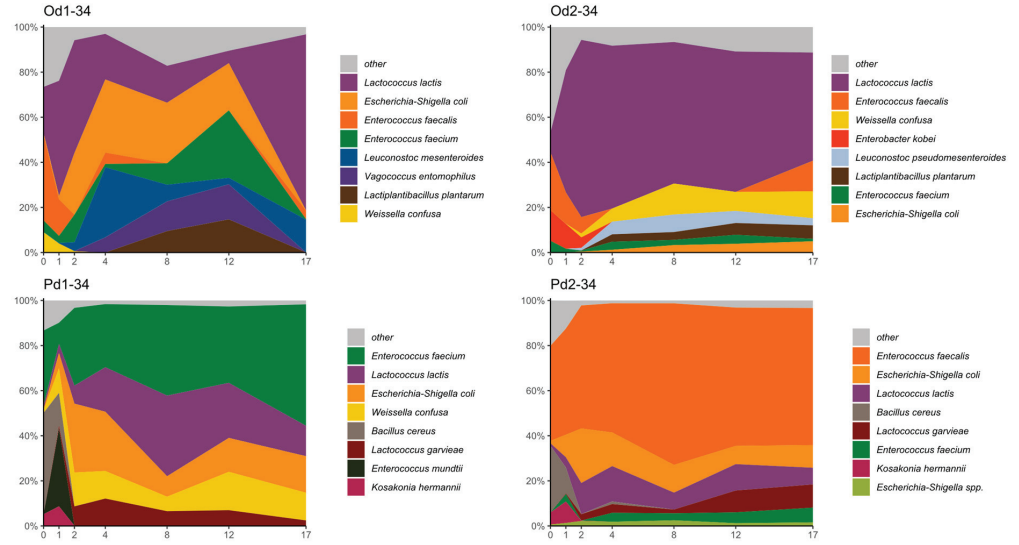

Figure 5. The dynamics in the microbial composition according to 16S rRNA NGS from backslopping over 17 cycles at 34 °C. The 0 signifies the macerated floral mass composition with the microbial biomass from the plant-based drinks, and further numbers indicate the backslopping points. Graphs names indicate: oat drink 1 cultivated at 34 °C (Od1-34), oat drink 2 cultivated at 34 °C (Od2-34), pea drink 1 cultivated at 34 °C (Pd1-34), and pea drink 2 cultivated at 34 °C (Pd2-34).

