Towards healthy and tasty plant-based milk alternatives: Food-grade microbial fermentation for improved nutritional value and flavor perception

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Summary

Plant-based milk alternatives are often nutritionally unbalanced, and their flavor profiles limit their acceptance. In this work, mono- and co-culture fermentation processes were developed to upgrade plant milks into more healthy and tasty products.

Here, chickpea milk, limited in L-lysine should be enriched in the essential amino acid by fermentation with food-grade microbes. Promising strains could be identified from more than 2,500 natural candidates based on their genomic repertoire. As an example, *L. paracasei* subsp*. paracasei* NCC 2511 and *B. amyloliquefaciens* NCC 156 accumulated high levels of L-lysine when grown in chickpea milk. Subsequently, a novel co-culture process was established to increase the vitamin B_{12} level in sunflower seed milk, a waste stream from the industrial sunflower oil production. Multi-substrate utilization strain *B. amyloliquefaciens* NCC 156 and vitamin B_{12} producing *P. freudenreichii* NCC 1177 emerged as perfect partners. After iterative optimization, co-fermentation of the two strains increased vitamin B_{12} to 17 µg $(100\ g)^{-1}$, 7-fold higher than the recommended daily uptake. Finally, the flavor metabolism of lactic acid bacteria during plant milk fermentation was studied. Notably, several of the food-grade approved strains delivered beneficial changes opening up the possibility to create new flavors and diversify plant milk products.

Zusammenfassung

In dieser Arbeit wurden Mono- und Co-Kultur-Fermentationsverfahren entwickelt, um Pflanzenmilch zu gesünderen und schmackhafteren Produkten aufzuwerten.

Dabei sollte Kichererbsenmilch, die wenig L-Lysin enthält, durch Fermentation mit Mikroben mit der essenziellen Aminosäure angereichert werden. Aus mehr als 2.500 natürlichen, lebensmitteltauglichen Kandidaten konnten anhand ihres genomischen Repertoires vielversprechende Stämme identifiziert werden. Zum Beispiel akkumulierten *L. paracasei* NCC 2511 und *B. amyloliquefaciens* NCC 156 hohe Mengen an L-Lysin, wenn sie in Kichererbsen-Milch gezüchtet wurden. Anschließend wurde ein neuartiges Co-Kultur-Verfahren zur Erhöhung des Vitamin B_{12}-Gehalts in Sonnenblumenkernmilch, einem Abfallprodukt der industriellen Sonnenblumenölproduktion, entwickelt. *B. amyloliquefaciens* NCC 156 und der Vitamin B_{12} produzierende *P. freudenreichii* NCC 1177 erwiesen sich als perfekte Kandidaten. Nach iterativer Optimierung erhöhte die Co-Fermentation der beiden Stämme den Vitamin B_{12}-Gehalt auf 17 µg $(100\ g)^{-1}$, das 7-fache der empfohlenen täglichen Aufnahme. Schließlich wurde der Geschmacksstoffwechsel von Milchsäurebakterien während der Fermentation von Pflanzenmilch untersucht. Mehrere der für Lebensmittel zugelassenen Stämme führten zu vorteilhaften Veränderungen, die die Möglichkeit eröffnen, neue Geschmacksrichtungen zu entwickeln und Pflanzenmilchprodukte zu diversifizieren.

1 Introduction

1.1 General introduction

Plant-based milk alternatives are water extracts of plants, which become increasingly popular for human nutrition. Over the years, the global market for these products has become a multi-billion dollar business and will reach a value of approximately 36 billion USD by 2026 (Rasika et al. 2021). Notably, 40% of the consumers are meanwhile willing to reduce the use of animal-based protein because of climate change concerns (Aschemann-Witzel et al. 2021). Meanwhile, many consumers demand plant-based milk alternatives due to sustainability aspects, health-related reasons, lifestyle and dietary choices.

To fully meet consumer expectations in terms of product quality, plant milks are intended to resemble animal milk. Unfortunately, plant-based milk alternatives are often nutritionally unbalanced and their flavor profiles limit acceptance. From the nutritional side, plant proteins often exhibit low quality, poor digestibility, and an undesired limitation in essential amino acids (Millward 1999). In particular, L-lysine, which is required for hormone formation, catalytic and structural proteins, and immune system support and is therefore, one of the most impacting nutrients (Aggarwal et al. 2022), often exhibits much lower abundance in many plant-based milks than in animal milks. As an example, chickpea-based milk only contains approximately half of the amount in a typical 10% dry matter formulation (Csapó et al. 2009a; Rachwa-Rosiak et al. 2015). In addition, certain vitamins such as vitamin B_{12}, are present at low levels or even absent. This vitamin is required for the development, and function of the central nervous system, healthy red blood cell formation, and DNA synthesis, and displays the most important micronutrient for vegans to be aware of (Guetterman et al. 2022; Mikkelsen and Apostolopoulos 2019). From the flavor and sensory side, plant-based milk alternatives are generally perceived as a product with displeasing taste. Their typically beany and grassy

flavor, as well as the bitter and seedy taste, are received as “off-flavor” by consumers, especially in countries without a tradition for this type of products (Diarra et al. 2005; Sethi et al. 2016; Tangyu et al. 2022).

Towards more valuable and tasty products, natural fermentation appears an appealing option to improve the nutrition, aroma, taste, and also the texture of plant milks and, moreover, deliver co-benefits such as increased nutritional value, sensory profile, stability, and microbial safety (Ayivi et al. 2020; Cichońska and Ziarno 2021b; Sethi et al. 2016; Tangyu et al. 2019). To date, plant-based milk fermentation mainly uses mono-cultures of microbes, such as LAB, bacilli, and yeasts, for this purpose. More recently, new concepts propose mixed-culture fermentation with two or more microbial species. However, at present, many of the selection of strains and strain combinations are still conducted in trial and error approaches. It seems difficult, or not even infeasible to predict the outcome, due to our still poor understanding of the underlying microbes and microbial interactions in different plant-based environments. Hereby, the understanding of the needs and capabilities of the microbes appears crucial to delivering food with predictable benefits as the results of natural processes catalyzed by safe microbes (Kohlstedt et al. 2014; Schwechheimer et al. 2018a; Schwechheimer et al. 2018b; Schwechheimer et al. 2018c). Possibilities for a more rational selection and combination of strains with predictable synergistic interaction with the help of systems biology approaches would be highly valuable towards smarter fermentation processes and better products with improved nutrition, flavor, and sensory quality.

1.2 Objectives

The present work aimed to develop potential mono- and mixed-culture fermentation processes enabling improvement of the nutritional value, focusing on L-lysine and vitamin B_{12}, flavor, and sensory acceptance of plant-based milks. To this end, first, a systematic workflow for product-derived strain selection should be developed with a pathway-based in-silico screening approach. Then for understanding mixed-culture for improving nutrition, the physiology of strains such as growth, sugar consumption, organic acid and amino acid metabolism, and B-group vitamin production, was supposed to be studied. The metabolic behavior and specific nutritional need and limitation of strains further provided the base for selection and understanding of mixed-cultures with predictable synergistic interaction. Additionally, flavor changes during plant milk fermentation were studied in detail, revealing species-related and plant milk-related differences and, notably, highlighting several well-performing strains that delivered a range of beneficial flavor changes.

2 Theoretical Background

Plant-based milk alternatives have been consumed for hundreds and thousands of years. These products are meant to give consumers an alternative choice of animal milk, an emulsion containing nutrients such as lipids, proteins, amino acids, vitamins, and minerals and produced by lactating mammals to provide nutrients for the growth and development of their sucklings (Haug et al. 2007; Mäkinen et al. 2016; Sethi et al. 2016). Today, milk alternatives are commercially obtained from a variety of plants, such as legumes, seeds, nuts, cereals, and pseudo-cereals (Mäkinen et al. 2016). Over the past years, the market for these plant-based milk alternatives has continually increased and, in the USA alone, reached an annual volume of approximately US$1.8 billion (**Fig. 1**). From a global perspective, the dairy alternative market size was valued at US$ 13 billion in 2018 and is estimated to be further expanded to US$ 36 billion by 2026 (Rasika et al. 2021). The increasing preference for plant-based milk alternatives is driven by different factors and consumer demands: health-related challenges such as lactose intolerance and milk allergies (Crittenden and Bennett 2005), consumer concerns about cow milk hormones and cholesterol (Epstein 1990), ethical disputes regarding the use of animals (Hughes 1995), environmental issues (Rotz et al. 2010), changes in lifestyle towards vegetarian and vegan food, presumably healthier diet (Craig 2010) and the marketed health-promoting properties of these products (Paucar-Menacho et al. 2010). Accordingly, leading dairy companies are adding plant-based milk alternative products to their portfolio.

However, plant-based milk alternatives often do not provide the full nutritional value of cow milk (Sethi et al. 2016) and suffer from undesirable off-flavors (Desai et al. 2002; Sethi et al. 2016; Vanga and Raghavan 2018). Therefore, commercial products positioned as plant-based milk alternatives are typically amended with additives such as vitamins, amino acids, minerals, and flavors (Sethi et al. 2016).

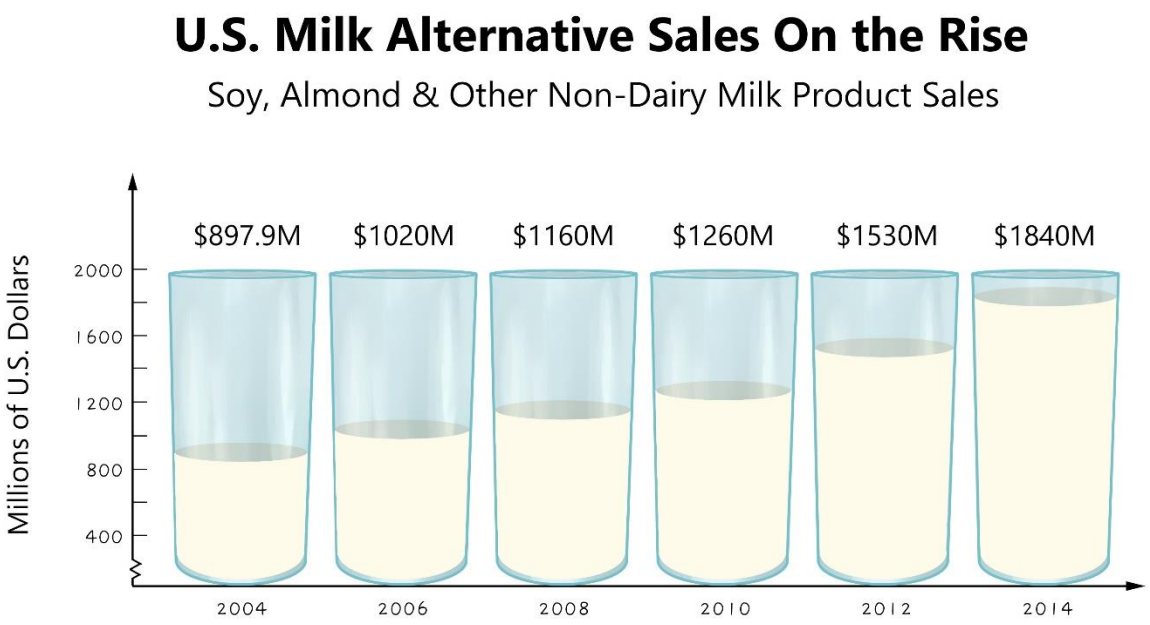

Fig. 1. U.S. market development for plant-based milk alternative products (soy, almond, and other non-dairy milk products). The data are taken from (Bloomberg Surveillance 2015).

2.1 Plant types

Due to a constantly increasing demand for non-dairy alternatives and growing interest to explore different functional properties, various plants are used to produce non-dairy milk alternatives (Sethi et al. 2016). The relevant plant sources can be classified into five types: (i) legumes (beans), (ii) nuts, (iii) seeds, (iv) pseudo-cereals, and (v) cereals (**Fig. 2**) (Sethi et al. 2016). Soy-based drinks are the dominating plant-based milk alternatives in the Western world (Mäkinen et al. 2016). In addition, drinks based on almond (Ginsberg and Ostrowski 2007), coconut (Seow and Gwee 1997), sunflower seeds (Fujisawa et al. 1986), chickpea (Rao et al. 1988), lupine (Ivanović et al. 1983), hemp (Vahanvaty 2009), sesame (Afaneh et al. 2011), quinoa (Pineli et al. 2015), pea (Li et al. 2004), and rice (Mitchell et al. 1990) are meanwhile available and contribute to the diversity of the plant-based milk alternative market.

Depending on the individual raw material, the corresponding drink differs significantly in composition and flavor.

2.2 Key quality criteria of plant-based milk alternatives

Compared to cow milk, plant-based milk alternatives are often nutritionally unbalanced and technically challenged, moreover, their flavor profiles limit acceptance. To improve the overall quality of plant-based milk alternatives, researchers and developers in academia and industry, have to overcome certain challenges (**Fig. 3**).

2.2.1 Physico-chemical properties of plant-based milk

Plant-based milk alternative manufacturing has a general layout of consecutive unit operations (**Fig. 4**). Generally, plant-based drinks are prepared by crushing plant material, followed by extraction of its soluble parts into water. The properties of the final product depend on the raw material and, furthermore, on the specifications of the individual steps for disintegration, homogenization, formulation, emulsification, and storage. Different strategies are applied to improve the homogenization and stability of plant-based milks towards the characteristics of animal milk, which is a natural emulsion. As an example, plant-based drinks from starchy materials (such as cereals or pseudo-cereals) easily gelate during sterilization (autoclaving or pasteurization), which causes technical problems in downstream processing (Mäkinen et al. 2016). Furthermore, the excessive lipid content of seeds and nuts (**Fig. 2, Fig. 3**) may lead to an undesired phase separation and reduced product stability, so these compounds are removed during processing (Briviba et al. 2016). More details on plant-based milk alternative manufacturing can be found in a recent excellent review (Mäkinen et al. 2016).

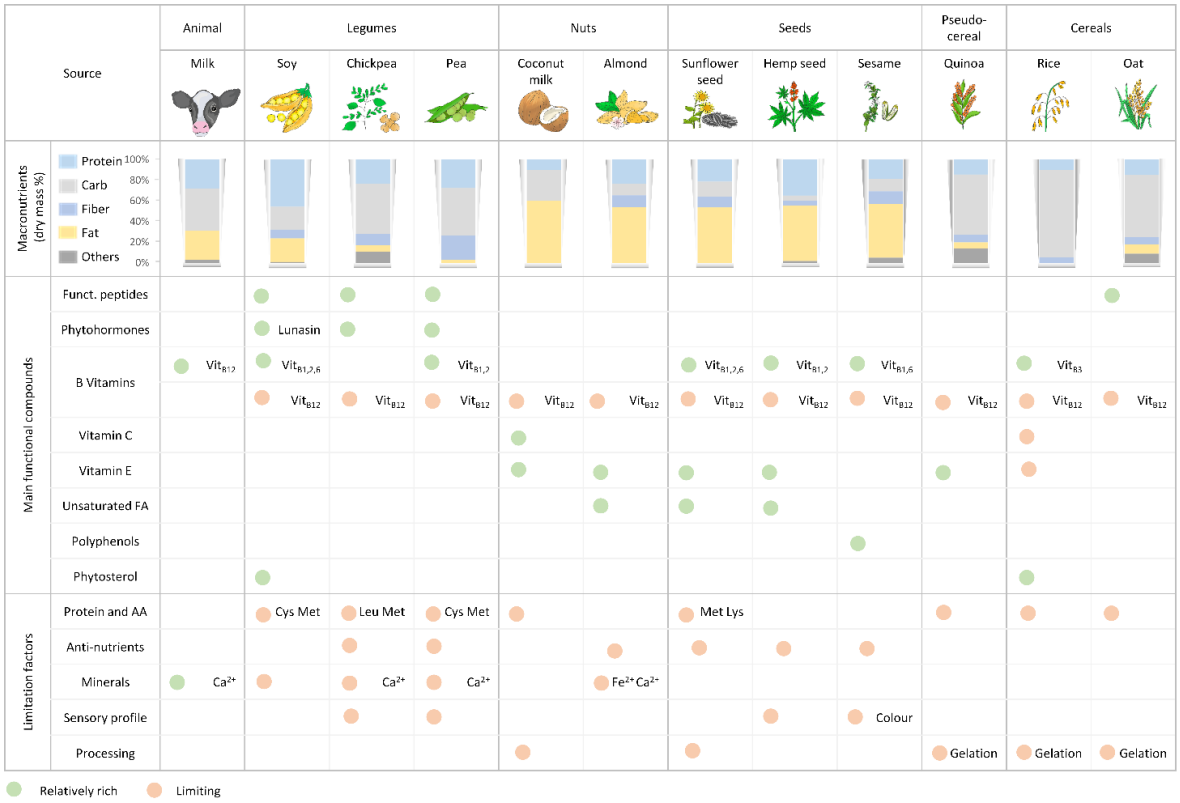

Fig. 2. Macronutrient composition, functional components, and limiting factors of common plants used for plant-based milk alternative production. The data are collected from previous work (Afaneh et al. 2011; Bernat et al. 2015; Callaway 2004; DebMandal and Mandal 2011; Duranti et al. 2008; Erbaş et al. 2005; Fernandez and Berry 1988; Hove 1974; Juliano and Hicks 1996; Lambo et al. 2005; Lampart-Szczapa et al. 2003; Lebiedzińska and Szefer 2006; Makinde and Akinoso 2013; Moneret-Vautrin et al. 1999; Noimark and Cox 2008; Önning et al. 1998; Paucar-Menacho et al. 2010; Ranhotra et al. 1993; Roy et al. 2010; Seow and Gwee 1997; Sethi et al. 2016; Škrbić and Filipčev 2008; Ulyatu et al. 2015; Vahanvaty 2009; Vanga and Raghavan 2018; Vidal-Valverde et al. 2003; Vilche et al. 2003; Villamide and San Juan 1998; Wood and Grusak 2007). The data of micronutrient composition are acquired from the National Nutrient Database for Standard Reference Release (NDB) (https://ndb.nal.usda.gov/ndb/). NDB identification of the selected materials selected is: milk (01212), soy (16111), chickpea (45041830), pea (45272128), lupine (16076), coconut milk (45117929), almond (12061), sunflower seed kernels (12036), Hemp seed (12012), sesame seed (12023), quinoa (20035), rice (20090), and oat (20132). Carb, carbohydrates except for fiber; Funct. peptides, functional peptides; Unsaturated FA, unsaturated fatty acids.

2.2.2 Protein and amino acids in plant-based milk

Without a doubt, the plants used offer certain attractive properties (**Fig. 2**). Some of the raw materials, such as legumes and seeds, have a protein content comparable to cow milk (although the amino acid quality is not comparable to the same extent).

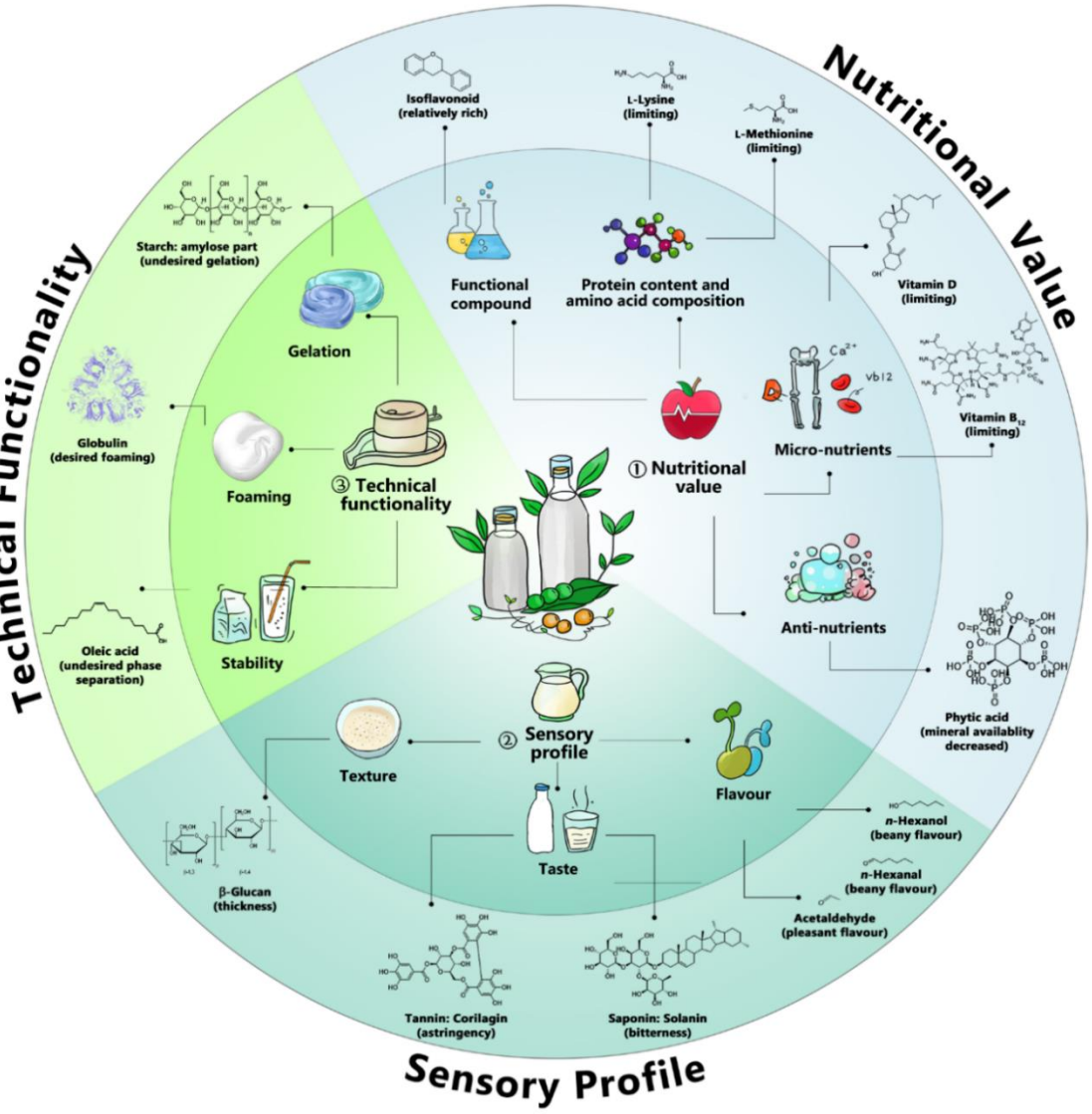

Fig. 3. Quality criteria and major functional molecule of plant-based milk alternative. Stereoviews of the soybean 11S globulin ribbon diagram were acquired from (Adachi et al. 2003).

However, the protein content of many plant-based drinks can be low. About 50% of commercial plant-based milk alternatives contain only a little or even no protein (< 0.5%), while only selected soybased milk analogs reach the higher level of cow milk (3.7%) (Jeske et al. 2017).

Additionally, plant proteins often exhibit low quality, poor digestibility, and an undesired limitation in amino acid composition (Millward 1999). This limitation also holds for the essential amino acid L-lysine, which is required for hormone formation, catalytic and structural proteins, and immune system support and is,therefore, one of the most impacting nutrients (Flodin 1997). Critically, it exhibits a much lower abundance in many plant-based milks, for example, chickpea-based milk only contains approximately half of the amount in a typical 10% dry matter formulation (Csapó et al. 2009a; Rachwa-Rosiak et al. 2015). Except this, sulfur-containing amino acids L-methionine and L-cysteine, and L-tryptophan are other amino acids typically underrepresented (Millward 1999).

2.2.3 Vitamins in plant-based milk

Plant-based milk alternatives are rich in certain vitamins (such as vitamin C, vitamin B_1, and, B_6) (Gernand et al. 2016) However, many other vitamins such as vitamin D and vitamin B_{12}, are present at low levels or even absent (**Fig. 2, Fig. 3, Table 1**), which seems partially responsible for the vitamin deficiency of people following a strict vegan diet (Pawlak et al. 2014). Moreover, vitamins are sensitive molecules and easily degraded during washing and heating steps, which further reduce their content.

Among all, lack of vitamin B_{12} (cobalamin) is one of the most severe limitations of plant-based products in general. Chemically, vitamin B_{12} is a cobalt-containing tetrapyrrole, one of the most complex small molecules made by nature (Warren et al. 2002). It is crucial for neurodevelopment, cell division and cell differentiation, and

Table 1. Nutritional comparison of cow milk and selected plant materials used for the production of plant-based milk alternatives.

(100 g)$^{-1}$	**Cow milk (dry)**	**Soybean (dry)**	**Sunflower seed (dry, partially defatted)**	**Oat (dry, partially debranned)**
Protein (g)	26	43	48	15
Ca^{2+} (mg)	912	140	114	55
Fe^{2+} (mg)	0.47	3.95	6.62	4.00
Thiamin (Vitamin B_1) (mg)	0.28	0.43	3.19	0.69
Riboflavin (Vitamin B_2) (mg)	1.20	0.76	0.27	0.12
Niacin (Vitamin B_3) (mg)	0.65	1.06	7.31	1.47
Vitamin B_6 (mg)	0.30	0.22	0.75	0.12
Total folate (µg)	37	205	222	32
Vitamin B_{12} (µg)	3.2	0.0	0.0	0.0
Vitamin D (D_2+ D_3) (µg)	0.5	0.0	0.0	0.0

The data are taken from the USDA National Nutrient Database for Standard Reference (https://ndb.nal.usda.gov/ndb/)

displays the most important micronutrient for vegans to be aware of (Guetterman et al. 2022; Mikkelsen and Apostolopoulos 2019; Stabler 2013; Yamada 2013). In addition, recent reports suggest that B_{12} might improve immunity against Covid-19 infections (dos Santos 2020; Shakoor et al. 2021).