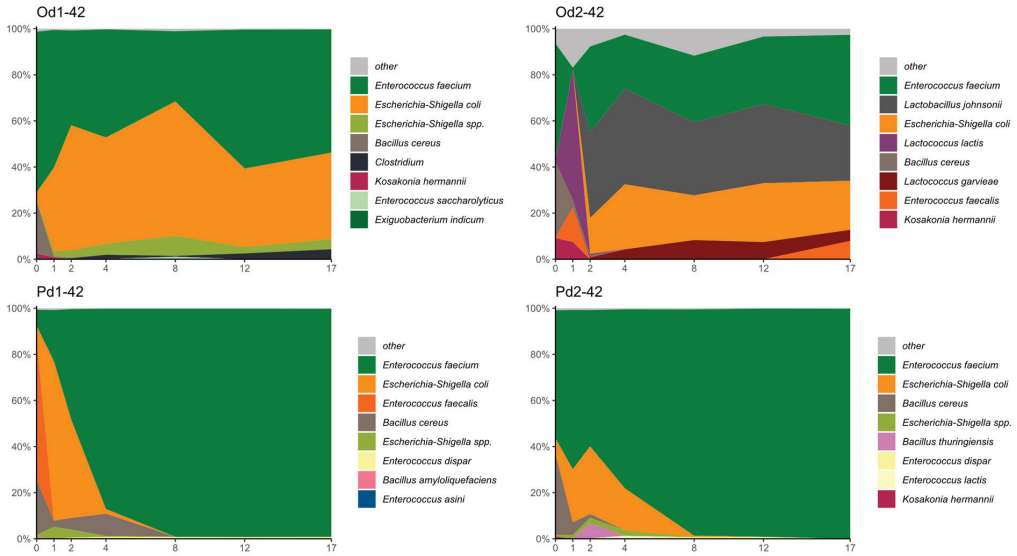

Figure 6. The dynamics in the microbial composition according to 16S rRNA NGS from backslopping over 17 cycles at 42 °C. The 0 signifies the macerated floral mass composition with the microbial biomass from the plant-based drinks, and further numbers indicate the backslopping points. Graphs names indicate: oat drink 1 cultivated at 42 °C (Od1-42), oat drink 2 cultivated at 42 °C (Od2-42), pea drink 1 cultivated at 42 °C (Pd1-42), and pea drink 2 cultivated at 42 °C (Pd2-42).

The bacterial distribution at backslopping at 34 °C (Figure 5) differed from that at 25 °C. New species such as *Escherihia-Shigella coli*, *Vagococcus entomophilus*, and others were more abundant. At the same time, *L. lactis* prevailed here for the oat drink matrix. The dynamics of consortia stabilization were also different compared to the lower fermentation temperature. For Od2, Pd1, and Pd2, the stable consortia formed already during the first cycles of backslopping, while Od1 continued to evolve even after the 12th Bs cycle. For this sample, the composition was highly diverse in the middle of the experiment, with *E. coli* having a slightly higher abundance than others. Similarly to the beginning of the experiment, the *L. lactis* started to dominate over others. Od2 fermented at the same temperature as Od1 showed the same results at the beginning of the experiment. Further, from the 2nd Bs cycle to the end of the experiment, *L. lactis* was the dominant species. The pea samples exhibited different dominant species than the oat samples. In pea drink 1 fermented at 34 °C (Pd1-34), *Bacillus cereus* and *E. faecium* were the most abundant species in the beginning. By the 8th Bs point, the *B. cereus* abundance was decreased, *L. lactis* increased, and *E. faecium* abundance was still high. At the end of the experiment, *E. faecium* still dominated, but the second place in terms of abundance was shared with *L. lactis*, *E. coli*, and *Weisella confusa*. In Pd2 fermented at 34 °C (Pd2-34), *E. faecalis* dominated throughout the experiment.

In general, the samples fermented at 42 °C in all environments were less diverse than samples fermented at 25 °C and 34 °C, except sample Od2 (Figure 6). In oat drink 1 fermented at 42 °C (Od1-42), the two most abundant species—*E. faecium* and *E. coli*—were prevalent throughout the experiment. *E. faecium* dominated at the beginning and end of the experiment, and *E. coli* dominated in the middle. Od2-42 had a highly diverse composition. *L. lactis* seems to have prevailed at the beginning of the experiment. Three species—*E. faecium*, *L. johnsonii*, and *E. coli*—were most abundant from the 4th Bs point till the end of the experiment. In pea drink 1 fermented at 42 °C (Pd1-42), *E. coli* was most abundant in the beginning. Interestingly, *E. faecium* was the only species detected in the sample from the 8th Bs point till the end. However, *E. faecium* was the dominant species throughout the

experiment. The same trend happened as in Pd1 from the 8th Bs point. Different incubation temperatures contributed to the emergence and dominance of specific bacterial species during the backslopping adaptation process. Notably, *L. lactis* and *Leuconostoc* spp. were more prevalent at lower temperatures, whereas *Enterococcus* species were more abundant at higher fermentation temperatures. The pattern may be explained by the creation of optimal growth conditions for each species. Despite the historical use of lactococci in the production of a wide range of dairy-based fermented products, plant-based isolates of these bacteria may offer desirable traits, potentially enhancing the flavor and aroma of traditional dairy products [29]. Moreover, their plant origin suggests potential for incorporation into plant-based matrices, opening new avenues for environmentally sustainable foods.

The dominance of *E. faecium* in 42 °C samples can likely be attributed to this species' high competitiveness, supported by bacteriocin production and resilience across a wide range of temperatures and pH levels [30]. However, although *E. faecium* strains may possess beneficial probiotic properties, strains of both *E. faecium* and *E. coli* can also exhibit virulence, raising safety concerns [30,31]. While LAB are generally recognized as safe (GRAS), the isolated microorganisms might carry potential risks and need to be further analyzed. Risk management for potential active novel starter cultures is regulated by the European Commission (EC) and assessed by the European Food and Safety Authority (EFSA) [32]. Therefore, it is essential to evaluate *Enterococcus* spp. in silico and thoroughly assess their antibiotic resistance, virulence characteristics, and biogenic amine production potential to ensure their safety before using them as starter cultures.

3.3. Establishment of Microbial Consortia During Prolonged Backslopping Propagation at Different Temperatures

In parallel with the metagenetic sequencing of samples, the consortia stability was measured by isothermal microcalorimetry (IMC). IMC is a unique method with high sensitivity which measures the heat production or absorption by metabolically active microorganisms in hermetically sealed ampoules [23] and thereby describes fine changes in their composition.

According to IMC, consortia Od1-25 and Od2-25 achieved stability by the 8th cycle (Figure S1), while the pea drinks stabilized four cycles earlier. At 34 °C, all plant drinks reached stability by the fourth cycle (Figure S2). For consortia cultivated at 42 °C, stability was also observed during the fourth cycle, except for Pd1-42, which settled on the eighth cycle (Figure S3). However, IMC was not the most reliable indicator to determine the evolved consortia stability because of the inconsistencies with the 16S NGS results. IMC graphs showed that the consortia stabilized many cycles before consistency was detected using the 16S NGS (Figures 4–6). This discrepancy may be due to IMC's measurements of the heat generated by total cell numbers, rather than the actual composition of consortia members [23,32]. Therefore, this method is more suitable for the determination of the growth of a single species or less diverse consortia or control of consortia growth in chemically defined media.

3.4. Changes in Free Amino Acids Metabolism During Backslopping Propagation

One of the important functions of novel plant starters is to modify the sensory, nutritional, and chemical properties of fermented food. Among others, plant-based food is poor in essential amino acids [33], and fermentation could improve this health-beneficial property [34,35]. To evaluate the changes and increases in the free amino acids (FAA) concentration during the backslopping propagation, the FAA analysis was performed (Figure 7).