2.2.4 Bioavailability of bioactive compounds in plant-based milk

A careful inspection reveals that many important nutrient compounds suffer from a low bioavailability. For instance, soy isoflavones mainly exist in the form of genistin and daidzin, which are glucosides of genistein and daidzein and far less bioavailable than the corresponding aglycone forms (Vacek et al. 2008; Xu et al. 1994). Moreover, plant-derived products can contain anti-nutritional factors. As an example, phytates and saponins form insoluble complexes with valuable minerals (such as Ca^{2+}, Mg^{2+},

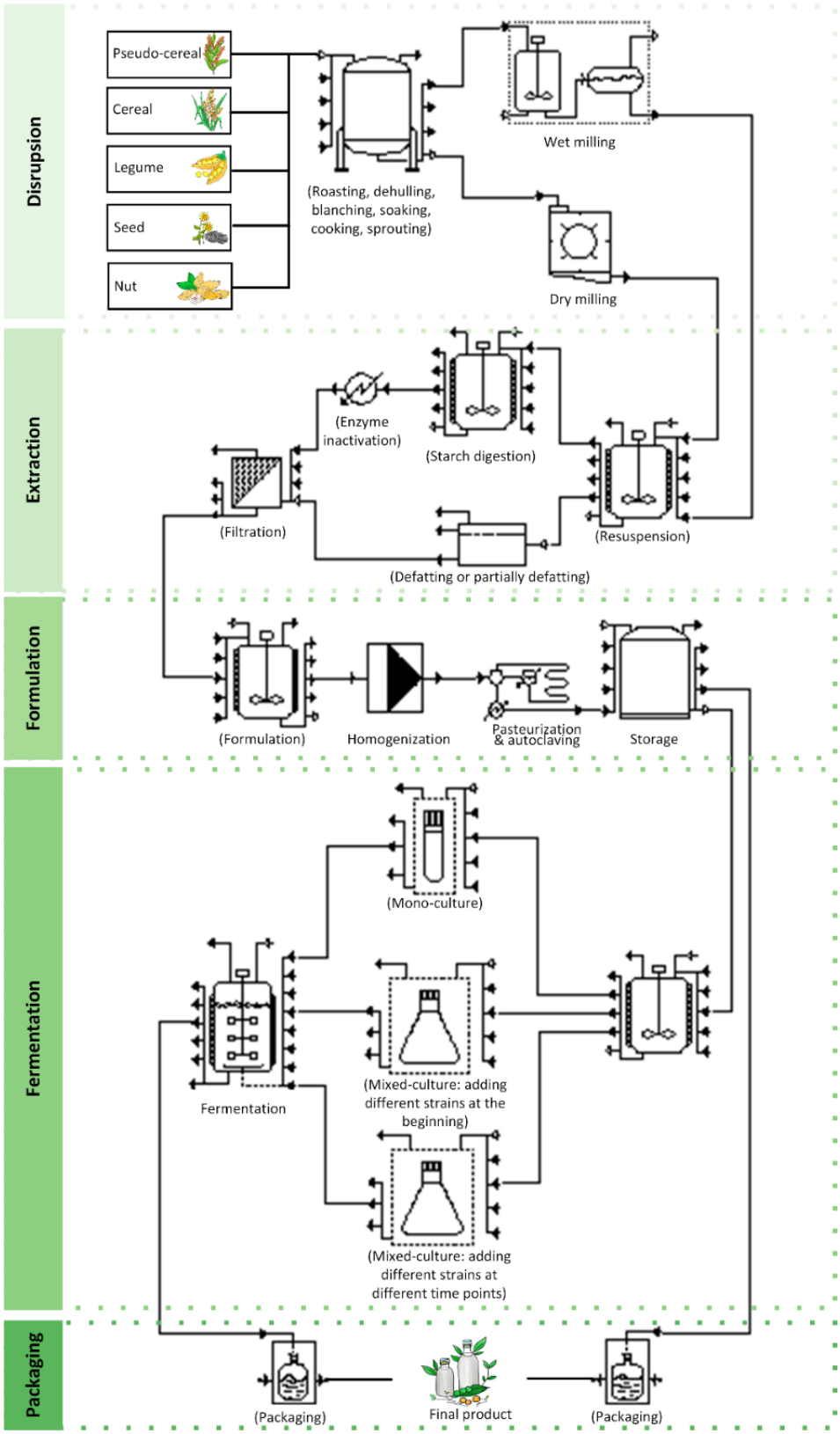

Fig. 4. Flow chart for the manufacture of fermented plant-based milk alternatives. The unit operations given in brackets are optional and depend on the chosen raw material and the expectation of the final product.

Fe^{2+}, Zn^{2+}), which decreases their bioavailability (Rekha and Vijayalakshmi 2010; West et al. 1978). Plant-based oligosaccharides, such as raffinose, stachyose, and verbascose, respectively, can only be digested by intestinal bacteria through fermentation, which results in flatulence, diarrhea, and other discomforts (Onyesom et al. 2005). The intestinal tract can furthermore be disturbed by trypsin and other protease inhibitors in plant-based milk alternatives, which interfere with protein and starch digestion by inactivating the digesting enzymes (Anderson and Wolf 1995).

2.2.5 Sensory profile of plant-based milk

It is approved by consumer and marketing studies that taste has a key impact on food selection (Glanz et al. 1998). In this regard, the natural taste of plant-based milk alternatives, unfortunately, exhibits only limited acceptance (Mäkinen et al. 2016). Although certain components of plant materials (such as soluble fibers) positively influence texture and mouthfeel (Vasquez-Orejarena et al. 2018), plant-based milk alternatives are still generally perceived as a product with displeasing taste, probably also because of previous experiences with less appealing products in the market (Wansink et al. 2005). Legume-based products tend to smell beany and earthy, which is regarded as undesired in countries without tradition for this kind of products. Volatile compounds such as *n*-hexanal and *n*-hexanol, which originate from oxidation of plant lipids, are mainly responsible for this type of off-flavor. Plant phenols (including anti-nutrients such as tannins and saponins), terpenes, glucosinolates, and flavonoids mediate a bitter, acrid, or astringent taste, depending on their molecular weight (Drewnowski and Gomez-Carneros 2000). Regrettably, certain bioactive (and therefore otherwise beneficial) compounds such as isoflavonoids are linked to an objectionable aftertaste (Matsuura et al. 1989). Additionally, a greenish, greyish, or brownish color, which corresponds to the color of the plant raw material, a chalky or sandy texture, and a thin mouthfeel due to insoluble particles, negatively influence the purchase willingness (Peyer et al. 2016).

2.2.6 Technical processing and fortification of plant-based milk

To solve some of the above-mentioned challenges, different manufacturing strategies have been developed. In early process steps, excess lipids (from nuts and seeds) and starch (from cereals and pseudo-cereals) are separated and/or enzymatically hydrolyzed to prevent phase separation, gelation, and increase product stability (Rustom et al. 1993). Homogenization is used to disrupt bigger particles and lipid droplets and uniform the particle size, which also improves stability (Briviba et al. 2016).

In order to overcome the known nutritional and sensory limitations, commercial plant-based milk alternatives are typically supplemented with sweeteners, artificial flavors, protein, amino acids, minerals (Ca^{2+}, Mg^{2+}, Fe^{2+}, Zn^{2+}), and vitamins (B_{12}, B_2, D, and E) (Sethi et al. 2016; Zhang et al. 2007). Moreover, extended mechanical and thermal pre-processing (e.g. roasting, dehulling, blanching, soaking, cooking, sprouting, microwaving, cold plasma) is applied to reduce anti-nutrients such as protease inhibitors (Jiang et al. 2013; Yuan et al. 2008), decrease, reduce, and mask off-flavor (Chang et al. 2019; Han et al. 2019; Jain et al. 2013; Varghese and Pare 2019), and improve mouthfeel and color (Dakwa et al. 2005; Kim et al. 1986). However, some anti-nutrients are very resistant. For example, phytates cannot be destroyed entirely even by heating to 100°C (Anderson and Wolf 1995).

2.3 Fermentation of plant materials

Leading food and beverage companies have committed to removing ingredients perceived as artificial from their products—clean label foods and beverages are not only a trend but are seemingly becoming an expectation. Accordingly, natural plant-based milk alternatives, which meet the nutritional quality and taste of animal-derived milk without blending, are of particular interest (Asioli et al. 2017). An

appealing option to reach this goal is fermentation. Since the early days of mankind, fermentation has been a natural approach to producing food, and today, fermented foods are more popular than ever before (Adler et al. 2013). During the production of coffee, bread, chocolate, wine, cheese, mixed pickles, kombucha, kimchi, and sauerkraut, food-grade microbes improve the nutritional value, aroma and taste, texture, and stability of foods and beverages and contribute to their microbial safety. In particular, the application of mono-culture fermentation to food products is well understood (Leroy and De Vuyst 2004; National Research Council 1992). Fermentation is applied to cereals such as maize, wheat, rice, and sorghum for a quite long time (National Research Council 1992). Plant materials support the growth of microorganisms (Espirito-Santo et al. 2014; Peyer et al. 2016; Sethi et al. 2016). LAB, bacilli, and yeasts (e.g. *Saccharomyces*), are most widely used for plant-based fermentation (Jeske et al. 2018; Steinkraus 1997). Being studied mainly as mono-cultures, these microbes have proven properties to enhance important nutritional and/or sensory attributes.

2.3.1 Improvement of protein and amino acid quantity and quality in plant materials by fermentation

Most importantly, fermentation can increase protein content by the growth of the fermenting food-grade microbes and by making more plant protein soluble and improving amino acid composition and availability. As an example, *Bifidobacterium* significantly increased the crude protein content of soy-based drinks (Hou et al. 2000). Moreover, fermentation of soybean meal with *Lactobacillus plantarum* resulted in a beneficial increase of essential amino acids such as L-lysine (Song et al. 2008). Similar to this, the increase of protein quality and digestibility and occasionally also the L-lysine content by fermentation were also observed in plant milks from peanut, soy, cowpea, and mung bean (Frias et al. 2008a; Frias et al.

2008b; Ghosh et al. 2013; Khalil 2006; Reyes-Moreno et al. 2004; Sanni et al. 1999; Santos et al. 2014).

2.3.2 Improvement of vitamin content in plant materials by fermentation

Notably, specific microbial strains synthesize vitamins during fermentation (LeBlanc et al. 2013), including vitamin K (Bentley and Meganathan 1982) and vitamins of the B group (LeBlanc et al. 2011). For example, yeast is well known for its ability to produce vitamin B_2 (Lindegren 1945). Remarkably, only a few bacteria can make vitamin B_{12} (Survase et al. 2006). The vitamin contains cobalt which is therefore ultimately required for biosynthesis (Roth et al. 1996). In addition, the lower-ligand B_{12} precursor dimethylbenzimidazole (DMBI), its starter unit riboflavin (vitamin B_2), nicotinamide (vitamin B_3), catalytically involved in DMBI formation, and oxygen availability have been shown to support vitamin B_{12} production (Hörig and Renz 1980; Roth et al. 1996), underlining the complexity of the biosynthetic process. *Propionibacterium freudenreichii* is the only generally regarded as safe (GRAS) approved bacterium, known to synthesize active B_{12} (Chamlagain et al. 2016), with a wide application in foods.

Compared to synthetic fortification, fortification by naturally vitamin-producing microorganisms is widely recognized as safer, more natural, and more environmentally friendly (LeBlanc et al. 2011).

2.3.3 Decrease of anti-nutrient content and improvement of mineral availability of plant materials by fermentation

Fermentation by itself or combined with other treatments like cooking, sprouting, and soaking can dramatically reduce the level of anti-nutrients such as tannins, phytates, and cyanides in plant-based food (Anderson and Wolf 1995; Onyesom et al. 2005; Soetan and Oyewole 2009; Wang et al. 2003). LAB are capable to produce phytases and furthermore provide the optimum pH conditions for these enzymes,

which then catalyze the hydrolysis of phytates into *myo*-inositol and phosphate, improving digestibility and increase of mineral bioavailability (Rekha and Vijayalakshmi 2010). As an example, fermentation of finger millet significantly reduced various undesired anti-nutrients (phytates, tannins, trypsin inhibitor), while simultaneously enhancing mineral extractability and digestibility (Antony and Chandra 1998).

2.3.4 Improvement of the bioactive component concentration and bioavailability in plant materials by fermentation

Fermentation is capable of increasing the concentration or bio-accessibility of functional (bioactive) compounds. The fermentation of soy using bacteria with β-glucosidase ability, enables the conversion of glucoside isoflavones into aglycone isoflavones of higher bio-activity and bio-accessibility (Pyo et al. 2005), which is also observed for seeds of kerandang, a flowering plant belonging to the legume family (Titiek et al. 2013). Correspondingly, *L. plantarum* is able to transform sesaminol tri-glucoside of sesame milk into bioactive sesaminol aglycone with enhanced radical scavenging activity (Ulyatu et al. 2015). It was also reported that LAB fermentation of soy releases bioactive peptides, which inhibit angiotensin-converting enzymes, related to desired antihypertensive effect (Hou et al. 2000).

2.3.5 Improvement of sensory profile of plant materials by fermentation

Fermentation can improve the sensory profile of plant-based milk alternatives (Mital and Steinkraus 1979). As an example, microbial fermentation decreased the beany flavor of plant material, probably due to deprivation of *n*-hexanal and n-hexanol (Wang et al. 2003). In addition, fermentation forms preferred volatile flavors. For example, diacetyl (2,3-butanedione), which provides a nice, butterscotch-like aroma, is emitted during cereal-based fermentation (Peyer et al. 2016). Acetaldehyde, delivering a pungent, fruity (green apple) flavor with sweet notes, increases in

concentration in peanut, cereal and soy during fermentation (Horáčková et al. 2015; Lee and Beuchat 1991; Sethi et al. 2016). The flavor and taste of plant-based milk alternatives are also affected by changes in the level of amino acids with taste profiles (Yamanaka et al. 1970). Many LAB species have been proven valuable to contribute to improved sensory profiles. As an example, species of *Streptococcus, Lactococcus, Lacticaseibacillus*, *Lactiplantibacillus*, *Lactobacillus, Limosilactobacillus,* and *Leuconostoc* affected the flavor of cereal-based milk (Kumar et al. 2020; Shin and Han 2015), chickpea milk (Tangyu et al. 2021), sunflower seed milk (Tangyu et al. 2022), soy milk (Beasley et al. 2003; Blagden and Gilliland 2005), mung bean milk (Liang et al. 2022), cowpea milk (Sanni et al. 1999), pea-based materials (Pei et al. 2022; Yousseef et al. 2016), as well as fruity and vegetable juice (Cui et al. 2019).

2.4 Mixed-culture fermentation can provide synergistic effects to enhance quality

An interesting and obviously potent concept uses mixed cultures to ferment plant materials. Generally, interactions between microbes in mixed cultures are numerous and complex, for example: competition (-/- interaction), mutualism (+/+ interaction), commensalism (+/0 interaction), amensalism (-/0 interaction), and parasitism (+/- interaction) (Sieuwerts et al. 2008). Desired interactions during mixed-culture fermentation mainly are of mutualistic and commensalistic nature, by which beneficial activities of at least one microbe are promoted (National Research Council 1992).

The cooperation between *Streptococcus thermophilus* and *Lactobacillus delbrueckii* subsp. *bulgaricus* during yogurt fermentation is a well-understood example of mutualism. The proteolytic *Lactobacillus* strain benefits non- proteolytic *S.*

thermophilus through the release of peptides and free amino acids as nitrogen sources. Conversely, *S. thermophilus* provides *L. delbrueckii* with growth-stimulating factors such as formic acid, pyruvic acid, folic acid, and carbon dioxide (Sieuwerts et al. 2008). In a mixed culture, the two strains stimulate each other in growth, acid production, and volatile compound formation (Sieuwerts et al. 2008). A synergistic effect on growth has also been observed for soy fermentation (Chumchuere and Robinson 1999), probably related to the different glycolytic activities of the strains involved. More specifically, microbial consortia can cooperate to enable multistep bio-transformations. A prominent example demonstrated a close cooperation of microbes in simulated cocoa pulp fermentation: LAB form lactate, while yeasts form ethanol during the early stage of the fermentation. Both nutrients in turn are crucial co-substrates for acetic acid bacteria, which then accumulate acetate, the key molecule to initiate the formation of aroma and flavor compounds (Adler et al. 2013). Demonstrated effects of mixed cultures comparing with mono-

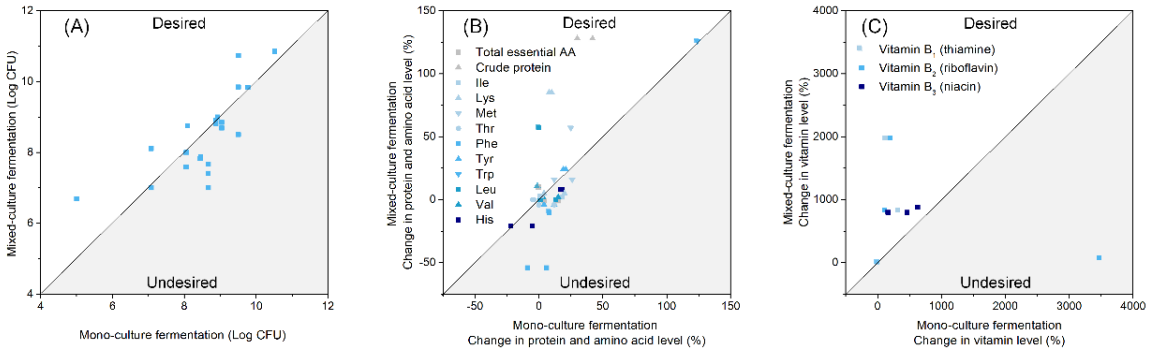

Fig. 5. Impact of mixed-culture fermentation on the quality of plant-based milk alternatives. Comparison between mixed-culture and mono culture fermentation: microbial growth (A); essential amino acid level (B), vitamin level (C). The white area covers desired synergistic effects of shown combinations (desired), while the grey area covers undesired effects, which results from mixed-cultures fermentation (undesired).

culture fermentation for the generation of plant-based milk alternatives are summarized in **Fig. 5** and **Fig. 6** and are described in more detail below.

2.4.1 Growth of strains during mixed-culture fermentation of plant materials

During soy fermentation, *Lactobacillus fermentum* NRRC 207 and *L. delbrueckii* subsp. *bulgaricus* NCDO 1489 exhibited more than 100 fold higher cell numbers when mixed with *S. thermophilus*, and both strains improved the growth of *S. thermophilus* as well (Chumchuere and Robinson 1999). Such a synergetic effect was also observed by other researches (Champagne et al. 2009; Mital et al. 1974). The combination of amylolytic and probiotic bacteria strains also reduced the fermentation time of rice by an elevated acidification rate (Espirito-Santo et al. 2014). In addition, certain yeasts benefit the growth of LAB through excreting specific nutrients (Liu and Tsao 2009; Rekha and Vijayalakshmi 2010). However, not all combinations are desired for the survival of starter cultures (Angelov et al. 2005). As an example, the viable count of *Bifidobacterium longum* R015 and *L. fermentum* decreased in specific mixed-culture fermentation (**Fig. 5A**) (Champagne et al. 2009; Chumchuere and Robinson 1999).

2.4.2 The effect of mixed-culture fermentation on protein and amino acid quantity and quality in plant materials

Protein content and essential amino acid composition can differ substantially between mono- and mixed-culture fermentation of plant-based milk alternatives (**Fig. 5B**). Co-fermentation of peanut using *Lactobacillus acidophilus* and *L. plantarum* significantly increased total protein level, and also L-lysine, L-methionine, and L-tryptophan content, as compared to the corresponding mono-culture fermentations (Sanni et al. 1999). Spontaneous co-fermentation of strains, originally existing in cowpea and chickpea, improved L-methionine levels approximately six fold (Zamora

and Fields 1979). However, in other cases, mixed-culture fermentation appeared inferior to mono-culture setups (Santos et al. 2014).

2.4.3 The effect of mixed-culture fermentation on vitamin quantity in plant materials

Moreover, mixed-cultures can impact vitamin formation. Co-fermentation of *L. plantarum* SM39 and *P. freudenreichii* DF13 showed a higher level of folate and vitamin B_{12}, which yielded up to 8400 ng L^{-1} of folate, otherwise only achievable with genetically modified strains (Hugenschmidt et al. 2011; Smid and Lacroix 2013). So far, only a few pioneering studies have investigated bacterial vitamin production during plant material fermentation (**Fig. 5C**). A mixture of *Lactobacillus* strains including *L. plantarum* improved riboflavin, thiamine, and niacin contents in cowpea milk, as compared to the mono cultures (Sanni et al. 1999). Similar effects were observed for soy, using a co-fermentation of *Saccharomyces boulardii* and *Lactobacillus casei* (Rekha and Vijayalakshmi 2010). However, many of the tested strain combinations only showed little or even adverse effects (Champagne et al. 2010; Zamora and Fields 1979). It has been speculated that the decrease in a specific vitamin may relate to the fact that the organism itself requires it for growth (Rekha and Vijayalakshmi 2010).

2.4.4 The effect of mixed-culture fermentation on decrease of anti-nutrients and increase of mineral availability of plant materials

Interestingly, mixed-cultures help to reduce anti-nutrients (**Fig. 6A**), which in turn enhances mineral availability (**Fig. 6B**). A mixed culture of *L. acidophilus* and *L. plantarum* was more effective than fermentation of the individual strains to eliminate phytic acid and trypsin inhibitor in cowpea (Sanni et al. 1999). Similarly, a mixed-culture of *S. thermophilus* CCRC 14085 *and Bifidobacterium infantis* CCRC 14603 dramatically decreased phytic acid (80%) and saponin (30%) in soy (Lai et al. 2013).

It was further found that a mixed *S. boulardii* and *L. plantarum* B4495 fermentation induced calcium bioavailability approximately six fold, as compared to the mono-culture fermentation (Rekha and Vijayalakshmi 2010).

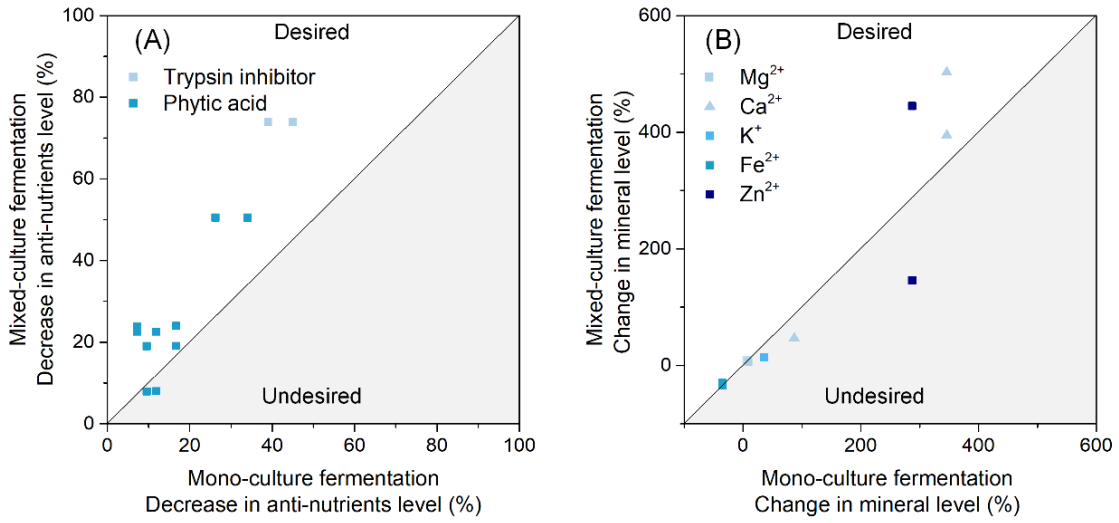

Fig. 6. Impact of mixed-culture fermentation on the quality of plant-based milk alternatives. Comparison the effect between mixed-culture and mono culture fermentation on eliminating anti-nutrients (A) and altering mineral contents (B).

Stachyose and raffinose are undesired flatulence factors of plant-based milk alternatives, especially legume-based (Desai et al. 2002). Generally, a combination of different strains was more efficient to degrade these carbohydrates than the pure cultures (Santos et al. 2014). Soy fermented by a mixed-culture of different LAB yielded a lower level of stachyose and raffinose and a preferred higher content of acetic acid, fructose, glucose plus galactose (Wang et al. 2003). Similar effects were also observed in other studies (Santos et al. 2014). However, the outcome is strongly dependent on the exact strain combination. As an example, *Streptococcus* induced adverse effects in *L. fermentum* and *Bifidobacterium longum* to metabolize stachyose (Champagne et al. 2009; Chumchuere and Robinson 1999).

2.4.5 The effect of mixed-culture fermentation on bioactive component concentration and bioavailability in plant materials

Fermentation of soy by bacteria, which possess β-glucosidase activity, enables biotransformation of isoflavones into the more bio-active aglycone form (Pyo et al. 2005). Mixed-cultures of *S. boulardii* together with five *Lactobacillus* species converted over 95% of the glucoside into the aglycone isoflavone (Rekha and Vijayalakshmi 2010). However, other strain mixtures revealed lower bioconversion efficiency as compared to pure cultures. For example, a weaker bioconversion was detected, when *S. thermophilus* was mixed with *B. infantis, B. longum,* and *Lactobacillus helveticus* (Champagne et al. 2010; Chien et al. 2006). This observation emphasizes again the importance of strain selection in mediating synergetic effects between the different cultures.

2.4.6 The effect of mixed-culture fermentation on sensory values of plant materials

Although mono-culture fermentation seems as efficient as mixed-culture fermentation in lowering the content of the off-flavor molecules *n*-hexanal and *n*-hexanol (Lee 2001), mixed fermentation appears more potent in generating preferred flavor enhancers. For example, acetaldehyde, a key compound of the desired yogurt flavor, is formed more extensively by a mixture of two or more cultures (Horáčková et al. 2015; Lee 2001; Liu et al. 2002). A mixed-culture of *L. delbrueckii* subsp*. bulgaricus* and *Streptococcus salivarius* subsp*. thermophilus* not only decreased the beany flavor of peanut milk, but also significantly increased whiteness, viscosity, gumminess, and smoothness (Lee 2001). An increase in luminosity and whiteness index values was also observed for almond fermentation by a mixed-culture of *Lactobacillus reuteri* and *S. thermophilus* (Bernat et al. 2015).

3 Material and Methods

3.1 Microorganisms

All microbial strains used in this work with NCC number were obtained from the NCC (Nestlé Culture Collection, Lausanne, Switzerland) (**Table 2**). *P. freudenreichii* DSM 4902 was obtained from the German Collection of Microorganisms and Cell Cultures (DSMZ, Braunschweig, Germany). All strains were food-grade approved based on the qualified presumption of safety (QPS) recommendation (EFSA Panel on Biological Hazards et al. 2017). They were maintained as frozen stocks in 30% glycerol (v/v) at -80°C.

3.2 Genome-based strain selection

The gene sequence information for strain selection was obtained from the NCC genome database (Gangoiti et al. 2017). The database was screened for the presence and absence of genes of interest using automated annotation. A proprietary database of enzyme domain profiles was used to screen all NCC genome proteins using NCBI's Reverse Position Specific Basic Local Alignment Search Tool (RPS-BLAST) algorithm, constrained by minimum query coverage (60%), minimum hit coverage (60%, (Marchler-Bauer et al. 2003). A particular enzyme domain was regarded as present when the corresponding E-value was below 1E-3.

3.3 Medium

3.3.1 Strain-specific pre-culture media

Depending on individual nutrient requirements, different media were used for pre-cultivation of the different strains (**Table 2**). *Limosilactobacillus reuteri, Limosilactobacillus fermentum,* and all *Propionibacterium*, *Lentilactobacillus*, *Levilactobacillus*, *Lacticaseibacillus*, *Lactiplantibacillus*, *Lactiactobacillus*, *Lactobacillus*, and *Leuconostoc*, were grown in de Mann-Rogosa-Sharpe (MRS) medium, containing per liter: 52.0 g of MRS broth (Sigma-Aldrich, Steinheim, Germany) and 1.0 mL of Tween-80 (Sigma-Aldrich) (De Man et al. 1960).

Fructilactobacillus sanfranciscensis and *Limosilactobacillus pontis* were grown in sanfrancisco medium (Vogel et al. 1994), which was modified in this work and contained 10.0 g of tryptone (Becton Dickinson, Franklin Lakes, NJ, USA), 7.0 g of yeast extract (Becton Dickinson), 7.0 g of glucose (Sigma-Aldrich), 7.0 g of fructose (Amresco, Solon, OH, USA), 7.0 g of maltose monohydrate (Sigma-Aldrich), 5.0 g of sodium acetate trihydrate, 5.0 g of di-ammonium hydrogen citrate, 2.5 g of KH_2PO_4, 2.0 g of Lab Lemco powder (Oxoid, Thermo Fisher, Rockford, IL, USA), 2.0 g of sodium gluconate, 0.5 g of L-cysteine hydrochloride, 0.4 g of $MgSO_4 \cdot 7H_2O$, 0.1 g of $MnSO_4 \cdot H_2O$, 0.05 g of $FeSO_4 \cdot 7H_2O$, and 1.0 mL of Tween-80 per liter.

All *Bifidobacterium* strains were grown in MRS-Cys broth containing 52.0 g of MRS broth, 0.5 g of cysteine hydrochloride, and 1.0 mL of Tween-80 (Sigma-Aldrich) per liter.

The strains of *Bacillus* were grown in modified tryptic soy broth (TSB) containing 17.0 g of tryptone (Becton Dickinson), 5.0 g of NaCl, 3.0 g of soytone (Becton Dickinson), 2.5 g of K_2HPO_4, and 1.0 mL of 30% silicone antifoam (Sigma-Aldrich) per liter.

Table 2. Growth conditions and media used to pre-culture the different strains.

Strains	Growth medium	T (°C)	Application*
Anaerobic cultivation			
Fructilactobacillus sanfranciscensis NCC 463	mHH	30	1
F. sanfranciscensis NCC 2572	mHH	30	1
F. sanfranciscensis NCC 2629	mHH	30	1
Limosilactobacillus pontis NCC 380	mHH	30	1
Bifidobacterium infantis NCC 365	MRS cystein	37	1
B. longum subsp. *infantis* NCC 283	MRS cystein	37	2
Propionibacterium freudenreichii NCC 1124	MRS	30	2
P. freudenreichii NCC 1138	MRS	30	2
P. freudenreichii NCC 1145	MRS	30	2
P. freudenreichii NCC 1151	MRS	30	2
P. freudenreichii NCC 1159	MRS	30	2
P. freudenreichii NCC 1177	MRS	30	2
P. freudenreichii NCC 1186	MRS	30	2
P. freudenreichii NCC 1197	MRS	30	2
P. freudenreichii NCC 1230	MRS	30	2
P. freudenreichii NCC 1236	MRS	30	2
P. freudenreichii DSM 4902	MRS	30	2
P. freudenreichii NCC 1124	MRS	30	2
Aerotolerant cultivation			
Lactobacillus helveticus NCC 1182	MRS	40	1
L. helveticus NCC 1104	MRS	40	1
L. helveticus NCC 158	MRS	40	1
L. helveticus NCC 1276	MRS	30	3
L. delbrueckii subsp. *bulgaricus* NCC 621	MRS	37	1
L. johnsonii NCC 2774	MRS	37	1
L. johnsonii NCC 2822	MRS	37	1
L. johnsonii NCC 2767	MRS	37	1
L. acidophilus NCC 2766	MRS	37	1
L. jensenii NCC 2867	MRS	37	1
Lentilactobacillus hilgardii NCC 1497	MRS	30	1
Lactiplantibacillus plantarum NCC 1385	MRS	30	1
L. plantarum NCC 2988	MRS	30	3
L. johnsonii NCC 533	MRS	37	3
Levilactobacillus brevis NCC 372	MRS	30	1
Limosilactobacillus reuteri NCC 1945	MRS	40	1
L. reuteri NCC 2613	MRS	40	1
L. reuteri NCC 2823	MRS	37	1
L. fermentum NCC 660	MRS	37	3
Lacticaseibacillus paracasei NCC 2537	MRS	30	1
L. paracasei subsp. *paracasei* NCC 2511	MRS	30	1, 2, 3
L. rhamnosus NCC 2891	MRS	37	3

L. rhamnosus NCC 4007	MRS	37	3
Streptococcus thermophilus NCC 1326	HJL	37	3
S. thermophilus NCC 1988	HJL	37	3
S. thermophilus NCC 2019	HJL	37	3
S. thermophilus NCC 2059	HJL	37	3
Lactococcus lactis NCC 2180	HJL	30	3
L. lactis NCC 2242	HJL	30	3
Latilactobacillus sakei NCC 1692	MRS	30	3
Leuconostoc mesenteroides NCC 2832	MRS	30	3
Aerobic cultivation			
Bacillus amyloliquefaciens NCC 156	BST	40	1, 2
B. amyloliquefaciens NCC 2770	BST	40	1
B. subtilis NCC 199	BST	40	1
B. licheniformis NCC 2940	BST	40	1
B. flexus NCC 2902	BST	40	1
B. flexus NCC 2903	BST	40	1
B. pumilus NCC 2962	BST	40	1
Corynebacterium stationis NCC 3013	BHI	30	1
C. stationis NCC 3016	BHI	30	1

*1, Strains used in l-lysine study (**Chapter 4.1**); 2, Strains used in vitamin B12 study (**Chapter 4.2**); 3, Strains used in flavor study (**Chapter 4.3**).

Corynebacterium isolates were cultivated in brain heart infusion cysteine (BHI-Cys) broth containing 37.0 g of brain heart infusion (Becton Dickinson) and 0.5 g of L-cysteine hydrochloride per liter.

Streptococcus and *Lactococcus* strains were grown in HJL medium, adjusted to a final pH value of 6.5, and containing per liter: 30 g of tryptone (Becton Dickinson), 10 g of yeast extract (Becton Dickinson), 10 g of lactose (Sigma-Aldrich), 2 g of beef extract (Life Technologies Corporation, Detroit, MI, USA), and 5 g of KH_2PO_4 (Stingele et al. 1996).

3.3.2 Differential agar media

Lactic acid bacteria differential (LBD) agar was used to differentiate *P. freudenreichii* strains from *L. paracasei* subsp. *paracasei* NCC 2511. It contained per liter: 10.0 g of casein enzymatic hydrolysate (Becton Dickinson), 3.0 g of casein acid

hydrolysate (Merck, Darmstadt, Germany), 1.5 g of enzymic digest of soybean meal (Becton Dickinson), 1.0 g of yeast extract (Becton Dickinson), 2.5 g of fructose (Amresco), 2.5 g of K_2HPO_4 (Sigma-Aldrich), 55 mg of bromocresol green (Sigma-Aldrich), and 15.0 g of agar (Becton Dickinson) (Adler et al. 2013). In addition, TSB agar was used to grow *B. amyloliquefaciens* NCC 156 for the estimation of colony forming units. It contained per liter: 17.0 g of tryptone (Becton Dickinson), 5.0 g of NaCl, 3.0 g of soytone (Becton Dickinson), 2.5 g K_2HPO_4, and 1.0 mL of 30% silicone antifoam, and 15.0 g of agar (Becton Dickinson) (Tangyu et al. 2021).

3.3.3 Plant-based milk alternative medium

Chickpea milk medium

A chickpea suspension was prepared by mixing 10% (w/w) chickpea flour (E. Zwicky AG, Müllheim-Wigoltingen, Switzerland) with deionized water. A two-step heat treatment was applied for sterilization. First, the suspension was stirred (250 rpm, 2 h, 75°C), which was followed by autoclaving (121°C, 15 min). Prior to fermentation, the suspension was manually homogenized. In ^{13}C tracer experiments, 98% [$^{13}C_4$] L-aspartate (Eurisotop, Gif-Sur-Yvette, France) was added to a final concentration of 11 mM, whereas controls received the same amount of naturally labeled L-aspartate. In further experiments, selected nutrients were added to chickpea milk from sterilized stocks before fermentation to investigate their impact: (i) citrate (10 mM), (ii) a mixture of the branched-chain amino acids valine, leucine, and isoleucine (5 mM each), and (iii) volatile aldehydes such as pentanal, hexanal, heptanal, octanal, nonanal, and benzaldehyde (5 mM each in separate experiments).

Low-pressure-pasteurized (LPP) sunflower seed milk medium

A suspension of sunflower seed press cake extract (6.8% w/w) was prepared by mixing 68 g of sunflower seed protein (All Organic Treasures) with 1 liter of deionized water. The suspension was pasteurized at 90°C for 6 h, while the

temperature was monitored. Then, the milk was aseptically filled into sterile plastic bottles (2 L) and kept at 4°C until use. Prior to cultivation, the LPP sunflower seed milk was manually homogenized. In selected supplementation studies, the pasteurized and homogenized sunflower seed milk was supplemented with one or several of the following additives: $CoCl_2$ (50 µM), riboflavin (40 µM), nicotinamide (27 mM), DMBI (100 µM), L-glutamate (0.6 mM), L-threonine (0.1 mM), glycine (0.3 mM), succinate (0.5 mM), lactate (1% w/w), and/or glucose (1% w/w). These were added from filter-sterilized stocks to the final concentrations given above. Non-inoculated controls were incubated under the same conditions and evaluated for sterility by plating on TSB and LBD agar.

Ultra-high-temperature (UHT) plant milk medium

Plant milk suspension was prepared by mixing the plant material with deionized water. The suspension was then homogenized and pre-heated to 75°C, immediately followed by UHT treatment in an automatized pilot-scale tubular heat exchanger (HT320 (ID920), OMVE, De Meern, The Netherlands). The suspension was homogenized and pre-heated to 75°C, immediately followed by continuous ultra-high temperature (UHT) treatment Hereby, the pre-warmed suspension was heated for 4 seconds to 143°C at a flow rate of 30 L h^{-1} and then efficiently cooled down to 4°C. All sterilization parameters were controlled by the integrated process control system. Finally, the milk was aseptically filled into sterile plastic bottles (2 L) and kept at 4°C until use. Prior to fermentation, the UHT plant milk was manually homogenized.

3.4 Cultivation

3.4.1 Pre-cultures

Strain specific-settings (medium and temperature) were used to propagate pre-cultures (**Table 2**). Anaerobic strains were grown anaerobically at in 20 mL tubes,

containing 10 mL of corresponding medium. For the first pre-cultivation, the tubes were inoculated from a glycerol stock (200 µL) and were then incubated overnight under a CO_2-enriched atmosphere (9-13%) (Anaerobic atmosphere generation bags, Merck). The first pre-culture of aerotolerant strains was grown at 30°C in 20 mL tubes, containing 10 mL of specific medium. For cultivation, the tubes were inoculated from a glycerol stock (200 µL), closed, and then incubated overnight, mimicking microaerobic conditions. Aerobic strains were grown aerobically. For this purpose, the microbe was cultivated overnight in 100 mL non-baffled shake flasks, filled with 10 mL growth medium, inoculated from a glycerol stock (200 µL), and incubated on a rotary shaker (30 ° C, 130 rpm, 80% humidity, Infors, Bottmingen, Switzerland). Independent of the strain, cells from the first pre-cultures were collected by centrifugation (5,000 ×*g*, 5 min, 4°C) and used as inoculum for a second pre-culture, which was then grown overnight under the same conditions, then served as inoculum for the main culture. In the flavor study, the second pre-cultures of all strains were cultivated anaerobically as described to increase their adaptability in anaerobic main-fermentation.

Seven selected strains were analysed for the correlation between optical density (OD) and colony forming units (cfu) during pre-cultivation. This yielded the following correlation factors f = cfu (OD_{600}^{-1}) during the exponential growth phase: 8.2E8 cfu (OD_{600}−1) (NCC 1177), 5.3E8 cfu (OD_{600}^{-1}) (DSM 4902), 8.2E8 cfu (OD_{600}^{-1}) (NCC 1197), 1.9 E8 cfu (OD_{600}^{-1}) (NCC 1186), 3.8E8 cfu (OD_{600}^{-1}) (NCC 1138), 2.0E8 cfu (OD_{600}^{-1}) (NCC 2511), and 3.6 E7 cfu (OD_{600}^{-1}) (NCC 156). These factors were used to infer the required amount of pre-culture needed to prepare an inoculum of desired size (see below).

3.4.2 Screening for vitamin B_{12} production

Strains of *P. freudenreichii* were grown in basic MRS medium and in supplemented MRS medium, additionally containing $CoCl_2$ (50 µM) and DMBI (100 µM). Dual-phase processes were conducted. Cultivation included a first anaerobic phase in 100 mL glass bottles (filled with 40 mL medium) which were inoculated from glycerol stocks and then incubated for 48 hours in anaerobic jars under CO_2 (9-13%). Afterward, the broth was transferred to 100 mL baffled shake flasks and incubated aerobically for further 24 hours on a rotary shaker (130 rpm, 80% humidity, 5 cm shaking diameter, Infors). Three biological replicates were carried out for each condition.

3.4.3 Chickpea milk fermentation

For screening of L-lysine production, 400 µL of the second precultures were collected and was used as inoculum for the main chickpea milk fermentation. Subsequently, *L. paracasei.* subsp. *paracasei* and *B. amyloliquefaciens* were analyzed for the correlation between optical density and colony forming units (cfu). The ideal amount of inoculum containing 2E7 cfu mL^{-1} of cells was used for the main culture for a detailed study. Depending on the studied microbe, the main fermentation was then conducted in 20 mL of medium in 200 mL glass bottles under a CO_2 atmosphere (obligate anaerobes) or ambient air (aerotolerant) and in 100 mL non-baffled shake flasks (obligate aerobes).

Potential evaporation loss during fermentation was monitored using non-inoculated controls and used to correct the obtained data (Schwechheimer et al. 2018a). The non-inoculated cultures further served as controls to verify the sterility of the raw material and monitor the occurrence of abiotic changes that potentially altered the composition. In selected experiments, the pH value and dissolved oxygen (DO) level were monitored in shake flasks with immobilized sensor spots and fluorescence

detection (PreSens Precision Sensing GmbH, Regensburg, Germany) (Wittmann et al. 2003). Each fermentation was conducted as biological triplicates.

3.4.4 Sunflower seed milk fermentation for vitamin B_{12} production

Cultivations were generally conducted at 30°C in sunflower seed milk, while other conditions were varied for optimization and testing, as described. Anaerobic incubations were done in 100 mL glass bottles, filled with 40 mL medium, and incubated in anaerobic jars under CO_2 (9-13%). Aerobic incubations were conducted in 100 mL non-baffled shake flasks, filled with 40 mL medium, and incubated on a rotary shaker (80% humidity, 5 cm shaking diameter, Infors). The shaking rate was usually set to 130 rpm but changed in selected experiments as described. Finally, dual-phase setups were conducted. After a first anaerobic bottled phase in the jar system, the broth was transferred to a shake flask for a second aerobic phase. The incubation time was varied as given below. The processes were inoculated as follows. The optical density of the corresponding pre-culture was measured. The obtained correlation between optical density and cfu (see above) was then used to calculate the pre-culture volume that contained the desired colony forming units for inoculation. This volume was collected, followed by washing the cells with water. Then, cells in the suspension were counted using a Neubauer counting chamber. On basis of the estimated cell concentration, an appropriate volume was then used as inoculum. The initial cell concentration was adjusted to 10^7 cells mL^{-1}, unless stated otherwise. In addition to monocultures, co-cultures were co-inoculated from individual pre-cultures in the same manner. Here, cells were added at different ratios, ranging from 1:1 to 1000:1, as given below. The corresponding conditions for each experiment are specified in the results section. Three biological replicates were carried out for each experiment. To minimize the impact of periodical sampling on culture conditions during time-resolved investigations, three complete incubations were sacrificed per data point, and

evaporation of culture volume was considered for data correction, as above described.

3.4.5 Oat milk, sunflower seed milk, pea milk, and faba milk fermentation for flavor studies

Anaerobic fermentations were conducted in 100 mL serum bottles (Becker et al. 2013; Lange et al. 2017), filled with 15 mL of the corresponding plant milk. The cultures were inoculated to a starting cell concentration of 2E7 colony formation units (cfu) mL^{-1}. The bottles were tightly sealed with an aluminum cap. Then, the air in the headspace was replaced by nitrogen (Becker et al. 2013; Lange et al. 2017). The inoculated plant milks were incubated on a rotary shaker (30°C, 130 rpm, 80% humidity, 5 cm shaking diameter, Infors). Three biological replicates were carried out for each fermentation, and non-inoculated fermentations were conducted as control.

3.5 Analytical methods

3.5.1 Quantification of colony forming units

Colony forming units (cfu) were determined by the plate serial dilution spotting method (Tangyu et al. 2021). Briefly, 1 mL culture samples were sequentially diluted using 0.85% NaCl (w/v), supplemented with 1.0 g L^{-1} of tryptone (Becton Dickinson). For cfu determination of *Lacticaseibacillus*, *Lactiplantibacillus*, *Lactiactobacillus*, *Lactobacillus*, and *Leuconostoc*, MRS agar was used. For cfu counting of *F. sanfranciscensis* and *L. pontis*, samples were spotted onto sanfrancisco agar. *Bifidobacterium* strains were counted on MRS-Cys agar. For *Bifidobacterium*, MRS-Cys agar was used. For cfu estimation of *Bacillus*, samples were spotted onto TSB agar. *C. stationis* was counted on BHI-Cys agar. For *Corynebacterium*, BHI-Cys

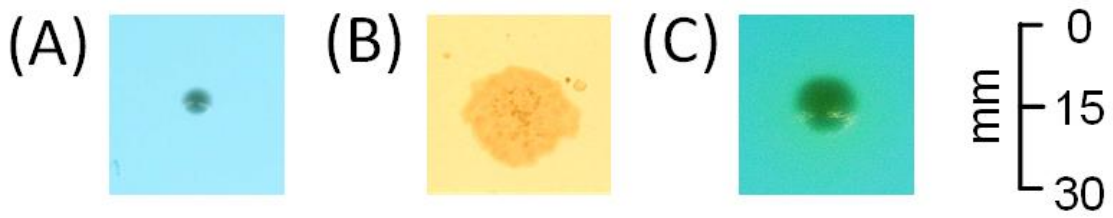

Fig. 7. The colony of strains used in the studies. *P. freudenreichii* NCC 1177 on LPD agar (A); *B. amyloliquefaciens* NCC 156 on TSB agar; *L. paracasei* subsp. *paracasei* NCC 2511 on LPD agar (C).

agar was used. For cfu estimation of *Streptococcus* and *Lactococcus*, samples were spotted onto HJL agar. For cfu determination of *P. freudenreichii* and *L. paracasei* LBD agar was used. On this agar, the strain-specific shape and color of colonies formed allowed for a clear differentiation (**Fig. 7**). All measurements were conducted in duplicate.

3.5.2 Quantification of moisture

Samples of 2 g were freeze-dried for 48 h. Afterward, the remaining total solids were quantified by measuring the residual weight.

3.5.3 Quantification of protein and amino acid

For the quantification of total amino acids, samples were homogenized (2 mL Precellys Lysing Kits filled with 1.4 mm ceramic balls, Bertin, Montigny-le-Bretonneux, France) using a homogenizer (5000 rpm, 3 × 90 s with 30 s breaks on ice in between). Approximately 10 mg of a homogenized sample was then hydrolyzed (24 h, 6 M HCl, 100°C), dried, resuspended in deionized water, and filtered (0.22 µm, Millipore, Merck, Darmstadt, Germany). The obtained amino acids were analyzed by HPLC (Agilent 1100 Infinity, Agilent Technologies, Waldbronn, Germany) with pre-column derivatization with *ortho*-phthaldialdehyde and 2-aminobutyrate as an internal standard (Krömer et al. 2005). In short, the analytes

were separated at 40°C and a flow rate of 1 mL min^{-1} on a reversed-phase column (Gemini 5 µm C18 110 Å, 150 × 4.6 mm, Phenomenex, Torrance, CA, USA) using the following gradient of eluent A (40 mM Na_2HPO4, 7.7 mM sodium azide, pH 7.8) and eluent B (45% acetonitrile, 45% methanol, 10% deionized water): 0-45% B from 0-45 min, 45-61% B from 45-47 min, 61-82% B from 47-48 min, 82-100% B from 48-48.5 min, 100% B from 48.5-50.5%, and 100-0% B from 51-53 min. The analytes were detected by fluorescence (340 nm/450 nm). L-cysteine, L-methionine, and L-tryptophan were degraded during the hydrolysis process so that these amino acids were not measurable, whereas L-glutamine and L-asparagine were converted into L-glutamate and L-aspartate, respectively, so that the obtained data reflected the lumped pools (Wittmann 2007). The total protein amount was calculated by summing up the level of the individual amino acids. For the quantification of free amino acids, 1 mL fermentation sample was centrifuged (20,000 ×*g*, 10 min, 4°C). The obtained supernatant was filtered (0.22 µm, Millipore) and analyzed as described above.

3.5.4 Quantification of sugars, organic acids, and alcohols

Disaccharides (sucrose and maltose) and larger oligomers (raffinose, stachyose, and verbascose) were quantified in filtered samples (0.22 µm, Millipore) using HPLC (Agilent 1260 Infinity Series, Agilent Technologies). The analytes were separated on a sulfonated spherical PS/DVB resin (VA 300/7.8 Nucleogel sugar Pb, Macherey-Nagel, Düren, Germany) with deionized water as the mobile phase (80°C, 0.4 mL min^{-1}) and detected via the refraction index. External standards were used for quantification. The concentrations of organic acids (lactate, acetate, pyruvate, citrate, α-ketoglutarate, isobutyrate, isovalerate) and alcohols (acetoin, ethanol, 2,3-butanediol) in filtered samples (0.22 µm, Millipore) were determined using HPLC (Agilent 1260 Infinity Series, Agilent Technologies) with ion exclusion (Aminex HPX-87H, 300 × 7.8 mm, Bio-Rad, Hercules, CA, USA), 12 mM H_2SO_4 as the mobile

phase (45°C, 0.5 mL min^{-1}), and refractive index detection (Lange et al. 2017). External standards were used for quantification.

3.5.5 Quantification of lipids

Analysis of the lipid (fat) content was conducted after hexane/isopropanol extraction (Caprioli et al. 2016). Briefly, 5 mL of hexane/isopropanol (3:2) was added to a 1 mL sample. The mixture was incubated for 24 h at 18°C and 230 rpm. After the addition of 5 mL of Na_2SO_4 (0.47 M), the extract was centrifuged at 4000 ×*g* for 10 min. The upper liquid phase was collected and extracted once more using 5 mL of hexane/isopropanol (7:2). Both lipid fractions were then combined and dried under nitrogen flow until a constant weight was reached. The remaining solids were weighed and used to calculate the lipid content.

3.5.6 Quantification of vitamin B_{12}

The analysis of vitamin B_{12} was based on the method proposed by AOAC International (AOAC 2019) which was slightly modified in this study (Giménez and Martin 2018). Briefly, 3 mL sample was mixed with 2.5 mL sodium acetate solution (0.4 M, pH 4.0) and 100 µL cyanide solution (1% w/v in deionized water), and 4.4 mL deionized water. For vitamin B_{12} extraction, the mixture was incubated for 20 min at 107 ºC. Solids were then removed by centrifugation (5000 x *g*, 30 min). A defined fraction of the obtained supernatant (3 mL) was subjected to an automated immunoaffinity clean-up (GX-271 ASPEC system, Gilson, Germany). The obtained eluate from the clean-up was evaporated to dryness and dissolved in 0.3 mL ultra-pure water (> 18.2 MΩ cm^{-1}). Subsequently, vitamin B_{12} was quantified using HPLC (Waters Acquity UPLC, Waters, Milford, MA, US) with separation on a silica bonded column (Acquity UPLC® HSS T3, 1.8 µm, 100 x 2.1 mm, Waters) using a gradient of water and acetonitrile as mobile phase and UV detection (550 nm). External

standards were used for quantification. Levels of vitamin B_{12} were given as (µg cyanocobalamin) $(100\ g)^{-1}$.