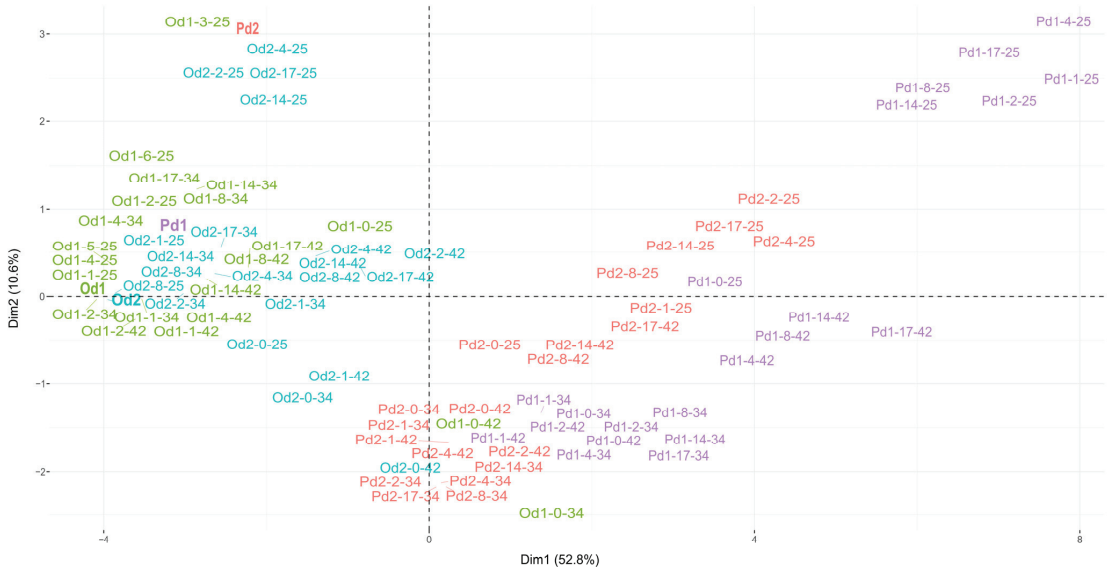

Figure 7. Principal component analysis using the free amino acids concentrations (mM) collected at the end of fermentation. Concentrations (mM) were centered and scaled. Each sample is encoded by the matrix followed by the Bs stage and temperature. Green, blue, purple, and red colors refer to oat drink 1 (Od1), oat drink 2 (Od2), pea drink 1 (Pd1), and pea drink 2 (Pd2) plant-based matrices, respectively. Standard deviations are shown (n = 2).

A principal component analysis of the measured FAA concentration revealed that Od1 and Od2 non-fermented plant drinks were mostly similar to fermented drinks. At the same time, Pd1 and Pd2 non-fermented plant drinks significantly differed from fermented samples. The Pd1 and Pd2 at 34 °C and 42 °C clustered together. This means that fermentation is more beneficial to the pea matrix than to the oat plant drinks. When comparing these results with the metagenetic profile, it was found that the predominant bacteria in pea drinks at high fermentation temperatures belong to the *Enterococcus* genus. Thus, most probably, these species have an impact on amino acid metabolism during pea protein fermentation. Previous studies have shown that *Enterococci* produce several aminopeptidases to release essential amino acids from casein [36], which is important for cheese ripening. A similar mechanism could be involved in the case of pea proteins.

In more detail, the amino acid concentrations of Gly, Glu, and Thr were higher at 34 °C and 42 °C in Od1 samples than in blank (non-fermented) matrices (Table S3). Additionally, Od1-42 contained more Tyr after fermentation. Od1-25 had lower concentrations of FAA than the non-fermented Od1-25. The amino acids His, Ser, Gly, Thr, and Pro showed higher concentrations in Od2 fermented samples than in blank samples at all temperatures. Also, there were some differences between temperatures. Additionally, some FAA increased at different temperatures, such as Arg and Glu at 25 °C, Asp at 34 °C, and Ala at 42 °C.

The pea matrices were more diverse at different temperatures, and in some cases, the fermentation influenced the opposite to oat matrices (Table S3). All amino acids were increased at the end of backslopping in Pb1-25 except Gln and Met. Asn, Gln, Arg, Glu, Lys, Met, and Trp decreased at 34 °C and other amino acids increased. At 42 °C, all amino acids had higher concentrations at the end of fermentation except Arg and Trp. The fermentation affected matrix Pb2 differently than the Pb1 matrix. At 25 °C, most of the FAA concentrations were increased after backslopping except Arg and Ala, which showed opposite results. At 34 °C and 42 °C, almost half of the FAAs were decreased compared to

the beginning. Interestingly, the amino acid methionine was not detected at all during the Bs experiment at 34 °C and 42 °C.

Overall, the concentration of free amino acids increased in pea-based matrices and decreased in oat-based matrices throughout the backslopping experiment. The concentrations of essential amino acids Ile, Leu, Phe, and Trp rose in all pea drinks by the end of fermentation, while only Pd1 backslopping at 25 °C showed increases in His and Lys concentrations. Additionally, Met levels rose in Pd1 drinks at all fermentation temperatures.