3.5.7 Quantification of thiamine, riboflavin, niacin, pyridoxine, and biotin

Samples were hydrolyzed in hydrochloric acid (100°C, 35 min), neutralized, and filtered (0.22 µm, Millipore). The subsequent analysis was based on method LI-00.610 (Bernat et al. 2015) using UHPLC-MS/MS with positive electrospray ionization and quantification via the peak area ratio of each analyte and an internal standard (Nestlé Quality Assurance Center, Dublin, Republic of Ireland).

3.5.8 Quantification of cobalt

The level of cobalt was quantified after sample work up by a high pressure asher (HPA-S High Pressure Asher, Anton Paar, IGZ Instruments, Zurich, Switzerland) using ICP-MS (Dubascoux et al. 2021; Hammer et al. 2005).

3.5.9 Estimation of sunflower seed milk protein digestibility and score

The nutritional quality of sunflower seed protein from non-fermented and fermented milk was assessed on the level of different parameters: i*n vitro* protein digestibility (IVPD), amino acid score (AAS), and *in vitro* protein digestibility corrected amino acid score (PDCAAS) (Schaafsma 2000). In short, IVPD, AAS, and PDCAAS were determined using the K-PDCAAS kit (Megazyme International, Bray, Co. Wicklow, Ireland), following the instructions of the manufacturer. Briefly, samples were sequentially digested by (i) pepsin under acidic conditions, (0.06 M HCl, pH 2), and (ii) trypsin/chymotrypsin under neutral conditions (1.0 M Tris, pH 7.4) to simulate the physiological conditions of gastric and intestinal digestion. Undigested protein was precipitated by the addition of trichloroacetic acid (40% w/v) and was then removed by centrifugation (15,000 ×*g*, 10 min). The amount of reactive α-amino acids obtained after the treatmentwas quantified after derivatization with ninhydrin into a

purple dye via absorbance measurement at 570 nm. From the data, the IVPD was determined by correcting for the relative reactivity of certain α -amino acids (L-proline, L-lysine, L-histidine, L-arginine). The AAS was inferred from the ratio between the amino acid amount in the sample and the amount recommended by the FAO (Jeske et al. 2018). Finally, the PDCAAS was calculated by multiplying AAS and IVPD.

3.5.10 Mass isotopomer analysis by GC-MS

In isotopic tracer experiments, the ^{13}C labeling patterns of L-lysine and L-aspartate were analyzed using GC-MS (Schwechheimer et al. 2018a). For ^{13}C labeling analysis of the total amino acids, fermentation samples were treated as described above. Approximately 10 µL of a homogenized sample was hydrolyzed (24 h, 6 M HCl, 100°C), filtered (0.22 µm, Millipore), dried under a nitrogen flow, and dissolved in 50 µL of *N,N*-dimethylformamide (1% (v/v) pyridine). For ^{13}C analysis of free L-lysine, a 1 mL sample was centrifuged (20,000 ×*g*, 10 min, 4°C). An aliquot of 50 µL of the obtained supernatant was filtered (0.22 µm, Millipore), dried under a nitrogen flow, and dissolved in 50 µL of *N,N*-dimethylformamide (1% (v/v) pyridine). The amino acids were then derivatized at 80°C for 30 min into the corresponding *t*-butyldimethylsilyl derivatives using 50 µL of *N*-methyl-*t*-butyldimethylsilyl-trifluoroacetamide (MBDSTFA, Macherey-Nagel). Mass spectrometric analysis was conducted on a GC-MS instrument (Agilent 5977 A MSD, Agilent Technologies) equipped with an HP-5MS column (30 m, 0.250 mm, 0.25 µm, Agilent Technologies) using helium as the carrier gas (1.7 mL min^{-1}) and the following temperature gradient: 120°C (0-2 min), 8°C min^{-1} (2-12 min), 10°C min^{-1} (12-24.5 min), and 325°C (24.5-27 min).

3.5.11 Headspace solid-phase micro extraction (HS-SPME)

Approximately 5 mL broth was collected into a 20 mL vial, supplemented with 1 g NaCl, and incubated for 20 min at 40°C and 400 rpm in the dark (PAL RSI 120, CTC Analytics, Zwingen, Switzerland). Afterward, the contained volatiles were trapped onto an SPME fiber for another 20 min at 40°C, using two fiber types, divinylbenzene/polydimethylsiloxane (65 µm, DVB/PDMS, Agilent Technologies) and divinylbenzene/carboxen/polydimethylsiloxane (50/30 µm, DVB/CAR/PDMS, Agilent Technologies), respectively. A blank sample (an empty vial) was run before each sequence to ensure the quality of the fiber and check for potential background interference. The fibers were replaced every 50 measurements to exclude memory effects.

3.5.12 Gas chromatography-mass Spectrometry (GC-MS) analysis of volatile flavor compounds

Volatiles were analysed by GC-MS (Agilent Technologies 8890 GC system), as described previously (Tangyu et al. 2021; Tangyu et al. 2022). Prior to injection, the loaded SPME fibre (see above) was transferred into the injector port (250°C) and maintained there for a 3 min desorption interval. The injection was then operated split-less. The volatiles were separated on an HP-5MS column (30 m, 250 µm, 0.25 µm, Agilent Technologies), using helium as carrier gas (0.5 mL min^{-1}). Chromatograms were recorded by monitoring the total ion current (TIC) over a mass range from 30 to 300 m/z, followed by deconvolution of the obtained signals (Agilent Chemstation, Agilent Technologies).

3.6 Data analysis

3.6.1 Processing of ^{13}C data

The [M-57] ion cluster of the derivatized amino acids (*m/z* 431 for L-lysine, *m/z* 418 for L-aspartate) was selected to determine the ^{13}C labeling pattern, as it represented the entire carbon backbone (Wittmann 2007). All samples were first measured in scan mode to exclude isobaric matrix interference with the analytes of interest and verify that the selected ion clusters were suited for ^{13}C quantification. Subsequently, labeling patterns were determined in duplicate using selective ion monitoring. The derived mass isotopomer distributions were corrected for natural isotopes (van Winden et al. 2002) and used to derive the SFL (Schwechheimer et al. 2018a). The SFL expressed the labeling pattern as the average ^{13}C enrichment of the included carbon. For method validation, naturally labeled samples were treated and analyzed.

3.6.2 Identification and grouping of volatile compounds

Detected volatiles were identified in two ways: First, the corresponding mass spectra were suspected to a mass spectra library search (NIST/EPA/NIH Mass Spectral Library 08). Compounds were regarded as identified when a match score > 75% to the corresponding library entry was obtained. For several analytes, additional conformation was obtained by analyzing external standards: hexanal, nonanal, benzaldehyde, phenylacetaldehyde, pentanal, octanal, 1-pentanol, 1-hexanol, and 1-octanol.

For data evaluation, volatiles were categorized chemically and functionally. On one hand, chemical groups distinguished between aldehydes, alcohols, ketones, organic acids, esters, furans, alkanes, alkenes, and others. On the other hand, flavor groups considered molecules of similar flavor such as (1) fruity, sweet, ethereal; (2) floral, nice green; (3) buttery, fatty, creamy, waxy; (4) cheesy, sour; (5) nutty, woody, toasted cocoa, terpenic, balsamic, camphoraceous; (6) green, grassy, beany,

herbal; (7) earthy, mushroom-like; and (8) pungent, spicy, sharp, phenolic. The aroma perception of the volatiles was collected from literatures (Akkad et al. 2019; Aznar et al. 2001; Boatright and Lei 1999; Bott and Chambers IV 2006; Cai et al. 2014; Costa et al. 2008; El Youssef et al. 2020; Fu et al. 2020; Högnadóttir and Rouseff 2003; Jirovetz et al. 2005; Liu et al. 2022; Lv et al. 2011; Maga and Katz 1979; Osorio et al. 2006; Ounamornas et al. 2017; Sabatini et al. 2008; Schmidt et al. 2008; The Good Scents Company Information System 2021; Van Opstaele et al. 2012; Verginer et al. 2010; Wu et al. 2016; Xiao et al. 2017; Xu et al. 2019; Yang et al. 2008; Zeng et al. 2008). Compounds without aroma description were classified as unknown and not considered further.

For each analyte i, the measurement yielded a peak area (A_i) that corresponded to the TIC of the eluting peak and reflected the absolute abundance of this compound. Its relative abundance a_i was obtained by normalization of A_i to the total peak area of all volatiles detected in the sample (A_{total}) according to **Eq. 1**.

$$a_i = \frac{A_i}{A_{total}} \times 100 \qquad \textbf{(Eq. 1)}$$

To evaluate the abundance of such a flavor group within the overall flavor profile, the individual peak areas of all detected analytes attributed to this flavor were summed up (**Eq. 2**), with g being the attribute, e. g. fruity or cheesy or other. Likewise, the relative abundance of such a flavor group within the overall profile was obtained (**Eq. 3**).

$$A_g = \sum A_i \qquad \textbf{(Eq. 2)}$$

$$a_g = \frac{A_g}{A_{total}} \times 100 \qquad \textbf{(Eq. 3)}$$

3.6.3 Functional evaluation of flavor profiles based on odor thresholds

To assess the relative impact of the different volatiles within a sample on the overall flavor note, odor threshold values of the individual compounds in air were taken into

account. The odor thresholds were collected from previous studies (Chemical Book Group 2016; Nagata and Takeuchi 2003; Tamura et al. 2001; van Gemert 2011; Wu et al. 2016; Xu et al. 2017a; Yan et al. 2020; Yang et al. 2008; Yang et al. 2019). In cases, where literature data had not been collected in air but water, a correction was required. In short, odor thresholds in water ($C^*_{i,\ aqueous}$, µg kg^{-1}) were converted into odor thresholds in air ($C^*_{i,\ air,}$ µg L^{-1}), considering the density of water (ρ= 0.997 kg L^{-1}), the ideal gas volume (V_m = 24.77 L mol^{-1}), the molecular weight of the compound (M_i, g mol^{-1}), and the air/aqueous partition coefficient (K_i) (**Eq. 4**). Hereby, the latter was acquired from previous work (Sander 2015) or estimated by the EPI Suite software (V 4.11) (**Appendix, Table S1**).

$$C^*_{i,air} = \frac{K_i \cdot \rho \cdot V_m \cdot C^*_{i,aqeous}}{M_i} \cdot 1000 \qquad \textbf{(Eq. 4)}$$

The calculation yielded individual odor thresholds at which each of the compounds could be sensed and which ranged from low (0.01 ppbv) to high (1.27×10^6 ppbv). The least recognizable among all detected compounds in this study was 2-ethyl-furan. Its odor threshold ($C^*_{2\text{-ethyl-furan, air}}$) was used as a reference to obtain relative odor thresholds ($c^*_{i,\ air}$), which were calculated for all other analytes (**Eq. 5**). This value described, how easy a compound could be sensed, relatively.

$$c^*_{i,air} = \frac{C^*_{i,air}}{C^*_{2-\text{ethyl}-\text{furan},air}} \cdot 100 \qquad \textbf{(Eq. 5)}$$

Then, for each compound, the relative abundance a_i was taken into account to infer its impact of it on the flavor within a mixture, named relative odor activity here (o_i) (**Eq. 6**).

$$o_i = \frac{a_i}{c^*_{i,air}} \qquad \textbf{(Eq. 6)}$$

Summing up the activity values for molecules that belonged to the same flavor group, allowed to generally extract the impact of a certain type of flavor (o_g), as shown above (**Eq. 7**).

$$o_g = \sum o_i \qquad \textbf{(Eq. 7)}$$

The comparison of the different notes then provided a rough estimate of the expected overall flavor note.

3.6.4 Principal component analysis (PCA) and hierarchical cluster analysis (HCA)

PCA and HCA were weighted by using unit variance and zero-mean normalization scaling, respectively (Wu et al. 2016). Moreover for PCA, growth was transformed to fold growth of cfu mL^{-1}, to account for significant differences. Subsequently, PCA and HCA were performed using SPSS (version 24.0).

3.6.5 Statistical analysis

All results displayed in Figures and Tables are shown as the mean values ± standard deviations (SDs). Statistical evaluation of the data was conducted by one-way analysis of variance (ANOVA), followed by Fisher's least significant difference (LSD) test and Duncan’s test. Differences in values were considered significant when the *p* value was less than 0.05 (+) and 0.01 (++). Statistical analyzes were performed using SPSS (version 24.0).

4 Results and Discussion

4.1 Genome-based selection and application of food-grade microbes for chickpea milk fermentation towards increasing L-lysine content

Chickpea (*Cicer arietinum L.)*, one of the oldest and most widely consumed legumes worldwide (Fu and Zhang 2013), is regarded an attractive source of milk-alternative consumer products (Kishor et al. 2017; Li et al. 2016) with good protein quality (Chavan et al. 1987; Ribeiro and Melo 1990).

Unfortunately, suspensions of chickpea flour (termed chickpea milk below due to their milk-like appearance) do not match animal milk in all desired characteristics, which is a limitation that is generally observed for plant-based milk alternatives (Sethi et al. 2016). This limitation also holds for the essential amino acid L-lysine, which is required for hormone formation, catalytic and structural proteins, and immune system support and is therefore one of the most impacting nutrients (Flodin 1997). Critically, it exhibits much lower abundance in chickpea-based milk than in animal milk, approximately half of the amount in a typical 10% dry matter formulation (Csapó et al. 2009b; Rachwa-Rosiak et al. 2015). Moreover, chickpea milk contains elevated levels of indigestible sugars such as raffinose and stachyose, which cause flatulence, diarrhoea, and other discomfort upon consumption (Onyesom et al. 2005). In addition, it exhibits a grassy and beany taste that does not meet consumer expectations (Aguirre et al. 2008).

In this chapter, we present a systematic workflow to fortify a chickpea milk using fermentation with natural food-grade microbes. To improve upon the low level of L-lysine, more than 30 strains were selected as candidates based on their genomic repertoire related to L-lysine metabolism. A screening round revealed that one-third (9) of the tested strains significantly accumulated L-lysine during chickpea milk

fermentation. ^{13}C isotope studies showed that the two best-performing strains, *Lacticaseibacillus paracasei* subsp*. paracasei* NCC 2511 and *Bacillus amyloliquefaciens* NCC 156, synthetize L-lysine *de novo*. Both microbes were used for a more detailed investigation of the fermentation process. They beneficially altered the amino acid and protein profile, utilized (indigestible) carbohydrates, and formed desired flavour molecules, which highlights them as microbes well suited for chickpea milk fermentation.

4.1.1 Nutritional characteristics of chickpea milk

Initial tests revealed that a one-step pre-treatment was not suitable to provide homogeneous, sterile chickpea milk for fermentation. Standard autoclaving (121°C, 15 min) resulted in undesired starch gelation and an inhomogeneous suspension, while pasteurization (63°C, 5 h) failed to avoid contamination in the subsequent fermentation studies (**Fig. 8**). After several tests, a suitable two-step heat pre-treatment was developed. It avoided starch gelation by an initial phase of stirring (75°C, 2 h), provided a sterile suspension by a subsequent autoclaving step (121°C, 15 min), and finally yielded a homogenous, easy to process suspension (**Fig. 8**).

From a chemical composition viewpoint, the unfiltered chickpea milk contained 16.2% (w/w, dry mass) protein, 8.7% soluble carbohydrates, 7.4% fat, 2.5% organic acid, and a remaining fraction accounting for 65.2% (e.g., starch, fibers, ash, and salt) (**Fig. 9**)，which well compared to previous studies (Jukanti et al. 2012; Kishor et al. 2017; Rachwa-Rosiak et al. 2015; Ribeiro and Melo 1990; Singh et al. 1983; Wallace et al. 2016). Among the nonessential amino acids, L-glutamate (3.3%) and L-aspartate (1.6%) were the most abundant. The major essential amino acids were L-leucine (1.4%) and L-phenylalanine (1.0%). As expected, the level of L-lysine (8.7

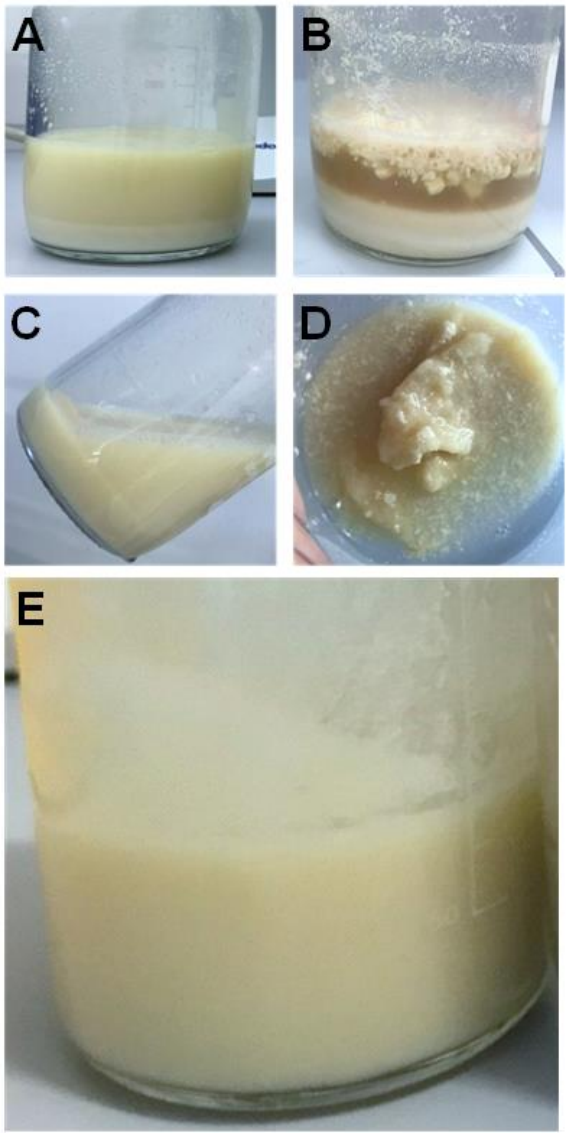

Fig. 8. Pre-treatment strategies for the processing of chickpea flour suspensions prior to microbial fermentation. Phase separation for the untreated milk (A), contamination of non-inoculated milk after pasteurization (63°C, 5 h) (B), gelation and phase separation after stronger heating (90°C, 30 min) (C), gelation and phase separation after autoclaving (121°C, 15 min), generation of homogeneous and sterile suspension after a two-step treatment that included combined heating and stirring (2 h, 75°C, 250 rpm) and autoclaving (121°C, 15 min) (E). The sterility of pasteurized milk and two-step treated milk, was evaluated by non-inoculated incubation for 48 h at 37°C.

mg (g dry mass)$^{-1}$) was only 30-70% of the value observed for animal milk (Guo et al. 2007). Soluble carbohydrates comprised digestible (sucrose, maltose) and

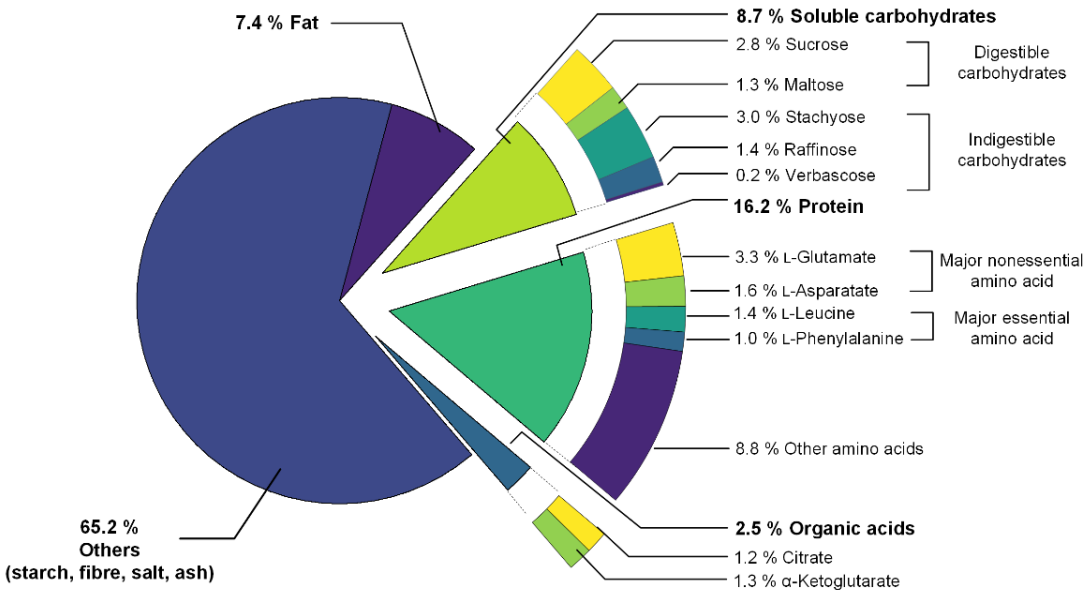

Fig. 9. Composition of non-filtered chickpea milk used for the fermentation studies. All values are related to the dry mass (w/w). n=3.

carbohydrates (raffinose, stachyose, verbascose). Stachyose (3.0%, w/w, dry mass), sucrose (2.8%), raffinose (1.4%), and maltose (1.2%) were the major soluble sugars. Verbascose was present in lower amounts, whereas the levels of glucose and fructose were negligible. In addition, chickpea milk contained organic acids such as 1.3% α-ketoglutarate (w/w, dry mass), 1.2% citrate, and trace amounts of succinate.

4.1.2 Genome-based selection of food-grade microbes for L-lysine production

Since the early days of humankind, fermentation has been a natural approach to producing food, and today, fermented foods are more popular than ever (Adler et al. 2013). Given the magnitude of available microbes for plant milk fermentation (or even food fermentation in general), a straightforward selection of the most appropriate microbes appears crucial. Previous approaches that successfully

increased the L-lysine content in chickpea (Reyes-Moreno et al. 2004), soybean (Frias et al. 2008a; Frias et al. 2008b; Ghosh et al. 2013; Santos et al. 2014), mung bean (Khalil 2006), and cowpea (Sanni et al. 1999) found the corresponding microbes mainly only spontaneously and occasionally. Here, potential L-lysine producers were systematically selected, based on their genomic repertoires.

Bacterial genomes of the NCC were analyzed for their features related to L-lysine, including L-lysine biosynthesis, L-lysine degradation, and pathways competing with L-lysine biosynthesis for carbon precursors (**Fig. 10**). A component from the pathway architecture for bacterial L-lysine biosynthesis, the *lysA* gene (encoding diaminopimelate decarboxylase) was inferred as a premium selection criterion to identify potential L-lysine producers. This enzyme catalyzes the terminal step of L-lysine synthesis, downstream of *meso*-diaminopimelate, where all synthetic routes converge (**Fig. 10**). Therefore, the presence of *lysA* was an essential (minimal) feature required for L-lysine formation. In total, 2,472 bacterial NCC genomes contained an annotated *lysA* gene, and most of them belonged to the orders *Lactobacillales*, *Bacillales*, *Bifidobacteriales*, and *Corynebacteriales*. Fewer than half of the genomes (1,117) additionally included a *dapF* gene, potentially indicating the succinylase and/or acetylase route to synthesize L-lysine (**Fig. 10**). Only 14 strains contained the *ddh* gene (encoding diaminopimelate dehydrogenase) as a key step of the dehydrogenase pathway. The selection was narrowed down by considering only strains approved for use in the food and feed chain according to the European Food Safety Authority (EFSA Panel on Biological Hazards et al. 2017). Among all potential L-lysine producers, i. e. all strains that contained *lys*A plus either *dap*F or *ddh*, 945 strains exhibited the qualified presumption of safety (QPS) status and therefore fulfilled this extra requirement. They belonged to five different families (*Lactobacillaceae, Bacillaceae, Bifidobacteriaceae, Corynebacteraceae,*

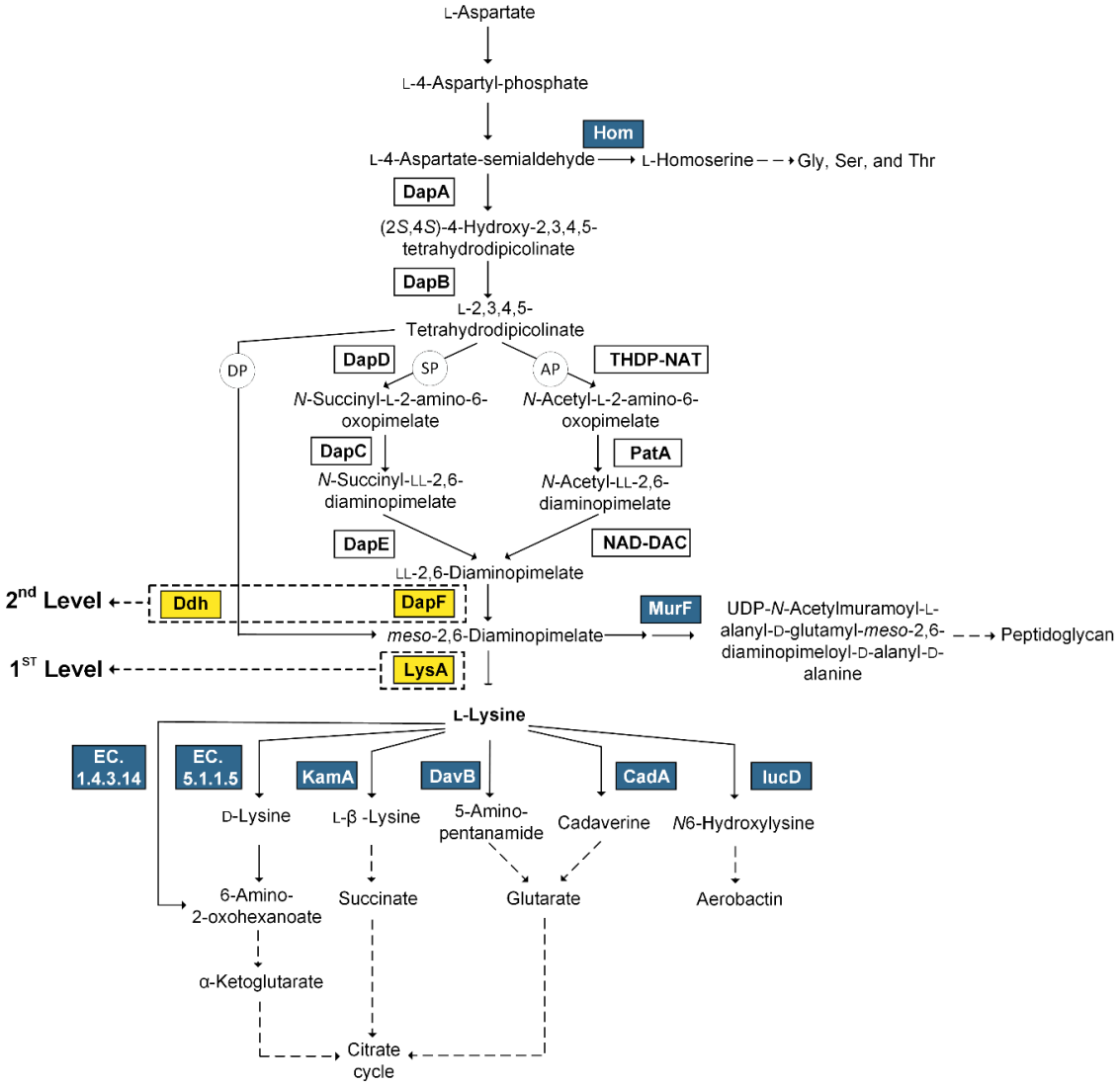

Fig. 10. Microbial pathways for the synthesis and degradation of L-lysine. Potential L-lysine overproducers were identified from the NCC database based on the presence and absence of key genes related to the well-established routes of L-lysine metabolism (Rezola et al. 2011). Enzymes shown in yellow were essential for L-lysine biosynthesis, whereas enzymes shown in blue were involved in competing pathways and L-lysine degradation (Chou et al. 2010; Revelles et al. 2005; Zhang et al. 2014). The presence of diaminopimelate decarboxylase (LysA) was regarded as essential for potential L-lysine-producing strains (level 1). Diaminopimelate epimerase (DapF) and diaminopimelate dehydrogenase (Ddh) were then used to infer the putative pathway involved (level 2). Abbreviations: DP, dehydrogenase pathway; SP, succinylase pathway; AP, acetylase pathway; DapA, 4-hydroxy-tetrahydrodipicolinate synthase; DapB, 4-hydroxytetrahydrodipicolinate reductase; DapD, tetrahydrodipicolinate succinylase; DapC, succinyl-amino-ketopimelate transaminase; DapE, *N*-succinyl-diaminopimelate

desuccinylase; DapF, diaminopimelate epimerase; Ddh, diaminopimelate dehydrogenase; *THDP-NAT*, tetrahydrodipicolinate acetylase; PatA, *N*-acetyl-amino-ketopimelate aminotransferase; NAD-DAC, *N*-acetyl-diaminopimelate deacetylase; LysA, diaminopimelate decarboxylase; Hom, homoserine dehydrogenase; MurF, UDP-*N*-acetylmuramoylalanyl-D-glutamyl-2,6-diamino-pimelate-D-alanyl-D-alanyl ligase; *EC* 1.4.3.14, L-lysine oxidase; *EC* 5.1.1.5, lysine racemase; KamA, L-lysine 2,3-aminomutase; DavB, lysine 2-monooxygenase; CadA, lysine decarboxylase; LucD, lysine N6-hydroxylase.

Carnobacteriaceae), including 12 genera and 33 different species. None of the strains that contained a *ddh* gene met the food safety constraint. *Carnobacterium* and *Pediococcus* were not further considered because they are mainly associated with producing bacteriocins for food preservation (Brillet et al. 2004; Porto et al. 2017), and the fermentation of materials other than milk such as wine, cheese, sausage, and cabbage (Wade et al. 2019), leaving *Lactobacillaceae* (757), *Bacillaceae* (34), *Bifidobacteriaceae* (119), and *Corynebacteraceae* (2) for the final selection. Accordingly, 31 potential producers (all containing *lysA* and *dapF* that covered the identified taxonomic diversity were selected for experimental studies. The selected strains (**Fig. 11**) represented 10 genera and 19 different species: (i) *Lactobacillus helveticus* (3), *Lactobacillus johnsonii* (3), *Lactobacillus acidophilus* (1), *Lactobacillus delbrueckii* (1), *Limosilactobacillus reuteri* (3), *Limosilactobacillus pontis* (1), *Lacticaseibacillus paracasei* (2), *Lactiplantibacillus plantarum* (1), *Fructilactobacillus sanfranciscensis* (3), *Levilactobacillus brevis* (1), *Lentilactobacillus hilgardii* (1); (ii) *Bifidobacterium longum* (1) and *Bifidobacterium infantis* (1); (iii) *Bacillus amyloliquefaciens* (2), *Bacillus flexus* (2), *Bacillus licheniformis* (1), *Bacillus pumilus* (1), and *Bacillus subtilis* (1); and (iv) *Corynebacterium stationis* (2). In addition, *Lactobacillus jensenii* NCC 2867, lacking *lysA*, was used as a negative control. Regarding oxygen sensitivity, the selected strains were classified as obligate anaerobes (6), aerotolerant (17), and obligate aerobes (9).

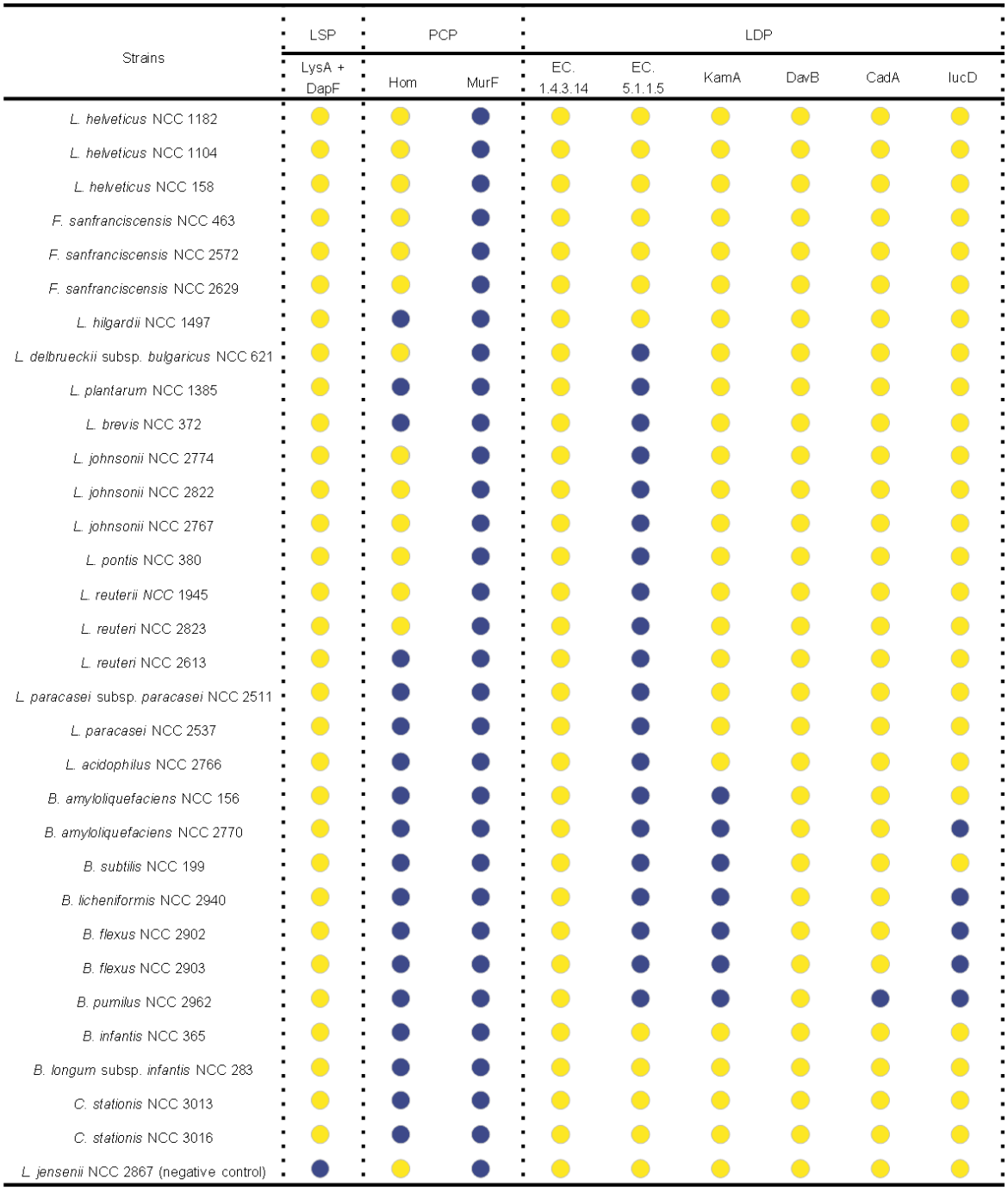

Fig. 11: Genomic repertoire of food-grade microbes linked to L-lysine metabolism: pathways for L-lysine biosynthesis (LSP), pathways competing with L-lysine biosynthesis for carbon precursors (PCP), and pathways for L-lysine degradation (LDP). The presence (yellow) and absence (blue) of corresponding key genes (**Fig. 10**) is indicated by color.

By strict filtering of genome sequences for key genes related to L-lysine synthesis and degradation, an initially high number of approximately 2,500 potential

candidates was narrowed down to 31 strains that possessed the key genes *lys A* and *dapF* for L-lysine biosynthesis, incomplete pathway sets for L-lysine degradation and fulfilled the QPS recommendation. The selected strains represented 10 different microbial genera and (given the low number) enabled straightforward experimental work.

4.1.3 Evaluation of the pre-selected microbes for chickpea milk fermentation

The selected 31 strains differed in their genomic repertoire regarding the number of completely and partially annotated L-lysine biosynthetic pathways (**Fig. 11**). They were next experimentally evaluated for their capability to ferment chickpea milk, and *L. jensenii* was included as a negative control (**Fig. 11**). Generally, the established fermentation process was highly reproducible, which enabled a clear evaluation of strain performance. In total, 60% of the strains (19 out of 32) were able to grow (**Fig. 12, Appendix, Table S2**). Depending on species and strain, the observed increase in the number of living cells ranged from 0.2 to 3.2 log cfu mL^{-1}. Generally, obligate aerobic and aerotolerant strains grew well, while obligate anaerobes showed weak or even no growth. The bacilli and corynebacteria showed the strongest growth. The cfu numbers for these strains increased at least tenfold (1 log) while the bifidobacteria grew only weakly. For the lactobacilli, the outcome was mixed. Some strains grew well (*L. plantarum, L. paracasei* subsp*. paracasei*, and two out of three *L. reuteri*), while others (*F. sanfranciscensis, L. helveticus, L. johnsonii, L. acidophilus*, the remaining *L. reuteri* and the negative control strain *L. jensenii*) did not grow.

Notably, 30% of the strains (9 out of 31 potential producers) increased the level of L-lysine (**Fig. 12**). The producers were exclusively found among four genera: *Lacticaseibacillus, Limosilactobacillus, Levilactobacillus,* and *Bacillus*. High and low producers were equally distributed between LAB and *Bacillus* groups. The best, third best, fifth best, and sixth best producers were LAB strains (*L. paracasei* subsp*.*

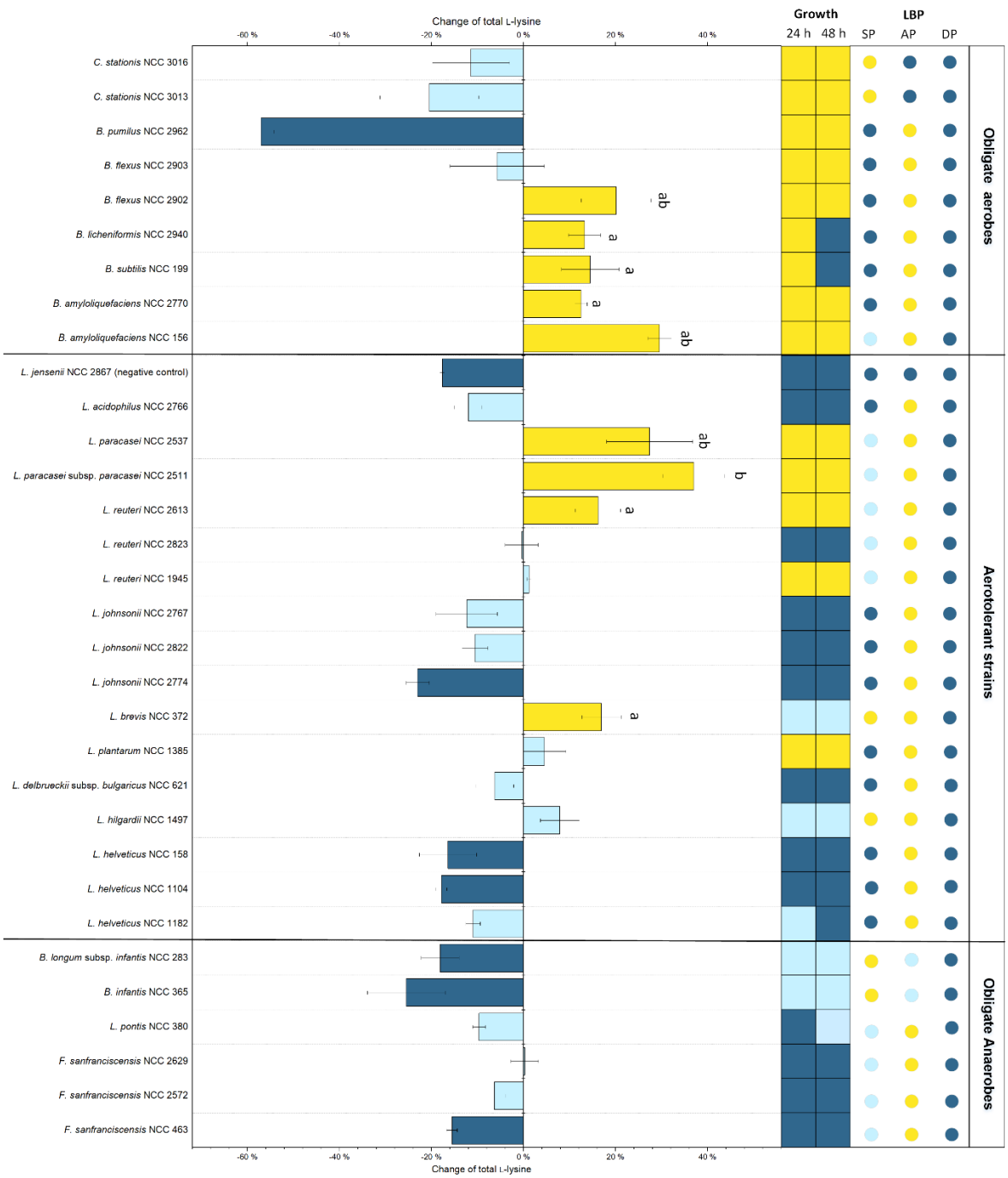

Fig. 12. Growth and L-lysine production of pre-selected food-grade strains during fermentation of chickpea milk. *Lactobacillus jensenii* was included as a negative control and is correspondingly annotated. The L-lysine concentration after 48 h of fermentation was significantly increased (yellow, $p < 0.05$), significantly decreased (blue, $p < 0.05$), or not significantly changed (light blue). The coloured squares represent the change in cfu after 24 h and 48 h: strong growth (yellow, increase > 1 log cfu mL^{-1}), weak growth (light blue, increase < 1 log cfu mL^{-1}), and no growth (blue, no change). In addition, the genomic repertoire of the strains for L-lysine biosynthesis is shown (**Fig. 10**): yellow, completely annotated pathway; light blue, partially annotated pathway; and blue, pathway not

annotated. Abbreviations: SP, succinylase pathway; AP, acetylase pathway; DP, dehydrogenase pathway. n=3.

paracasei NCC 2511, *L. paracasei* NCC 2537, *L. reuteri* NCC 2613, and *L. brevis*. NCC 372), while the second, fourth, seventh, eighth and ninth highest L-lysine levels were achieved by *Bacillus* strains (*B. amyloliquefaciens* NCC 156, *B. flexus* NCC 2902, *B. flexus* NCC 2940, *B. subtilis* NCC 199, *B. licheniformis* NCC 2940, and *B. amyloliquefaciens* NCC 2770). *L. paracasei* subsp*. paracasei* NCC 2511 and *B. amyloliquefaciens* NCC 156 increased the L-lysine content by 37% and 30%, respectively. In contrast, none of the *Bifidobacterium* and *Corynebacterium* isolates increased the L-lysine level. Several isolates degraded L-lysine. *B. pumilus* NCC 2962 decreased the L-lysine content by over 50%. Fifteen strains did not alter the content significantly.

As a valuable proof-of-concept, 30% of all experimentally tested strains exhibited the desired phenotype in increasing the L-lysine content, and it appears promising to extend this genome-based selection approach to other plant milk fermentation processes and traits. Although our study could not yield a complete picture, it provided at least systematic insight into the pathways that supported L-lysine production the most. Among the strains that exhibited growth and L-lysine accumulation, the majority (79%) possessed a completely annotated acetylase pathway (AP), while 32% exhibited a complete succinylase pathway (SP), and two isolates had both routes in parallel. None of the strains used here contained the dehydrogenase pathway (DP). The different biosynthetic routes are widely abundant in nature: there are SP-using classes and species such as *Escherichia coli*, *Corynebacterium*, *Bacillus,* and *Lactobacillus* (Dogovski et al. 2012; Hao et al. 2011; Schrumpf et al. 1991), AP users such as certain *Bacillus* and *Lacticaseibacillus* (Cahyanto et al. 2006; Hao et al. 2011), and DP users such as

Corynebacterium and some *Bacillus* species (Dogovski et al. 2012). The AP and SP both supported L-lysine accumulation in chickpea milk, with a slightly better performance from the AP.

Among catabolic pathways and potential L-lysine withdrawal and degradation, there was no clear trend. The competing routes to homoserine (Becker et al. 2008; Nelofer et al. 2007) and peptidoglycan (Smith 2006; Xu et al. 2014) did not have an impact. However, *B. pumilus* NCC 2962, the only strain tested that possessed a lysine decarboxylase (EC. 4.1.1.18)-encoding gene (**Fig. 11**), dramatically decreased the L-lysine content so that future strain selection might consider potentially negative effects of this enzyme. Taken together, AP and SP genes appear to be genomic key features of well-performing L-lysine-producing strains. Based on performance, future focus should be given to strains of *Lacticaseibacillus, Limosilactobacillus, Levilactobacillus,* and *Bacillus.* Given that these genera are among the most widely used microbes for plant-based fermentation (Jeske et al. 2018; Steinkraus 1997), there seems striking potential ahead.

L. paracasei subsp*. paracasei* NCC 2511 and *B. amyloliquefaciens* NCC 156 appeared most promising for further studies regarding their superior capability to overproduce L-lysine. The inspection of their genomes revealed that they possessed a rich repertoire to potentially improve also other important traits of the plant milk. They contained several genes, functionally assigned to catalyze flavor formation, such as alcohol dehydrogenase (Liu et al. 2008), aldehyde dehydrogenase (Liu et al. 2008), and enzymes of branched-chain amino acid metabolism (Smit et al. 2009) (**Table 3**). Moreover, genes encoding carbohydrate degrading enzymes, known to be involved in the removal of indigestible sugars (Savoy de Giori et al. 2010), were present. Therefore, both strains were subjected to a more detailed characterization.

4.1.4 Fermentation of chickpea milk using *B. amyloliquefaciens* NCC 156

The physiology of *B. amyloliquefaciens* NCC 156 during chickpea milk fermentation was monitored over a period of 48 h (**Fig. 13A-G**). The microbe went through several distinct phases of an obviously altered metabolism. The initial phase was the main phase of growth. The cells immediately started to proliferate, and the cfu number increased almost 1000-fold during the first 8 h (**Fig. 13A**).

Table 3. The number of locus in *B. amyloliquefaciens* NCC 156 and *L. paracasei* subsp. *paracasei* NCC 2511 encoding functional enzymes invoving in flavor formation,pyruvate, and butanoate metabolism.

Function	Enzyme name and orthology	Enzyme Commision number	*B. amyloliquefaciens* NCC 156	*L. paracasei* subsp. *paracasei* NCC 2511
Flavor formation	Alcohol dehydrogenase (NAD)	*EC* 1.1.1.1	1	1
	Alcohol dehydrogenase ($NADP^+$)	*EC* 1.1.1.2	3	1
	Aldehyde dehydrogenase	*EC* 1.2.1.3	1	0
	Acetaldehyde dehydrogenase	*EC* 1.2.1.10	0	1
	Branched-chain aminotransferase	*EC* 2.6.1.42	0	1
	Leucine dehydrogenase	*EC* 1.4.1.9	1	0
	Valine dehydrogenase (NAD^+)	*EC* 1.4.1.23	1	0
	L-Amino-acid oxidase	*EC* 1.4.3.2	1	0
Pyruvate metabolism	Lactate dehydrogenase	*EC* 1.1.1.27	2	2
	Pyruvate-formate lyase	*EC* 2.3.1.54	0	1
Butanoate metabolism	Acetolactate-synthase	*EC* 2.2.1.6	3	1
	Acetolactate-decarboxylase	*EC* 4.1.1.5	1	1

Notably, the initial phase (0-16 h) was also the major phase of L-lysine production (42.8% increase), which appeared growth coupled (**Fig. 13C**). TCA cycle-related intermediates (citrate, α-ketoglutarate) were utilized as major substrates during this initial phase (**Fig. 13F**). Carbohydrates were also taken up but at only a rather low rate (**Fig. 13DE**). Dissolved oxygen was quickly consumed and became limiting after approximately 10 h, while the pH value significantly dropped (**Fig. 13B**). Acetate was the only by-product formed in significant amounts (**Fig. 13G**). Then, growth and L-lysine accumulation slowed (16-30 h) (**Fig. 13AC**). Likely linked to limited oxygen availability (**Fig. 13B**), fermentation products such as lactate and 2,3-butanediol emerged (**Fig. 13G**). With the depletion of citrate and α-ketoglutarate, NCC 156 switched to consuming mainly sucrose, while raffinose and stachyose degradation continued (**Fig. 13DEF**). After approximately 30 h, sucrose was used up, triggering an accelerated use of stachyose and raffinose (30-48 h). The level of maltose, on the other hand, increased (**Fig. 13DE**). Overall, *B. amyloliquefaciens* NCC 156 showed an outstanding capability to degrade indigestible carbohydrates. Stachyose (94.8%) and raffinose (88.6%) were almost completely consumed (**Fig. 13E**).

Remarkably, the formed L-lysine was largely present as a free amino acid. The extracellular L-lysine level increased almost 9-fold to a final value of 1.4 mM after 24 h of fermentation. The pH value increased towards the end and finally again reached the starting value of approximately 6 (**Fig. 13B**). At the end of the fermentation, acetate (40 mM), acetoin (30 mM), 2,3-butanediol (19 mM), and maltose (11 mM) were the main products (**Fig. 13DG**). Ethanol, isobutyrate, and isovalerate, often detected fermentation products for other strains of *Bacillus*, were not detected. Regarding essential amino acids, the microbe increased the total levels of L-phenylalanine (18.6%), L-valine (11.1%), and L-leucine (10.0%) in addition to L-lysine, while the nonessential amino acids L-glutamate/L-glutamine, L-alanine, L-

serine, and L-arginine – all fuelling the EMP pathway and the TCA cycle, respectively – were degraded (**Fig.14**).