Previous studies have shown that fermentation enhances the proteolytic activity of pea-based but not oat-based matrices [34,35]. Kütt et al., 2023 reported that Glu, Asn, and Asp concentrations decreased, while His and Ser levels increased after fermentation in oat drinks. On the other hand, our results show an increase in the Glu concentration in Od1 at 34 °C and 42 °C. Another difference from published results was the observed increase in Thr release [34]. Kaleda et al., 2020 found that fermentation elevated levels of Ala, Cys, His, Ile, Leu, Lys, Phe, Pro, Ser, Tyr, and Val while decreasing Arg, Asn, and Asp in a pea–oat protein blend, where the pea protein content was twice as high as the oat protein content. Consistent with this, our results also showed a decrease in Arg concentration in pea matrices at 34 °C and 42 °C. All mentioned studies used LAB species such as *Streptococcus thermophilus*, *Lactobacillus acidophilus*, and *Lactobacillus delbrueckii* subsp. *bulgaricus* for fermentation [11,34,37], which were not present in the microbial communities in our study.

3.5. Chemical and Prelimenary Sensory Analysis

In addition to free amino acid analysis, we performed pH and organic acid measurements, essential tests for evaluating the food fermentation process. The pH values of selected Bs points remained below 5, with the lowest observed value being 4.0 (Table S4), showing successful acidification of the matrices. For dairy alternative products such as yoghurt, shelf-life is shorter at pH 4.6 compared to pH 4.2 or lower [11].

The observed pH decrease during backslopping was supported by an increase in organic acid concentrations. Measured levels of acetic and lactic acids showed that acid concentration changes differed according to temperatures and environments. In general, the lactic acid concentration was higher than acetic acid, pointing out the dominance of LAB in the fermentation environment. The most significant increase in acetic acid concentration was observed in oat drinks at 25 °C, potentially linked to the high abundance of *Leuconostoc* species in both drinks, as these bacteria can produce acetate [38].

For all samples, the lactic acid levels slightly increased during the backslopping cycles, with the most substantial change occurring in Od2. Interestingly, at 25 °C, the lactic acid concentration sharply decreased in all drinks at the 8th Bs point, then increased steadily until the end of the experiment (Figure S4). The largest decreases were detected in Od1 and Od2. At the same time, the acetic acid concentration continued to rise in Od1, while Od2 showed similar fluctuations as lactic acid. These drastic changes in acid concentrations coincided with shifts in microbial composition (Figure 4). Specifically, in drinks fermented at 25 °C, *L. lactis* showed a marked decline from the 8th Bs in Od1 and disappeared entirely in Od2, while *Leuconostoc* species became dominant in both drinks from this point onward.

At 34 °C, the lactic acid concentration increased consistently in all drinks, while the acetic acid concentration remained stable or showed minimal changes (Figure S4). From a microbiological perspective, the microbial composition at this temperature was more diverse, with fewer drastic shifts (Figure 5), correlating with less dynamic alteration in organic acid profiles.

At 42 °C, lactic acid concentrations remained stable in Od1 and Pd2 throughout the backslopping process, while Od2 and Pd1 gradually produced more lactic acid with each Bs cycle (Figure S4).

Sensory evaluation is one of the most important parameters for the food industry during new product development. Consumers tend to choose food according to positive perceptions and familiar tastes. Thus, for the final decision-making and selection of samples