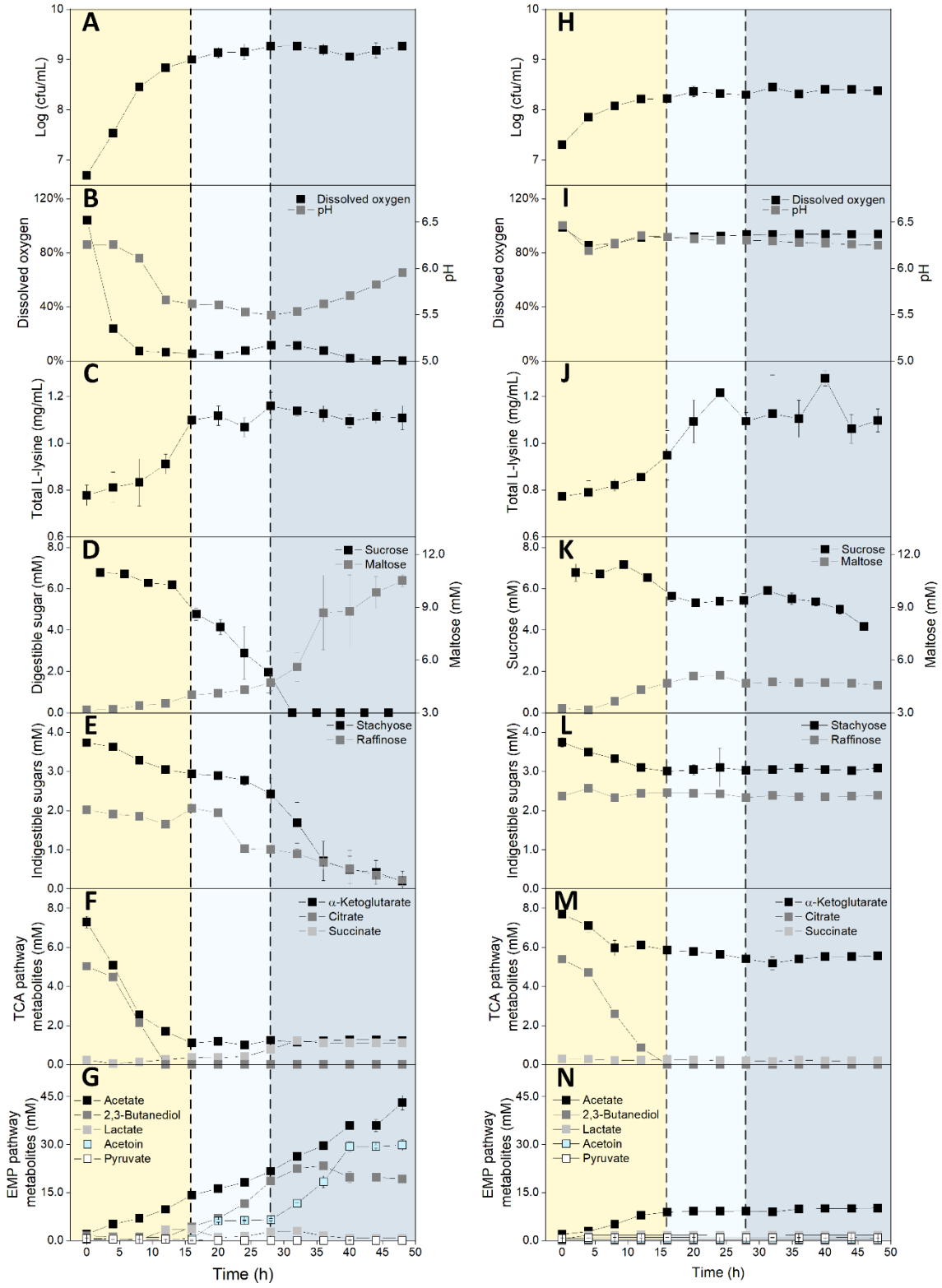

Fig. 13. Time-resolved fermentation of chickpea milk using *B. amyloliquefaciens* NCC 156 (left, A-G) and *L. paracasei* subsp. *paracasei* NCC 2511 (right, H-N). The data comprise living cell number (cfu) (A, H), dissolved oxygen level and pH value (B, I), and the concentrations of total L-lysine (C, J), sucrose, maltose (D, K), raffinose, stachyose (E, L), citrate, α-ketoglutarate, succinate (F, M), acetate, acetoin, 2,3-butanediol, lactate, and pyruvate (G, N). n=3.

4.1.5 Fermentation of chickpea milk using *L. paracasei* subsp. *paracasei* NCC 2511

L. paracasei subsp. *paracasei* NCC 2511 showed a completely different fermentation behavior than *B. amyloliquefaciens* NCC 156 (**Fig. 13H-N**). Its growth was much weaker, and changes in pH and dissolved oxygen were also less pronounced (**Fig. 13HI**). Citrate was the preferred carbon source. It was completely depleted during the first hours, together with some of the α-ketoglutarate (18.2%) (**Fig. 13KM**). Sucrose (38.3%) and stachyose (16.2%) were partially consumed,

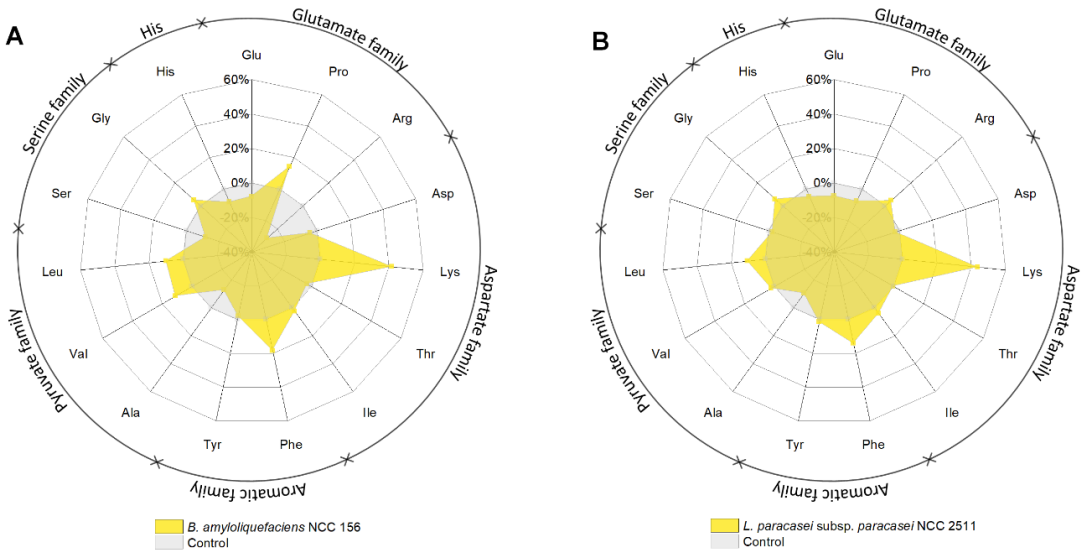

Fig. 14. Relative changes in the amino acid profile during fermentation of chickpea milk using *B. amyloliquefaciens* NCC 156 (A) and *L. paracasei* subsp. *paracasei* NCC 2511 (B). n=3.

while the raffinose level did not change, indicating a weaker ability of *L. paracasei* subsp.*paracasei* NCC 2511 to ferment carbohydrates (**Fig. 13KL**). Acetate (10 mM) and lactate (2 mM) accumulated as by-products during the initial phase (**Fig. 13N**). Considering the generally lower metabolic activity, the high increase in total L-lysine (45.7%) during the first 24 h was remarkable for this strain. Interestingly, the formed L-lysine remained inside the cells and/or protein bound because the level of the free amino acid did not change significantly (data not shown). *L. paracasei* subsp*. paracasei* NCC 2511 slightly changed the amino acid profile (**Fig. 14**). The levels of L-phenylalanine (14.0%) and L-leucine (11.0%) increased, while L-glutamate/L-glutamine, and L-alanine were partially consumed.

4.1.6 Metabolic pathway analysis: L-Lysine biosynthesis

It appeared interesting to study the L-lysine pathway in more detail. As inferred from the genomic repertoire, it contained L-aspartate as a central intermediate (**Fig. 15**). Monitoring of the incorporation of ^{13}C from [$^{13}C_4$] L-aspartate, added as a tracer substrate to the fermentation process, into L-lysine was used to elucidate biosynthesis *in vivo*. For this purpose, 11 mM [$^{13}C_4$] L-aspartate was added to medium. After 20 h of fermentation, the ^{13}C enrichment of total L-lysine was analyzed by GC-MS. In a control study, the medium was supplemented with the same amount of naturally labeled L-aspartate, and the ^{13}C enrichment of total L-lysine was analyzed. The ^{13}C enrichment of total L-lysine in the control was 1.1 ± 0.1% , reflecting the expected natural ^{13}C abundance (Schwechheimer et al. 2018a). Notably, it was significantly increased to a value of 1.9 ± 0.0% in the tracer study ($p < 0.01$) which indicated that [$^{13}C_4$] L-aspartate had been converted into L-lysine (**Fig. 15**). The ^{13}C enrichment of extracellular L-lysine was even more increased (5.6 ± 0.1%), underlining that cells excreted large amounts of the free amino acid, formed from L-aspartate, into the media (**Fig. 15**). Parallel analysis of the ^{13}C enrichment of L-aspartate revealed a substantial decrease from a high initial value of 38.5 ± 1.2%

(0 h) to 2.7 ± 0.1% (20 h) indicating that the tracer was largely used up during this time (**Fig. 15**). In a parallel study, *de novo* biosynthesis of L-lysine was also observed

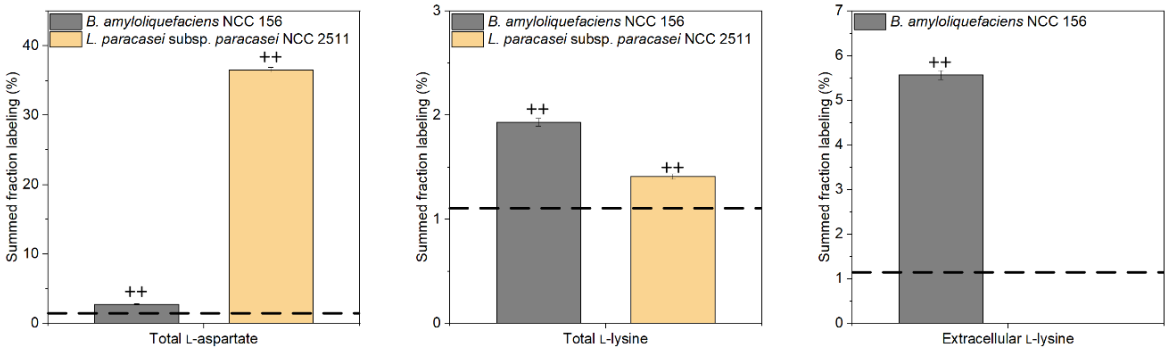

Fig. 15. Metabolic flux analysis of L-lysine biosynthesis in *B. amyloliquefaciens* NCC 156 (yellow) and *L. paracasei* subsp. *paracasei* NCC 2511 (blue) during chickpea milk fermentation. The carbon flux through of L-lysine biosynthesis was investigated by monitoring the incorporation of ^{13}C from [$^{13}C_4$] L-aspartate, added as a tracer to the medium, into L-lysine. As a control, naturally labeled L-aspartate was supplemented to the same amount instead. The data show the ^{13}C enrichment in total L-lysine, free extracellular L-lysine, and total L-aspartate after 20 h of fermentation, measured by GC-MS. The given summed fractional labeling (SFL) reflects the average enrichment of ^{13}C in each of the analytes. The corresponding values from the control experiment for total L-aspartate (1.1 ± 0.1%), total L-lysine (1.1 ± 0.1%), and free extracellular L-lysine (1.1 ± 0.1%) matched the theoretical values expected from natural ^{13}C abundance. They are shown as dashed lines. The significance of ^{13}C enrichment in each analyte was determined by comparison of the measured value against the naturally labeled control, and is marked accordingly (*, $p < 0.05$, ** $p < 0.01$). n=3.

for *L. paracasei* subsp. *paracasei* NCC 2511. Using [$^{13}C_4$] L-aspartate as a tracer, the ^{13}C enrichment of total L-lysine after 20 h was again significantly increased (1.4 ± 0.0% , $p < 0.01$) (**Fig. 15**). At a first glance, it appeared less pronounced. The weaker increase in ^{13}C enrichment in L-lysine than in NCC 156, however, partially resulted from the fact that the added [$^{13}C_4$] L-aspartate was only slightly used, i. e. the ^{13}C enrichment of total L-aspartate after 20 h (36.5 ± 0.4%) was almost as high as the starting value (38.5 ± 1.2%) indicating only little consumption (**Fig. 15**).

As shown above, citrate appeared to be the preferred carbon source of both strains, and, for *B. amyloliquefaciens*, its consumption happened simultaneously with L-lysine formation, suggesting a potential link (**Fig. 13**). In additional studies, citrate (10 mM) was spiked into the medium. For *B. amyloliquefaciens* NCC 156, this resulted in 27% increased L-lysine production to 1.8 ± 0.2 mM after 24 h, while the growth of the microbe was stimulated to $10^{9.5}$ cfu mL^{-1}. For *L. paracasei* subsp. *paracasei*, no significant impact on growth or L-lysine formation was observed (data not shown).

The genome of *L. paracasei* subsp. *paracasei* contained a potential citrate degradation route via citrate lyase, while that of *B. amyloliquefaciens* NCC 156 did not (**Table 4**). The latter observation suggested that, in this microbe, citrate was apparently converted via the TCA cycle towards oxaloacetate and the precursor of L-lysine instead (**Fig. 16A**).

Table 4. The number of locus in *B. amyloliquefaciens* NCC 156 and *L. paracasei* subsp. *paracasei* NCC 2511 encoding functional enzymes invoving in carbohydrate degradation and citrate metabolism.

Function	Enzyme name and orthology	Enzyme Commision number	*B. amyloliquefaciens* NCC 156	*L. paracasei* subsp. *paracasei* NCC 2511
Carbohydrate degradation	α-Galactosidase	*EC* 3.2.1.22	3	1
	β-Fructosidase	*EC* 3.2.1.23	3	1
	α-Amylase	*EC* 3.2.1.1	2	0
	Pullulanase	*EC* 3.2.1.41	1	0
	Neopullulanase	*EC* 3.2.1.135	1	1
Citrate metabolism	Citrate CoA-transferase (citrate lyase α-subunit)	*EC* 2.8.3.10	0	1
	Citrate CoA-lyase (citrate lyase β-subunit)	*EC* 4.1.3.34	0	1
	Citrate lyase synthetase	*EC* 6.2.1.22	0	1

4.1.7 Metabolic pathway analysis: Fermentation by-products and flavor molecules

The citrate supplemented cultures (see above) were now evaluated for citrate-related effects on the formation of flavor molecules and fermentative by-products. Both microbes differed in the genomic repertoire regarding these parts of metabolism (**Table 4**). Citrate contributed to the formation of different organic acids in *L. paracasei* subsp*. paracasei*, including acetate, lactate, pyruvate, and α-ketoglutarate, when spiked into chickpea milk (**Fig. 16AB**). In addition, acetoin and 2,3-butanediol were obviously produced from citrate. *B. amyloliquefaciens* formed higher amounts of short-chain fatty acids (such as isovalerate and isobutyrate) from citrate. In a series of further experiments, different chickpea milk contained aldehydes (**Fig. 17, Fig. 18**) – flavor compounds themselves and precursors for other flavors as well – were individually spiked into the cultures. Both microbes efficiently reduced pentanal, hexanal, heptanal, octanal, nonanal, and benzaldehyde into the corresponding alcohols (**Fig. 16C**). *L. paracasei* subsp*. paracasei* NCC 2511 further revealed a significant capability to oxidize five-carbon to nine-carbon aldehydes into the corresponding acids. *B. amyloliquefaciens* NCC 156 also and nonanal. Moreover, we tested flavor-related effects by supplementing branched-chain amino acids (**Fig. 16AB**). *B. amyloliquefaciens* NCC 156 degraded leucine, isoleucine, and valine (spiked into the medium) into short-chain fatty acids, whereas *L. paracasei* subsp*. paracasei* did not metabolize these amino acids.

4.1.8 A rich set of microbial aroma compound conversions contributes to an improved flavor profile during chickpea milk fermentation

Flavors are a major attribute that consumers consider when buying plant milk-derived products (Lv et al. 2011). Unfortunately, plant-based milk alternatives are generally perceived as having a displeasing taste, probably because of previous

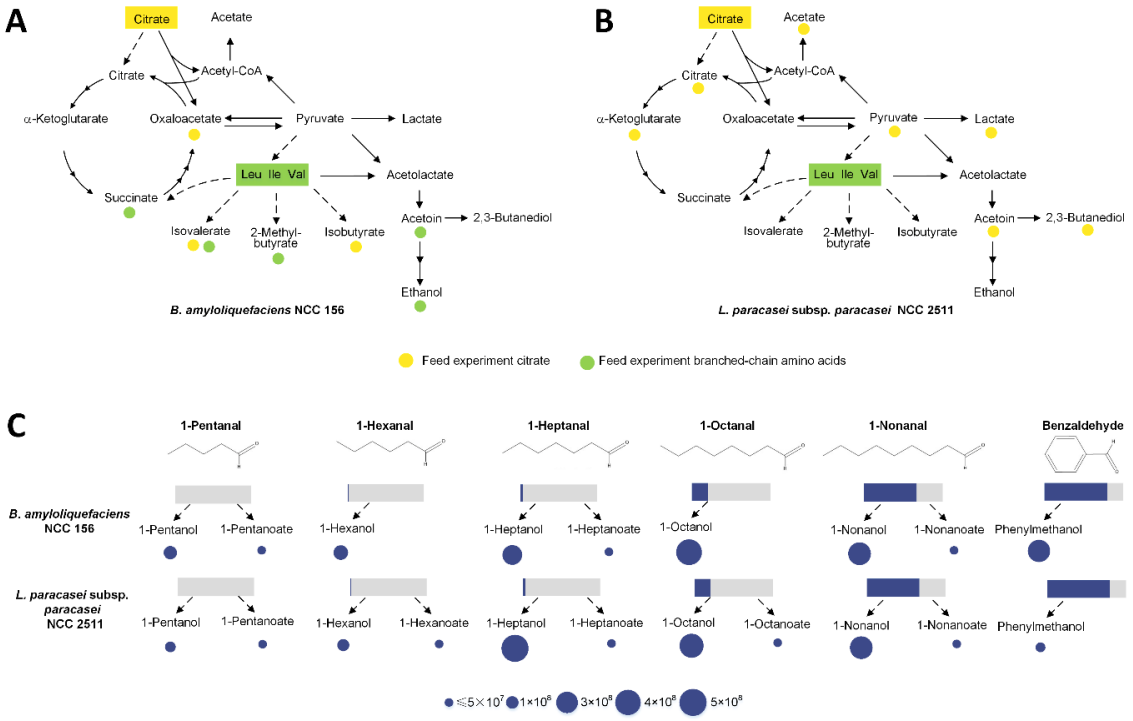

Fig. 16. Metabolic pathway analysis in *B. amyloliquefaciens* NCC 156 and *L. paracasei* subsp. *paracasei* NCC 2511. Metabolism of citrate and branched-chain amino acids (A, B) and of short- to medium-chain flavour aldehydes (C). The data reflect changes in metabolite abundances during 24 h of fermentation in comparison to those in non-fermented chickpea milk incubated under the same conditions (control). In separate experiments, citrate (yellow, 10 mM), a mixture of the branched-chain amino acids valine, leucine, and isoleucine (green, 5 mM each), and flavour aldehydes (blue, 5 mM, each tested individually in separate experiments) were spiked into chickpea milk. The evaporation of aldehydes (assessed from non-inoculated controls) is indicated as relative loss by the grey colour. The formation of alcohols and acids from a particular aldehyde is visualized by the size of the corresponding circle associated with the products ($p < 0.05$). n=3.

experiences with less-appealing products in the market (Wansink et al. 2005). As shown here, unfermented chickpea milk contained various volatiles associated with an undesired taste (**Fig. 18**), a typical drawback for these types of plant-based raw materials (Bott and Chambers IV 2006). Prominent beany and earthy off-flavors are caused by medium-chain aldehydes such as pentanal, hexanal, and heptanal

(Blagden and Gilliland 2005), which originate from the oxidation of plant lipids and are also found in high amounts in other legume-based milks (Blagden and Gilliland 2005).

It can therefore be regarded as beneficial that *L. paracasei* subsp*. paracasei* NCC 2511 and *B. amyloliquefaciens* NCC 156 largely eliminated these aldehydes (**Fig. 16C**). As shown by spiking experiments, the major routes of elimination seemed to be one-step reductive and oxidative biotransformations into the corresponding alcohols and acids, respectively, mostly yielding a strong upgrade in flavor properties into sweet and fruity notes (Chua et al. 2017), although multistep bioconversion and *de novo* formation found in other microbes appeared possible as well (Wittmann et al. 2002). Other flavor-related conversions involved entire pathways of carbon core metabolism, underlining the complexity involved. Citrate and branched-chain amino acids were identified as important precursors. Citrate metabolism in *L. paracasei* subsp*. paracasei* provided acetate, lactate, acetoin, and 2,3-butanediol, whereas valine, leucine, and isoleucine degradation delivered isobutyrate, isovalerate, and 2-methylbutanoate. Based on the discovered link between the chickpea ingredient and specific flavors, screening for plant-based materials rich in such precursors to stimulate the formation of desired flavor notes appears promising (Ayseli and Ayseli 2016). As demonstrated, elevated citrate levels would also provide more L-lysine. An interesting possibility also seems to be the direct addition of such compounds to fermentation to generate beneficial fermentation products. Maltol is also formed, and it contributes to a sweet caramel flavor and is released by the cleavage of antinutrient saponins during fermentation (Hubert et al. 2008). The metabolic origin of the removal of other compounds, such as 1-octen-3-ol and 2-pentyl-furan, remains to be elucidated, but their degradation surely reduces the undesired earthy and beany taste of chickpea flour (Xu et al. 2019). Clearly, the overall aroma of (fermented) legume-based milk is not due to

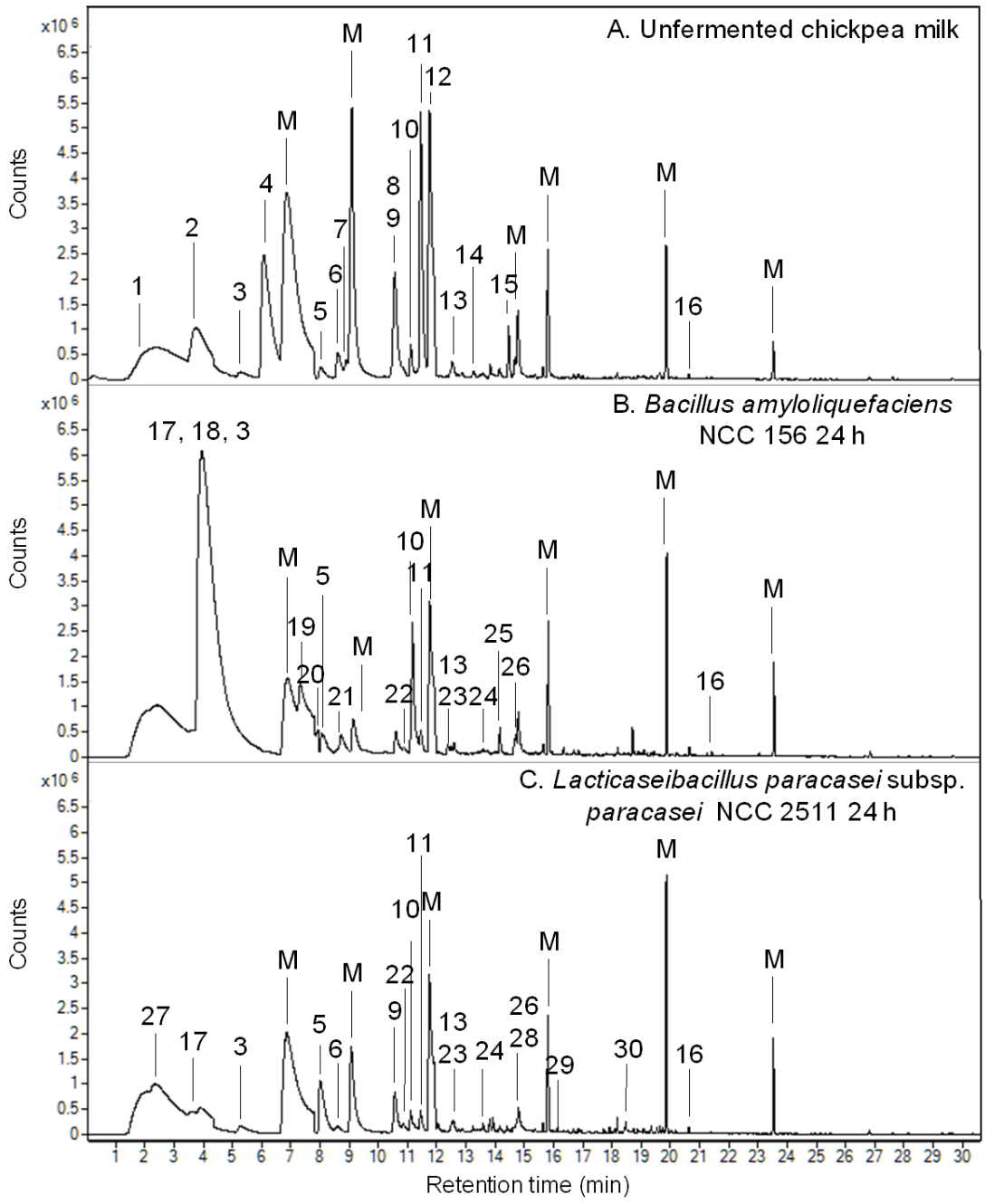

Fig. 17. GC-MS-based analysis of volatile compounds in unfermented chickpea milk (A), chickpea milk fermented for 24 h with *B. amyloliquefaciens* NCC 156 (B) and chickpea milk fermented for 24 h with *L. paracasei* subsp*. paracasei* NCC 2511 (C). The peak numbers refer to the identified analytes: 1, acetone; 2, pentanal; 3, 1-pentanol; 4, hexanal; 5, 1-hexanol; 6, 2-heptanone; 7, heptanal; 8, cyclooctane; 9, benzaldehyde; 10, 1-octen-3-ol; 11, 2-pentylfuran, 12, octanal; 13, phenyl-methanol; 14, *trans*-2-octenal; 15, 1-nonanal; 16, γ -heptyl-butyrolactone; 17, acetoin; 18, isobutyric acid; 19, 2-methyl-hexanoic acid; 20, 2-methyl-butanoic acid; 21, acetoin acetate; 22, 1-heptanol; 23, ethyl-1-hexanol;

24, 1-octanol; 25, 2-nonanone; 26, maltol; 27, 2,3-butanedione; 28, 2-phenylethanol; 29, 1-nonanol; 30, nonanoic acid; M, peaks from sample matrix and instrumental background noise.

single chemicals but rather their exact mixture (Bott and Chambers IV 2006). As such, the overall decrease in off-odour aldehydes plus the formation of favored sweet and fruity alcohols, acids, ketones, and phenols is an important indication that sensory properties were significantly improved, but more work, e.g., using sophisticated electronic aroma sensing and personal aroma detection by skilled and trained sensory panelists, is needed to fully capture the underlying changes (Blagden and Gilliland 2005; Schindler et al. 2012; Vong and Liu 2017).

4.1.9 A multi-benefit chickpea milk derived by *B. amyloliquefaciens* NCC 156 and *L. paracasei* subsp*. paracasei* NCC 2511

Plant-based milk alternatives preferably exhibit the technical, nutritional, and organoleptic properties of cow milk, so researchers and developers in academia and industry must overcome certain challenges (Tangyu et al. 2019). From a technical viewpoint, gelation of typically starchy plant materials during sterilization causes problems in downstream processing (Mäkinen et al. 2016). Conventional autoclaving and pasteurization failed as pre-treatment strategies due to the reasons mentioned above. The developed two-step heat treatment, however, provided a homogeneous and stable emulsion and was a valuable strategy for processing chickpea milk prior to fermentation at a small scale. On the fermentation side, several goals had to be addressed: (i) improvement of the naturally low nutritional value of plant milks limited in amino acids (Millward 1999), (ii) improvement of digestibility to avoid flatulence, diarrhea, and other discomforts (Onyesom et al. 2005), and (iii) improvement of the typically unpleasant earthy and beany taste (Wansink et al. 2005). It is therefore an important outcome of this study that

Compound	RT (min)	Flavor and odor properties[a]	Abundance			
			Control 0 h	Control 24 h	*Bacillus amyloliquefaciens* NCC 156	*Lacticaseibacillus paracasei* subsp. *paracasei* NCC 2511
Favored volatiles						
2,3-Butanedione	2.52	Buttery[AB], sweet[B], creamy[B]				
Acetoin	3.95	Pleasant[B], buttery[B], creamy[BC], sweet[D], toasted[D]				
1-Pentanol	5.32	Fermented[B], balsamic[E], alcoholic[F]				
Acetoin acetic acid	8.52	Fruity[B], creamy[B],sweet[B]				
2-Heptanone	8.60	Fruity[G], fora[A], sweet[G]				
1-Octanal	11.76	Fruity[F],citrus[FG], sweet[H]				
Phenylmethanol	12.58	Fruity[B], floral[BI], sweet[B], honey[C]				
Maltol	14.61	Sweet[B], caramellic[B]				
1-Nonanol	16.14	Flora[B], rose[C]				
2-Phenylethanol	14.71	Flora[B], rose[B], sweet[B]				
γ-Heptyl-butyrolactone	20.62	Fruity[B], creamy[B]				
Neutral volatiles						
Acetone	1.93	Fruity[B], solvent[B], ethereal[B]				
Isobutyric acid	5.18	Buttery[B], cheesy[B], sour[B], rancid[B]				
2-Methyl-hexanoic acid	7.85	Cheesy[B], fruity[B], oily[B], sour[C]				
2-Methyl-butanoic acid	7.93	Sour[B], cheesy[B], dirty[B], fermented[B]				
1-Hexanol	8.03	Fruity[BE], lemmon[C], herbaceous[H], nice green[EG]				
1-Heptanal	8.90	Citrus[D], fatty[D], flora[G], rancid[DF]				
Cyclooctane	10.45	Camphoraceous[I]				
Benzaldehyde	10.52	Nutty[H], bitter[H], almond[ACH], sweet[C]				
2-Ethyl-1-hexanol	12.48	Citrus[B], fatty[B], rose[D], green[D]				
1-Octanol	13.59	Fruity[H], flora[H], waxy[B], aldehyde[BC]				
2-Nonanone	14.15	Fruity[G], flora[H], green[D], hot milk[D], soap[D]				
1-Nonanal	14.46	Fruity[H], citrus[CEFG], flora[EFG], fatty[G], green[E]				
Nonanoic acid	18.46	Sweet[F], terpenic[F], cinnamon[F], cheesy[B], waxy[B]				
Unfavored volatiles						
1-Pentanal	3.74	Green[F], grassy[F], nutty[AG], woody[H]				
1-Hexanal	6.07	Green[FGH], grassy[CDFH], nutty[A], fat[D], oxidized oil[J]				
1-Heptanol	10.86	Green[G], mushroom[F], rancid[C]				
1-Octen-3-ol	11.11	Mushroom[ACFGH], fungi[D]				
2-Pentyl-furan	11.45	Beany[ACH], green[D], grassy[J], nutty[AH]				
trans-2-Octenal	13.24	Green[D], vegetable[F], nutty[D], fatty[D]				

○ < 2.5 x 10^6 ○ 2.5 x 10^6 - 5 x 10^7 ○ 5 x 10^6 - 2 x 10^7 ○ 2 x 10^7 - 1 x 10^8 ○ 1 x 10^8 - 2.5 x 10^8

Fig. 18. Aroma development during fermentation of chickpea milk with *L. paracasei* subsp. *paracasei* NCC 2511 and *B. amyloliquefaciens* NCC 156. The data reflect changes in the abundance of volatiles during 24 h of fermentation in comparison to their abundance in non-fermented chickpea milk, incubated under the same conditions (control). Volatile compound analysis was conducted using GC-MS, and compounds were identified based on their mass spectra using the NIST library. The given abundance reflects the mean peak areas from triplicate experiments. Compound classification into favoured volatiles with flora, fruity, sweet, and creamy aroma properties (yellow), neutral volatiles with concentration-dependent desired and undesired aroma properties (light blue), and unfavoured volatiles potentially contributing to the beany, green, and mushroom flavour (dark blue) relates to previous studies and databases on flavour (Kaczmarska et al. 2018;

Li et al. 2014; Lv et al. 2011; Vara-Ubol et al. 2004). RT = retention time. The assigned flavour properties are taken from previous studies and databases: A (Boatright and Lei 1999), B (The Good Scents Company Information System 2021), C (Xu et al. 2019), D (Verginer et al. 2010), E (Sabatini et al. 2008), F (Lv et al. 2011), G (Yang et al. 2008), H (Zeng et al. 2008), I (Sell 2006), J (CAMEO Chemicals 2015). n=3.

B. amyloliquefaciens NCC 156 and *L. paracasei* subsp. *paracasei* NCC 2511 addressed several of these major criteria well. As shown, they increased the L-lysine level by up to 43% (**Fig. 13CJ**), improved the amino acid profile (**Fig. 14**), largely removed indigestible sugars (**Fig. 13EL**), and improved the flavor (**Fig. 18**). Notably, the L-lysine content of chickpea milk fermented by *B. amyloliquefaciens* NCC 156 and *L. paracasei* subsp. *paracasei* NCC 2511 increased from 54 mg/g to up to 65 mg/g protein, surpassing the recommended L-lysine content for 6 months to 3-year child (57 mg/g), according to Food and Agriculture Organization (Consultation and FAO Expert 2011). It indicated that L-lysine is no longer the limiting amino acid of chickpea milk after fermentation.

Based on their performance, they are well-performing microbes for chickpea milk fermentation. The multiple benefits delivered, and the food-grade approval associated with both strains suggest their great potential for use in industry.

In this regard, *B. amyloliquefaciens* NCC 156 and *L. paracasei* subsp*. paracasei* NCC 2511 stand in a prominent line with related microbes. For example, *B. amyloliquefaciens* strains are found in fermented legume-based foods, including doenjang and meju (Dakwa et al. 2005; Lee et al. 2016a), and are often regarded as probiotics due to their beneficial effects on functional food products (Urdaci and Pinchuk 2004). Selected bacilli improved the L-lysine content in soy-based food (Ekwealor and Ebele 2003; Frias et al. 2008b; Teng et al. 2012). *L. paracasei* subsp*. paracasei* NCC 2511 belongs to the group of facultative heterofermentative LAB, which are also widely used in dairy and plant-based milk fermentation (Lee et al. 2013; Lin et al. 2004; Wei et al. 2007) and known to exhibit probiotic properties

(Chiang and Pan 2012; Chuang et al. 2011) and increase L-lysine levels, as shown for soy meal (Song et al. 2008), soybean flour (Frias et al. 2008b), and cowpea milk (Sanni et al. 1999).

The efficient consumption of (also indigestible) oligosaccharides and the accumulation of maltose by *B. amyloliquefaciens* NCC 156 (obviously resulting from degraded starch) revealed its high glycosidic activity involving α-galactosidase, β-fructosidase, and α-amylase (**Fig. 13DE, Table 4**). The enzymatic portfolio, also observed in other bacilli (Aderibigbe and Odunfa 1990; Ouoba et al. 2007), appeared valuable since it improved the digestibility and acceptability of the fermented product. *L. paracasei* subsp*. paracasei* NCC 2511 also exhibited β-fructosidase, α-galactosidase, and α-amylase activity but to a weaker extent (**Fig. 13KL, Table 4**). According to previous studies, the glycolytic capacity among strains of *L. paracasei* subsp*. paracasei* and other lactobacilli is rather strain-specific (Chen et al. 2011; Giraud et al. 1991; Lee et al. 2013; Li et al. 2016; Morlon-Guyot et al. 1998; Sanni et al. 2002).

Interestingly, the two microbes utilized nutrients differently. As a striking example, citrate stimulated the growth and L-lysine production of *B. amyloliquefaciens* NCC 156, which is an interesting finding given that sugars are mainly known to drive L-lysine overproduction (Becker et al. 2008). In contrast, citrate triggered aroma development in *L. paracasei* subsp*. paracasei* NCC 2511. Hereby, the organic acid was obviously converted into four-carbon dicarboxylic acids such as succinate and oxaloacetate via the TCA cycle and potentially also via citrate lyase and then triggered the production of acetate, acetoin, acetate, and smaller amounts of lactate (Hugenholtz 1993; Zotta et al. 2017). *B. amyloliquefaciens*, lacking a citrate lyase pathway, instead used acid for growth.

4.2 Co-cultures of *Propionibacterium freudenreichii* and *Bacillus amyloliquefaciens* cooperatively ferment sunflower seed milk to high levels of vitamin B_{12} and multiple co-benefits

Sunflower seeds (*Helianthus annuus*) emerge as an attractive source for plant-based milk alternatives, given their high ecological sustainability, a natural richness in protein, magnesium, and potassium, and a relatively low abundance in anti-nutritional factors and toxins (Alagawany et al. 2015; Rauf et al. 2020; Škrbić and Filipčev 2008; Villamide and San Juan 1998). In addition, they are nut-free and suit consumers with celiac disease and food allergies. Of particular interest as raw material are the (defatted) press cakes of sunflower seeds, cheap residuals from sunflower oil manufacturing that offer attractive eco-friendliness and economic benefits (de Oliveira Filho and Egea 2021). It is therefore no surprise that sunflower seed suspensions (termed sunflower seed milk in this study due to their milk-like appearance) have found broad application in milk, yogurt, smoothies, and butter, among other products (de Oliveira Filho and Egea 2021; Fleming and Sosulski 1977; Taha et al. 1986). Admittedly, sunflower seed milk does not match animal milk in all desired characteristics, a drawback that is commonly reported for plant-based milk alternatives and requires upgrading (Sethi et al. 2016).

One of the most severe limitations of plant-based products in general, and sunflower seed milk specifically is the lack of vitamin B_{12} (cobalamin). Chemically, vitamin B_{12} is a cobalt-containing tetrapyrrole, one of the most complex small molecules made by nature (Warren et al. 2002). It is crucial for neurodevelopment, cell division, and cell differentiation, and displays the most important micronutrient for vegans to be aware of (Martin 2013; Stabler 2013). Notably, only a few bacteria can make vitamin B_{12} (Survase et al. 2006). The vitamin contains cobalt which is therefore ultimately required for biosynthesis (Roth et al. 1996). Other undesired features of sunflower seed milk are low levels of essential amino acids, especially L-lysine (Flodin 1997),

the presence of indigestible sugars such as stachyose and raffinose (Onyesom et al. 2005), and a bitter, seedy taste (Bao et al. 2020; Heenan et al. 2008). To overcome B_{12} limitation in plant-based materials, direct supplementation of limiting ingredients is a common way (Allen et al. 2010; Watanabe et al. 2014). However, this adds substantial extra costs, and does no longer meet the expectations of consumers, who more and more expect naturally derived, clean-label food and beverages without artificial blending (Tangyu et al. 2019). At this point, fermentation offers an appealing option to naturally increase the nutritional quality and taste of plant-based milk. In particular, mono-culture fermentation of plant-based materials is well established and has been used to increase e.g. vitamin B_{12} levels (Assis et al. 2020; Chamlagain et al. 2016; Xie et al. 2019). Often, however, fermentative synthesis of the vitamin is enabled by undesired non-natural supplementation (Hedayati et al. 2020; Hugenschmidt et al. 2010; Hugenschmidt et al. 2011; Rauf et al. 2020; Xie et al. 2019).

In this chapter, following the great work from the first chapter, we developed a novel co-culture process for the natural fermentation of sunflower seed milk to increase the vitamin B_{12} level. An initial screening round identified potential strains of *P. freudenreichii*, the only generally regarded as safe (GRAS) approved bacterium, known to synthesize active B_{12} (Chamlagain et al. 2016), for fermentative production of the vitamin in sunflower seed milk. Subsequently, metabolic activities of the best performing strain *P. freudenreichii* NCC 1177 were analyzed and provided a detailed signature profile. *B. amyloliquefaciens* NCC 156 and *L. paracasei* subsp. *paracasei* NCC 2511, two microbes found well-performing in chickpea milk, revealed rather complementary features suggesting to co-culture them with the *Propionibacterium* to promote its growth and vitamin B_{12} biosynthetic power. After iterative optimization rounds, co-culture fermentation of *P. freudenreichii* NCC 1177 and *B. amyloliquefaciens* NCC 156 was identified as the best setup. Co-cultivation of

sunflower seed milk by the two strains increased the level of vitamin B_{12} up to 17 µg (100 g)$^{-1}$, almost eight-fold more than initially observed for the *Propionibacterium* alone. Furthermore, the co-culture increased the levels of vitamins B_3 and B_6, and the essential amino acid L-lysine, improved the protein digestibility corrected amino acid score (PDCAAS), decreased indigestible carbohydrate levels, and improved flavor. The developed co-culture approach seems valuable to upgrade sunflower seed milk and potentially also other plant milk materials.

4.2.1 Sunflower seed milk composition

Sunflower seed milk was prepared as an aqueous solution of defatted sunflower seed flour. For batches at a small scale, sterilization of the milk using low-pressure-pasteurization (LPP) provided a sterile, homogenous suspension. Protein represented the largest fraction (57.6% w/w dry mass), followed by soluble carbohydrates (9.3%), and only a low share of fat (0.8%) (**Fig. 19A**). The remaining fraction (32.3%) was attributed to e. g. fibers, ash, and salt. The most abundant non-essential amino acids were L-glutamate (12.6%) and L-aspartate (5.4%). The major essential amino acids were L-leucine (3.8%) and L-valine (3.0%). Soluble carbohydrates comprised digestible (sucrose) and indigestible sugars (raffinose, stachyose). Hereby, sucrose (7.2%) and raffinose (2.9%) showed the highest level, while stachyose was present in a low amount (0.2%). L-Lysine was the most limiting essential amino acid. Related to the protein content, the level of L-lysine was 40 mg (g protein)$^{-1}$, which represented only 69% of the FAO recommended value of 58 mg (g protein)$^{-1}$ (Jeske et al. 2018). In addition, the milk was also ultra-pasteurized at ultrahigh temperature and pressure to represent industrially preferred treatments (Munekata et al. 2020). We therefore processed a 50 kg batch of UHT sunflower seed milk at pilot scale, including pre-warming to 75°C, heating to 143°C for 4 seconds, followed by efficient cooling. Overall, the chemical composition of UHT

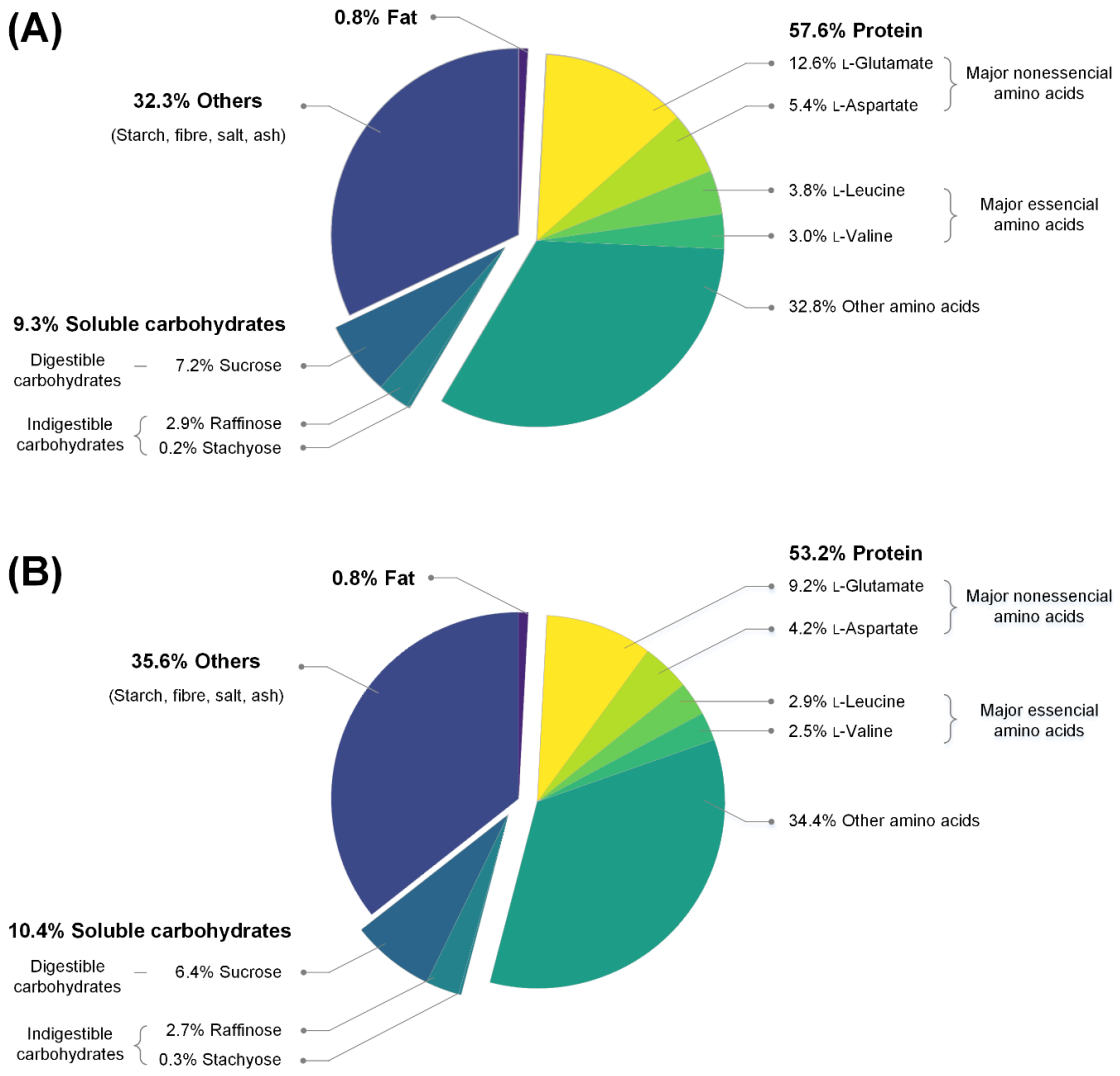

Fig. 19. Composition of lab-scale pasteurized sunflower seed milk and ultra-high temperature processed sunflower seed milk. All values are related to the dry mass (w/w). n = 3

sunflower seed milk was comparable to that of LPP sunflower seed milk (**Fig. 19B)**. Visual inspection revealed a less brownish color of the UHT plant milk, indicating less pronounced Maillard reactions by the shorter heating.

4.2.2 Evaluation of propionibacteria for their vitamin B_{12} production capacity

In an initial round of experiments, food-grade strains of *P. freudenreichii* were screened in MRS medium for their suitability to synthesize and secrete vitamin B_{12}.

Because anaerobic conditions were crucial for the growth of the cells (Thierry et al. 2011), while aerobic conditions had proven beneficial to supporting vitamin B_{12} biosynthesis (Ye et al. 1999), dual-phase cultures were conducted. After two days

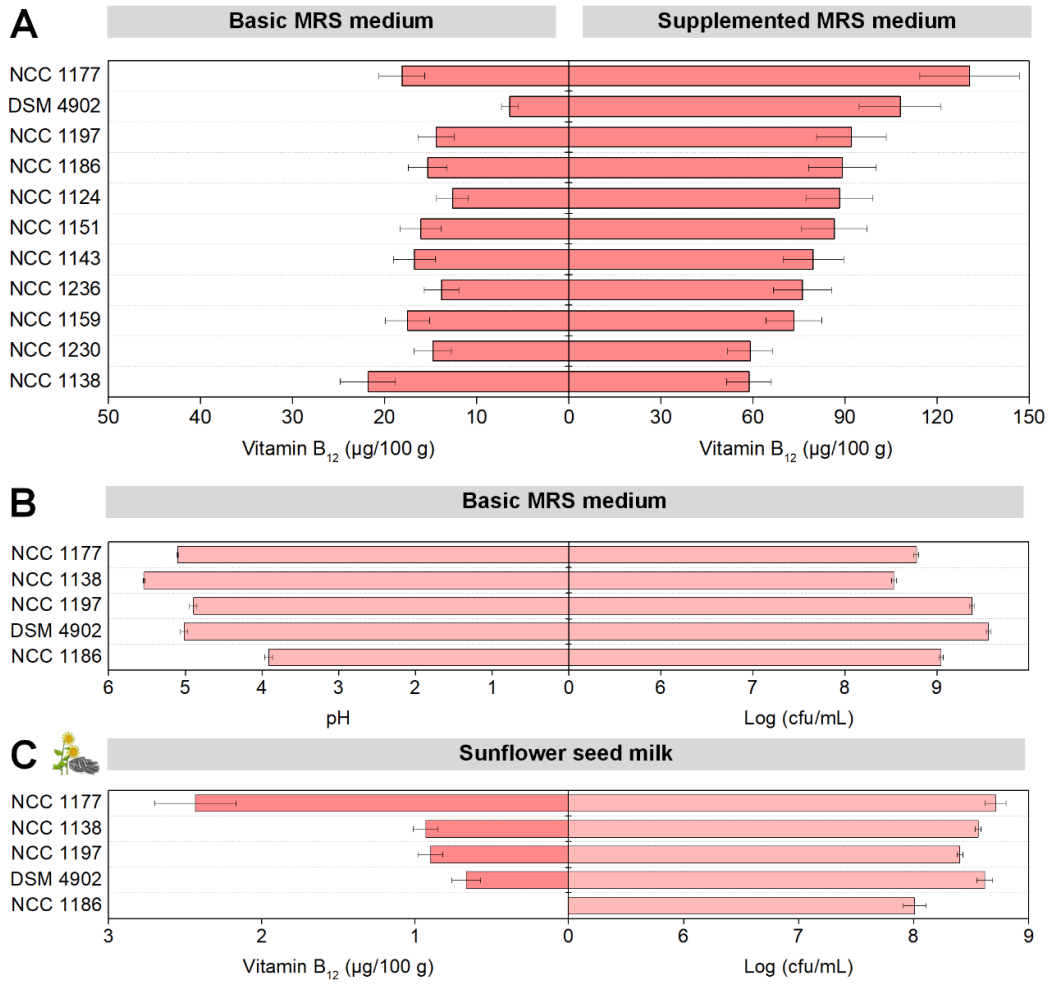

Fig. 20. Screening of food-grade strains of *P. freudenreichii* for vitamin B_{12} synthesis. The data comprise the final titer of vitamin B_{12} in basic MRS medium and in MRS medium, supplemented with 50 µM $CoCl_2$ and 100 µM dimethylbenzimidazole (DMBI) (A). In addition, pH, and growth of selected *P. freudenreichii* strains in MRS medium (B), and vitamin B_{12} production and growth of selected *P.* freudenreichii strains in pasteurized sunflower seed milk medium is shown (C). *P. freudenreichii* DSM 4902 was used as a positive control. The process was carried out as dual phase cultivation with an initial anaerobic phase (48 h), followed by an aerobic phase (24 h). n = 3.

of anaerobic fermentation, the cells were shifted to aerobic conditions for another day. All strains formed the desired vitamin (**Fig. 20A**), whereby the B_{12} level ranged between 6.4 and 21.8 µg (100 g)$^{-1}$. Vitamin B_{12} production in MRS medium was enhanced up to almost 150 µg (100 g)$^{-1}$ by the addition of $CoCl_2$ (50 µM) and DMBI (100 µM) (**Fig. 20A**). Interestingly, the strains differed quite substantially in the extent, to which B_{12} production was activated by the stimulant mixture. Based on the data, five strains appeared most interesting to be taken further: *P. freudenreichii* NCC 1177 (the best producer in supplemented MRS medium), NCC 1138 (the best producer in basic MRS medium), NCC 1197, and NCC 1186 (two strains with pronounced activation potential), and *P. freudenreichii* DSM 4902 (previously reported to be a vitamin B_{12} producer) (Chamlagain et al. 2016).

To better understand their needs and capabilities, we assessed the formed cell concentration, the residual sugar level, and the final pH value. It turned out that the four NCC strains did not completely use up glucose which was likely due to inhibiting acidification of the medium to a pH value around 5.0 – 5.5 (**Fig. 20B**). In contrast, strain NCC 1186 appeared more acid tolerant. The medium pH dropped to 3.9 while the sugar was fully depleted which corresponded to the fact that this strain reached the highest cfu number. Taken together, it seemed that the observed differences in vitamin B_{12} production were not simply due to differences in growth. As an example, NCC 1186 yielded much less of the vitamin than NCC 1177, despite better pH tolerance, sugar uptake, and growth. Based on the data, the five strains were taken further to assess their capability to produce vitamin B_{12} in sunflower seed milk.