for further strain isolation, preliminary sensory analysis was performed. It quickly provided additional knowledge regarding positive evaluation of the backslopping process and information about potential sensory perception and the successful course of the study. The sensory evaluation was conducted after each backslopping cycle by half of the panel members. The evaluators characterized the odor, taste, and texture of the samples. The sensory parameters changed throughout the backslopping process, and the most promising drinks were obtained from the 17th (last) Bs cycle in Od1-25, Pd1-34, Pd1-42, and Od2-42 (Table S5). In general, Pd2 had an unpleasant taste, odor, and texture despite having the highest concentration of sugar. The other combinations were perceived as too bitter and sour, and the textures were watery. Od1-25 had a sour cream-like odor, a good sour–sweet balance, and a texture similar to cow-based yoghurt. The next sensorially best sample was Pd1-42. It had a sweet and caramelly odor, tasted like cow milk-based yoghurt, and had an oat porridge-like texture. Pd1-34 had a sweet and caramelly odor, a watery texture, and a similar taste to mild unflavored plant-based yoghurt. Od2-42 also had a good sour–sweet balance, but the texture was the least similar to real cow milk-based yoghurt. It was sensorially described as an oat drink which had a good sour–sweet balance. The caramel and sweet odor in all Pd1 samples came from the plant-based drink due to the addition of natural flavorings (Table S1). Other plant-based drinks did not contain additional flavors (Table S1). The consortia of the most promising plant-based drinks described above were selected for strain isolation following identification by MALDI-TOF MS.

3.6. Strain Isolation

Sensory characteristics such as taste, odor, and texture (Table S5), along with bacterial profiles identified through 16S rRNA sequencing, were used to select candidates for strain isolation. Four samples (Od1-25, Pd1-34, Pd1-42, and Od2-42) from the last backslopping cycle (Bs17) were chosen as promising candidates for plant-based starter cultures. Bacterial strain isolation was performed using a standard outplating technique, and colonies were identified by MALDI-TOF mass spectrometry. To maximize diversity in candidate selection, we used two different growth media, MRS and M92 (common for LAB), and tested all previous incubation temperatures and under both aerobic and anaerobic conditions (Figure 2).

In this study, a total of 65 isolates were analyzed by MALDI-TOF MS; out of them, eight isolates could not be identified. The most abundantly identified species were *L. lactis*, *L. mesenteroides*, and *E. faecium* (Table 1). All these LAB species are mostly used in industrial dairy fermentation as main starter contributors [39], where a part of consortia are responsible for antimicrobial activity [40] or expressing probiotic properties [41]. They are generally recognized as safe (GRAS) and may be beneficial to a host's health [41].

More specifically, isolates from Od1-25 fermentation grown on MRS medium contained 30% *L. mesenteroides* followed by 15% *L. pseudomesenteroides* and 10% *L. citreum*, although 45% of the sample composition could not be identified by the MALDI-TOF MS approach (Table 1). Bacterial isolates from the Pd1-34 fermentation on MRS medium included *L. lactis*, *E. faecium*, and *W. cibaria* in equivalent proportions (16.7%), along with 8.3% *L. garvieae*, while 41.7% of the isolates were not identified. The results for Pd1-34 on M92 medium differed from those on MRS medium; *L. garvieae* was the most abundant at 40%, followed by *E. faecalis* and *L. lactis* with 20% each, and *E. faecium* at 10%, with 10% remaining unidentified. The majority of Od2-42 isolates on MRS consisted of *E. faecium* (60%), along with 30% *Lb. johnsonii* and 10% *E. faecalis*. In contrast, Od2-42 isolates on M92 contained 30% *E. faecalis* and *E. coli*, followed by 20% *E. faecium*, with 20% of isolates remaining unidentified. Only *E. faecium* was isolated from Pd1-42 on M92 medium.

Table 1. The species identified according to MALDI-TOF-MS from the last backslopping point (Bs17) compared with 16S NGS results.

Matrix	Temp, °C	Cultivation Media	MALDI-TOF MS			16S	
			Species	Count	%	Species	%
Od1-25	25	MRS	*Leuconostoc mesenteroides*	6	30.0	*Leuconostoc mesenteroides*	48.7%
			Leuconostoc pseudomesenteroides	3	15.0	*Leuconostoc pseudomesenteroides*	22.7%
			Leuconostoc citreum	2	10.0	*Lactococcus lactis*	11.4%
			Unidentified	9	45.0	*Vagococcus entomophilus*	6.4%
				0	0,0	*Escherichia-Shigella coli*	6.4%
						Leuconostoc citreum	2.2%
						Raoultella ornithinolytica	0.8%
						Lactococcus unclassified	0.4%
						Other	1.0%
Pd1-34	34	MRS	*Enterococcus faecium*	2	16,7	*Enterococcus faecium*	53.9%
			Lactococcus lactis	2	16.7	*Escherichia-Shigella coli*	16.2%
			Weissella cibaria	2	16.7	*Lactococcus lactis*	13.5%
			Lactococcus garvieae	1	8.3	*Weissella confusa*	12.3%
			Unidentified	5	41.7	*Lactococcus garvieae*	2.5%
						Escherichia-Shigella unclassified	1.2%
		M92	*Lactococcus garvieae*	4	40.0	*Lactococcus unclassified*	0.1%
			Lactococcus lactis	2	20.0	*Latilactobacillus unclassified*	0.1%
			Enterococcus faecalis	2	20.0	Other	0.3%
			Enterococcus faecium	1	10.0		
			Unidentified	1	10.0		
Od2-42	42	MRS	*Enterococcus faecium*	6	60.0	*Enterococcus faecium*	39.3%
			Lactobacillus johnsonii	3	30.0	*Lactobacillus johnsonii*	24.1%
			Enterococcus faecalis	1	10.0	*Escherichia-Shigella coli*	21.3%
			Unidentified	0	0.0	*Enterococcus faecalis*	7.9%
						Lactococcus garvieae	4.8%
		M92	*Enterococcus faecalis*	3	30.0	*Escherichia-Shigella unclassified*	1.8%
			Escherichia coli	3	30.0	*Latilactobacillus unclassified*	0.4%
			Enterococcus faecium	2	20.0	*Lactobacillus unclassified*	0.2%
			Unidentified	2	20.0	Other	0.3%
Pd1-42	42	M92	*Enterococcus faecium*	5	100.0	*Enterococcus faecium*	98.9%
			Unidentified	0		*Enterococcus dispar*	0.9%
						Enterococcus saccharolyticus	0.1%
						Lactococcus lactis	0.1%
						Other	0.1%

When comparing the MALDI-TOF MS results with 16S sequencing data, bacterial isolates from Od1-25 on MRS and Pd1-42 on M92 showed the same bacterial species and proportions across both approaches. These findings suggest that extreme conditions, such as lower or higher fermentation temperatures, which favor a narrower range of species, may be more selective [42]. Greater differences appeared when incubation was performed at an intermediate temperature (approx. 35 °C) on different media (MRS and M92). These results were expected, as certain species preferentially grow on MRS while others thrive on M92 [43]. Nevertheless, all major species identified on both media via agar plates were also detected in 16S rRNA gene sequencing (Table 1). For example, in Pd1-34 incubated at 34 °C on MRS, the dominant species were *E. faecium*, *L. lactis*, and *W. cibaria*, whereas on M92, *L. garvieae* predominated. All these species, however, were confirmed by 16S sequencing. Comparing MALDI-TOF MS and 16S sequencing highlights the complementary strengths of each method: 16S sequencing, though more costly, is highly selective and capable of identifying even trace bacterial species within a sample. Conversely, plate incubation combined with MALDI-TOF MS allowed us to isolate and identify preferred species, paving the way for developing non-dairy starter cultures tailored for plant-based products.

Overall, by exposing the initial plant inoculum to different temperatures and matrices, we diversified the final consortia and increased the potential to isolate unique strains with distinctive characteristics. Backslopping selected the LAB species that were able to survive and evolve over several cycles, indicating the robustness and potential scalability

of these strains. The propagation of these species must continue in the future in plant-based feedstocks in order to be able to dominate in the environment and not lose their abundance in the starter composition. To evaluate and characterize possible functional properties of the isolates, whole genome sequencing was performed on several selected strains. We sequenced 11 genomes, including those from the *Leuconostoc*, *Enterococcus*, *Lactobacillus*, and *Lactococcus* genera (strain list in Table S6). The next step will be high throughput in silico genome mining based on comparative genomic evaluation to identify starter cultures with desirable technological properties, such as utilization of available carbohydrates, production of proteolytic enzymes, or synthesis of exopolysaccharides. Additionally, safety-related traits including the absence of antibiotic resistance genes, intact prophage regions, or biogenic amine production genes will be assessed.

4. Conclusions

This study demonstrated the potential of traditional backslopping technology in plant-based matrices to adapt and evolve lactic acid bacteria of plant origin, yielding promising candidates for non-dairy starter cultures. By applying this technique, we successfully enriched LAB populations from an initial consortium of plant-associated and soil bacteria derived from horseradish, blackcurrant leaves, oat florets, and green peas. Although LAB initially represented less than 0.1% of the microbial population, they became the dominant species in all oat and pea drinks by the final backslopping cycle, with one to three LAB species prevailing in each consortium. In this way, future culture development can benefit from different natural floral consortia, where thousands and thousands of microbial species grow that are best suited to ferment the plant-based matrices. This study is the first of its kind to demonstrate how novel dairy alternatives should be fermented with autochthonous lactic acid species and exhibit potential starter culture survival and evolution through backslopping cycles.

Using 16S sequencing, we tracked LAB species throughout backslopping propagation in oat and pea matrices at different temperatures and isolated LAB from four endpoint drinks (Od1-25, Pd1-34, Pd1-42, and Od2-42) that exhibited favorable sensory properties. Eleven LAB species, belonging to the *Leuconostoc*, *Enterococcus*, *Lactobacillus*, and *Lactococcus* genera, were successfully isolated and identified via MALDI-TOF MS analysis, followed by whole-genome sequencing.

These results highlight backslopping as an effective method for bacterial stain enrichment and adaptation from natural plant sources, advancing the development of functional non-dairy starter cultures. Future work should focus on genomic analysis of the strains to assess safety factors, including virulence and antibiotic resistance profiles, and to identify functional traits that enhance product quality. These foundational data will support the design and modulation of effective starter consortia for innovative, plant-based fermentation applications.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/fermentation10120663/s1, Table S1. The list of ingredients and nutritional values of plant based+ drinks (Oat drink 1, oat drink 2, pea drink 1 and pea drink 2); Table S2. List of components and concentrations of the media from this study; Figure S1. Microcalorimetry growth curves during backslopping cycles in all environments at 25 °C; Figure S2. Microcalorimetry growth curves during backslopping cycles in all environments at 34 °C; Figure S3. Microcalorimetry growth curves during backslopping cycles in all environments at 42 °C; Table S3. Comparison of free amino acids concentration (mM) measured at the end of fermentation; Table S4. Measured pH and ORP from selected Bs points during prolonged backslopping propagation; Figure S4. Lactic acid and acetic acid concentration changes during backslopping experiments in all plant-based drinks at 25 °C, 34 °C, 42 °C; Table S5. Sensory characteristics and pH measurements from backslopping point 17; Table S6. Principal information of genomes from the isolates.

Author Contributions: Conceptualization, M.A. and M.-L.K.; methodology, M.A., A.K., J.K. and T.J.; software, T.J.; validation, M.A. and J.K.; formal analysis, M.A., A.K., I.S. and T.J.; investigation, M.A., A.K., J.K. and T.J.; resources, T.J. and M.A.; data curation, M.A.; writing—original draft preparation, M.A.; writing—review and editing, M.A., J.K., A.K., I.S. and M.-L.K.; visualization, T.J.; supervision, J.K. and M.-L.K.; project administration, T.J. and M.A.; funding acquisition, T.J. All authors have read and agreed to the published version of the manuscript.

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