4.2.3 *P. freudenreichii* NCC 1177 performs best in sunflower seed milk

The five most promising strains from the screening (**Fig. 20AB**) were now evaluated for their fermentation performance in the sunflower seed milk. When fermented in sunflower seed milk for three days – two days under anaerobic conditions, followed by one day under aerobic conditions – strain NCC 1177 showed by far the best

performance (**Fig. 20C**). The microbe produced 2.4 µg (100 g)$^{-1}$ vitamin B_{12}. In quantitative terms, NCC 1177 accumulated 160% more of the vitamin than NCC 1138, the second-best strain, and up to threefold more than the other three strains. NCC 1177 was also the best grower, as indicated by the high level of living cells. For NCC 1186, weak growth appeared somewhat linked to insignificant vitamin B_{12} formation. The other three strains were medium growers and medium B_{12} producers. The observed differences indicated that growth and B_{12} production in the sunflower seed milk were linked. As an example, NCC 1177 produced 2.5-fold more vitamin B_{12} than NCC 1197, while reaching a 2-fold higher cfu number. This observation suggested that good growth in the sunflower seed milk seemed, at least, one prerequisite for high vitamin B_{12} levels. The best-performing strain, NCC 1177, was selected for further studies.

4.2.4 Limiting factor of Vitamin B_{12} biosynthesis of *P. freudenreichii* NCC 1177

Next, we elucidated the potential to stimulate vitamin B_{12} production of strain NCC 1177 in sunflower seed milk. We tested supplementation with cobalt and DMBI, given their positive effects on vitamin B_{12} synthesis in MRS medium. Furthermore, we tested a range of other supporting ingredients, namely the DMBI precursor riboflavin (vitamin B_2), nicotinamide (vitamin B_3), previously shown to catalytically enhance DMBI formation (Hörig and Renz 1980), different amino acids, incorporated during vitamin B_{12} biosynthesis (L-threonine, L-glutamate, and glycine), and succinate as a donor for succinyl-CoA at the start of the B_{12} pathway. In addition, glucose and lactate were tested as carbon sources to stimulate growth of the *Propionibacterium* and thereby enhance the growth-associated vitamin B_{12} biosynthesis (Hedayati et al. 2020; Lee et al. 1974) (**Table 5**).

The addition of cobalt did not have any significant impact on vitamin B_{12} synthesis (**Table 5**). In addition, growth remained unchanged as well. Likewise, the extra

Table 5. Growth and vitamin B_{12} production of *P. freudenreichii* NCC 1177 during sunflower seed milk fermentation: Impact of different supplements added to the main fermentation process. The fermentation in the supplemented plant milk was carried out at 30°C for 72 hours, including an initial anaerobic phase (48 hours), followed by an aerobic phase (24 hours). In addition, a non-supplemented process was conducted as control. The plant milk was pasteurized prior to fermentation. The vitamin B_{12} level and the cfu number reflect the final values at the end of the fermentation. n=3.

Supplementation	Vitamin B_{12} (µg (100 g)$^{-1}$)	Cell count (log cfu mL^{-1})
No supplementation (control)	2.4 ± 0.3	8.7 ± 0.1
50 µM $CoCl_2$	2.1 ± 0.1	8.6 ± 0.0
40 µM Vitamin B_2	2.9 ± 0.4	8.8 ± 0.1
40 µM Vitamin B_2, 27 µM vitamin B_3	2.9 ± 0.1	8.7 ± 0.0
100 µM Dimethylbenzimidazole (DMBI)	2.3 ± 0.3	9.0 ± 0.1
600 µM L-Glutamate, 100 µM L-threonine, 300 µM glycine, 500 µM succinate	2.2 ± 0.1	8.7 ± 0.0
1% (w/w) Lactate	5.7 ± 0.2	9.4 ± 0.0
1% (w/w) Glucose	3.4 ± 0.2	9.3 ± 0.0
50 µM $CoCl_2$, 40 µM vitamin B_2, 27 µM vitamin B_3, 100 µM DMBI 600 µM L-glutamate, 100 µM L-threonine, 300 µM glycine, 500 µM succinate, 1% (w/w) lactate	12.3 ± 0.6	9.4 ± 0.0

addition of 50 µm $CoCl_2$ to the MRS pre-culture medium to eventually pre-load the cells with cobalt for better fermentation performance did not reveal any stimulating effect (**Table 6**) neither did have the effect of higher concentrations of cobalt (**Table 6**). Obviously, the natural cobalt level of the plant milk (8 µg/kg) was sufficient. Supplementation of the milk with riboflavin (vitamin B_2) increased vitamin B_{12} production by more than 20%, whereas DMBI and nicotinamide addition had no effect. Notably, lactate and glucose were found highly beneficial as extra carbon sources. Their addition resulted in 2.4-fold and 1.4-fold more vitamin B_{12}, whereby the growth of NCC 1177 was markedly increased. In contrast, a mixture of L-threonine, L-glutamate, glycine, and succinate, added at a low level to check for a growth-decoupled contribution to the B_{12} pathway itself, did not trigger vitamin B_{12} production. A combination of all supplements worked best. The mixture resulted in a more than five-fold increased vitamin B_{12} level (12.3 µg (100 g)$^{-1}$, indicating

synergistic effects between a stimulation of growth and a supplementation of vitamin B_{12} precursors (**Table 5**).

Table 6. Growth and vitamin B_{12} production of *P. freudenreichii* NCC 1177 during sunflower seed milk fermentation: Impact of cobalt supplementation during pre-culturing of the microbe. In short, the second pre-culture of strain NCC 1177, conducted under anaerobic conditions in MRS medium, was supplemented with different levels of $CoCl_2$. In addition, a non-supplemented pre-culture was grown as control. The subsequent main fermentation in the plant milk was carried out at 30°C for 72 hours, including an initial anaerobic phase (48 hours), followed by an aerobic phase (24 hours). The vitamin B_{12} level and the cfu number reflect the final values at the end of the fermentation. n=3.

$CoCl_2$ (µM)	Vitamin B_{12} (µg (100 g)$^{-1}$)	Cell count (log cfu mL^{-1})
0	2.4 ± 0.3	8.7 ± 0.1
5	2.5 ± 0.1	8.7 ± 0.0
50	2.1 ± 0.1	8.8 ± 0.1
250	2.0 ± 0.1	8.9 ± 0.1

4.2.5 Metabolic profiling unravels capabilities and inabilities of *P. freudenreichii* NCC 1177

At this stage, it appeared important to study the lifestyle of strain NCC 1177 in more detail and identify the specific needs of the microbe. Cultures of the microbe in sunflower seed milk were conducted under (micro) aerobic (24 h) and under anaerobic (48 h) conditions and analyzed for cell growth and changes in various metabolites: sugars, organic acids, amino acids, and vitamins. Non-inoculated sunflower seed milk served as a control. Under anaerobic conditions, NCC 1177 grew well from 10^7 to almost 10^9 cfu 's (**Fig. 21, Appendix, Table S3**). The strain formed 0.9 µg (100 g)$^{-1}$ vitamin B_{12}.

Interestingly, the microbe had a strong demand for biotin (vitamin B_7) but did not

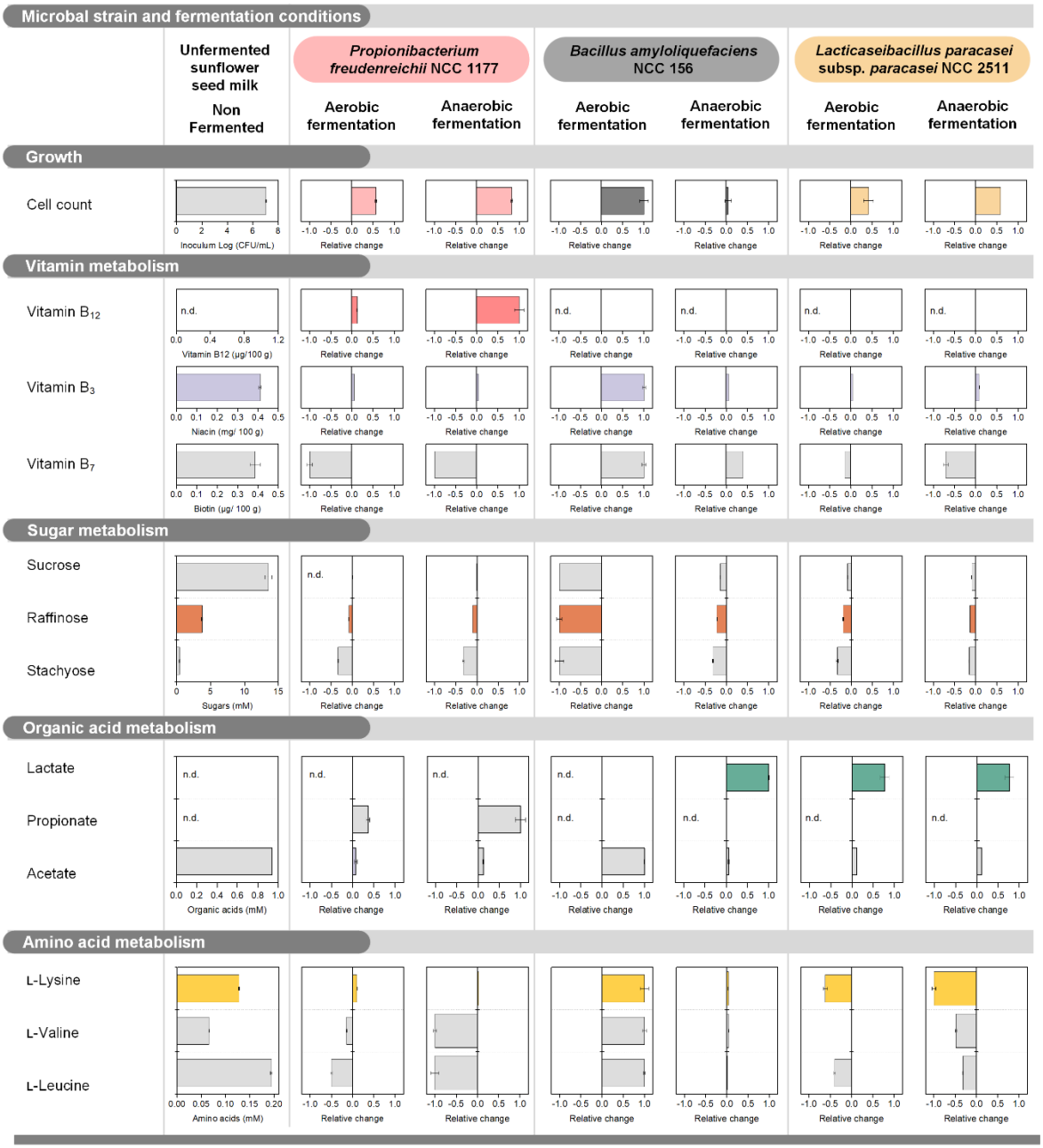

Fig. 21. Metabolic signature profile of *P. freudenreichii* NCC 1177, *B. amyloliquefaciens* NCC 156, and *L. paracasei* subsp. *paracasei* NCC 2511 during aerobic and anaerobic cultivation of pasteurized sunflower seed milk. The data shown display the most relevant characteristics regarding growth, the content of vitamins, sugars, organic acids, amino acids, and flavor formation. The changes of metabolite levels are given as relative values. For each parameter, the maximum absolute concentration change (increase or decrease), observed among all conditions, was set to a value of 1. The changes of the other conditions were normalized to this maximum to allow a straightforward comparison. All corresponding absolute values are provided in **Table 7**, whereby the maximum change, used for the normalization is highlighted. In addition, the starting values

for non-fermented milk are shown. The fermentation was carried out at 30°C either anaerobically (48 h) or aerobically (24 h). n = 3

touch niacin (vitamin B_3), also present in plant-based milk. Furthermore, strain NCC 1177 did not efficiently consume the available sugars. It was not able to utilize sucrose, the most abundant sugar, and consumed only minor shares of raffinose and stachyose. Likewise, acetate remained largely constant. Lactate, an otherwise suitable carbon source was not available in the sunflower seed milk (**Appendix, Table S3**). Instead, the growth of NCC 1177 seemed to rely largely on selected amino acids: L-isoleucine, L-alanine, L-serine, glycine, L-proline, and most strongly L-aspartate were consumed, well matching the catabolic amino acid spectrum of the microbe (Brendehaug and Langsrud 1985). Propionic acid was observed as a prominent by-product, and its occurrence nicely reflected the fermentative routes present in propionibacteria (Brendehaug and Langsrud 1985; Gonzalez-Garcia et al. 2017). These can form propionic acid from amino acids such as L-aspartate via the Wood-Werkman cycle to generate ATP and regenerate oxidized co-enzymes (Falentin et al. 2010).

In comparison, growth under aerobic conditions was much weaker. Cells were sufficiently supplied with dissolved oxygen during the whole cultivation, and the pH value remained relatively stable at around 5.7 (**Fig. 22A**). The amino acids L-aspartate and L-proline were the main growth substrates. Raffinose and stachyose were partially consumed, while sucrose and acetate were not used. Regarding vitamin metabolism, vitamin B_7 was required and vitamin B_{12} was formed. One could conclude at this point that the growth of the *Propionibacterium* suffered from its weak capability to use the organic compounds in the sunflower seed milk as growth substrates. It goes without saying that anaerobic conditions were found optimal. Propionic acid was observed as a prominent by-product.

4.2.6 *B. amyloliquefaciens* NCC 156 and *L. paracasei* subsp. *paracasei* NCC 2511 emerge as potential partners for co-cultures

We now wondered: could another microbe cover the specific needs of *P. freudenreichii* NCC 1177? In previous work (see above), *B. amyloliquefaciens* NCC 156 and *L. paracasei* subsp. *paracasei* NCC 2511 were identified as well-performing strains for chickpea milk fermentation (Tangyu et al. 2021). Although this outcome was based on a different raw material than that used here, the multiple benefits discovered for the two strains (broad substrate range, production of essential amino acids, removal of indigestible sugars) appeared appealing to test them as potential partners for the *Propionibacterium*. To this end, they were both cultivated in sunflower seed milk, once under aerobic and once under anaerobic conditions. *B. amyloliquefaciens* NCC 156 performed best under aerated conditions (**Fig. 21, Appendix, Table S3**). The strain exhibited strong growth (from 10^7 to 10^9 cfu's) and efficiently utilized all major sugars: sucrose, raffinose, and stachyose. Interestingly, it accumulated significant amounts of vitamins B_3 and B_7 (**Fig. 21**). Furthermore, the microbe formed substantial levels of free amino acids (**Table S3**). Due to the obviously high oxygen demand of *B. amyloliquefaciens* NCC 156, the dissolved oxygen level sharply decreased to 0% within 5 hours and remained low until the end of the process (**Fig. 22B**). The pH value dropped from 5.8 to 4.9 after 10 h and increased back to the starting value afterward. Under anaerobic conditions, the growth of NCC 156 was diminished. Small amounts of sucrose (2.0 mM), stachyose (0.5 mM) and raffinose (0.1 mM), were mainly fermented into lactate (1.6 mM) and acetate (1.2 mM) (**Appendix, Table S3**). Vitamins were accumulated, although much less than in the aerated process.

In comparison *L. paracasei* subsp. *paracasei* NCC 2511 grew almost equally well under both conditions (**Fig. 21, Appendix, Table S3**). Under aerobic conditions, it degraded all three sugars and formed lactate as the main by-product plus acetate.

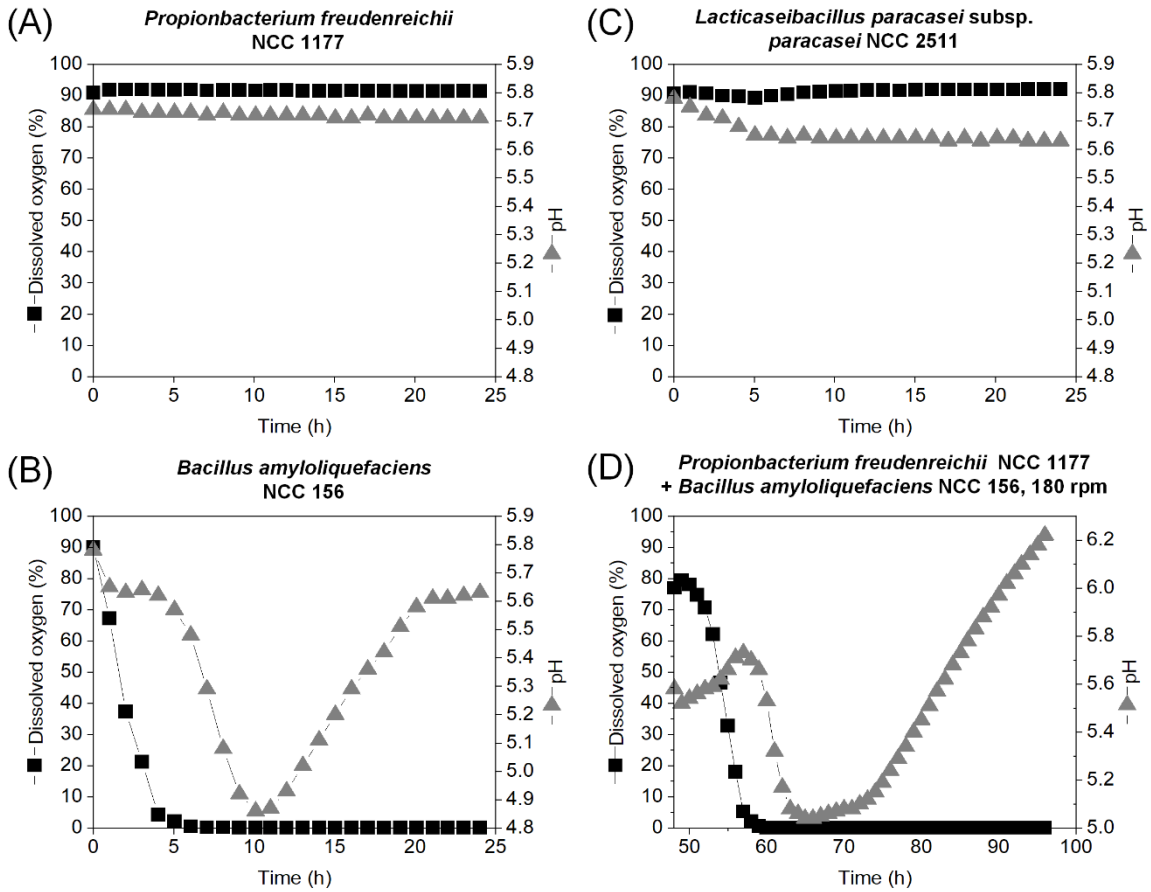

Fig. 22. The dissolved oxygen and pH during the aerobic fermentation for single strain of P*. freudenreichii* NCC 1177 (A), *B. amyloliquefaciens* NCC 156 (B), *L. paracasei* subsp. *paracasei* NCC 2511 (C), and co-fermentation of two strains (D). n=1.

The pH value slightly decreased from 5.8 to 5.5, whereas the dissolved oxygen level remained almost at full saturation (**Fig. 22C**). Moreover, NCC 2511 exhibited a need for vitamin B_7, and largely consumed free amino acids. The most pronounced differences, observed under anaerobic conditions, comprised a shift in substrate use from sugars to amino acids. Again, lactate and acetate were the major products formed.

Neither *B. amyloliquefaciens* NCC 156 nor *L. paracasei* subsp. *paracasei* NCC 2511, of course, formed vitamin B_{12} under any condition.

4.2.7 Co-cultures of *P. freudenreichii* NCC 1177 and *B. amyloliquefaciens* NCC 156 as perfect synergistic partners

As shown, the metabolic signatures of the different microbes appeared highly complementary. As an example, *B. amyloliquefaciens* NCC 156 formed lactate, free amino acids, and vitamin B_7, which were apparently all utilized by the *Propionibacterium* (**Fig. 21, Appendix, Table S3**). NCC 156 furthermore supplied vitamin B_3, potentially stimulating B_{12} production (**Appendix, Table S3**). NCC 2511 formed lactate too. These patterns provoked an interesting question. Could co-culturing of *P. freudenreichii* NCC 1177 with the *Bacillus* and/or the *Lactocaseibacillus* enhance its growth and eventually boost vitamin B_{12} production? Therefore, *P. freudenreichii* NCC 1177 was co-cultured in LPP sunflower seed milk with each strain individually (double co-cultures) and with both strains together (triple co-culture). Like the initial studies, the 72-hour cultures comprised two phases, an initial anaerobic phase (48 hours), followed by an aerobic phase (24 hours).

Grown together with *P. freudenreichii* NCC 1177, *B. amyloliquefaciens* NCC 156 improved vitamin B_{12} production by the *Propionibacterium* remarkably (**Fig. 23A**). In both co-cultures that contained the *Bacillus*, the final level of the vitamin was around 9 µg $(100\ g)^{-1}$, almost four-fold higher than in the fermentation with the *Propionibacterium* alone. The boost in vitamin B_{12} observed in the co-culture was almost as high as observed for NCC 1177 upon full supplementation with cobalt, various precursors, and biosynthetic stimulants (**Table 5**).

How did *B. amyloliquefaciens* NCC 156 boost vitamin B_{12} production? Clearly, it stimulated the growth of the *Propionibacterium* during both phases of the fermentation. The latter achieved a tenfold higher cfu level by the presence of NCC 156 (**Fig. 23B**).

This effect was observable in the double and the triple culture in which both strains were combined. First, NCC 156 formed lactate readily available carbon source for

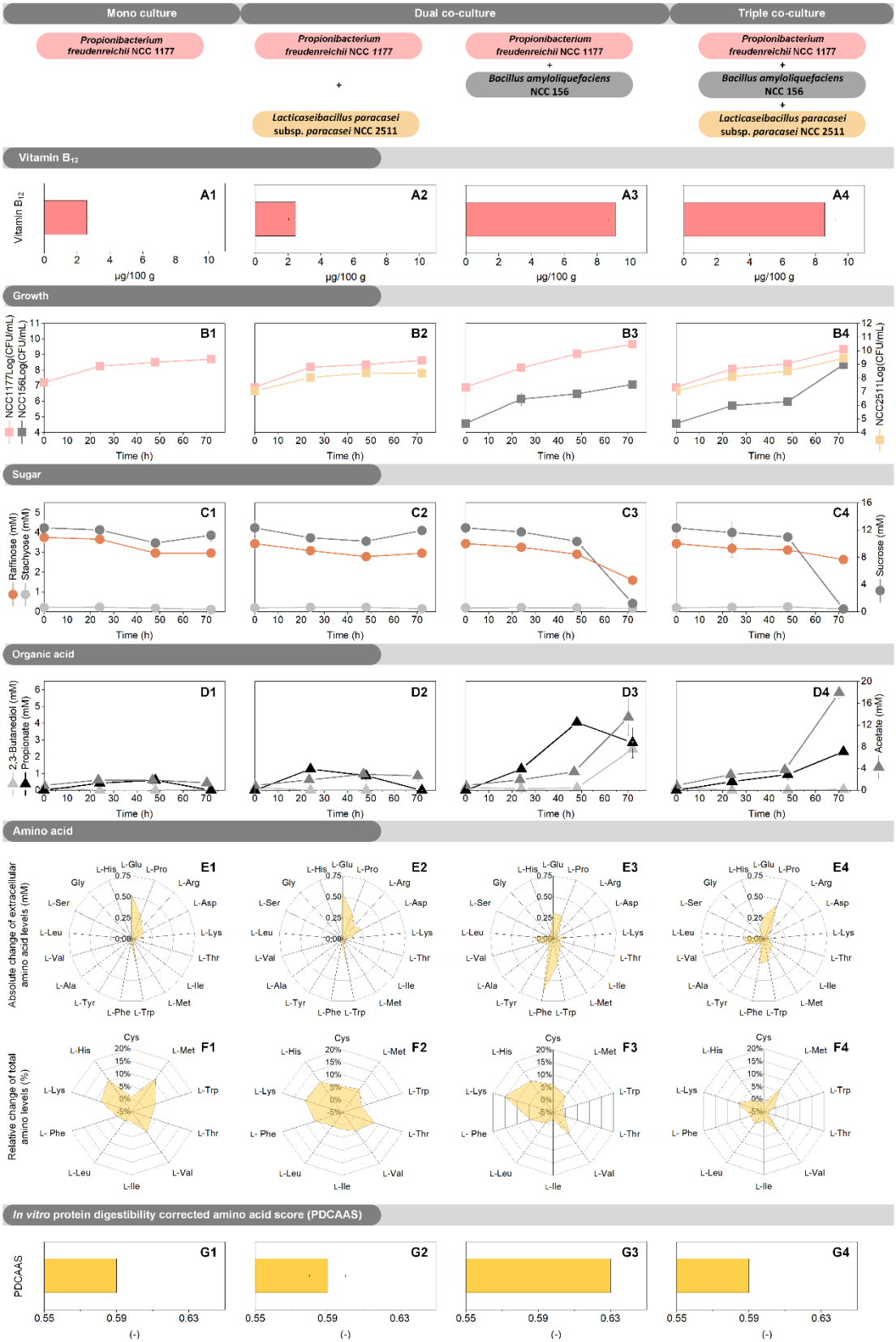

Fig. 23. Performance of co-cultures on sunflower seed milk. The data comprise time-resolved changes for *P. freudenreichii* NCC 1177 (column 1), a dual co-culture of *P. freudenreichii* NCC 1177 and *L. paracasei* subsp. *paracasei* NCC 2511 (inoculated at 1:1

ratio, column 2), a dual co-culture of *P. freudenreichii* NCC 1177 and *B. amyloliquefaciens* NCC 156 (inoculated at 1000:1 ratio, column 3), and a triple co-culture of *P. freudenreichii* NCC 1177, *B. amyloliquefaciens* NCC 156, and *L. paracasei* subsp. *paracasei* NCC 2511 (inoculated at 500:500:1 ratio, column 4). Shown are vitamin B12 content (A), living cell number (B), sugar content (C), organic acid content (D), extracellular amino acid content (E), total amino acid content (F), and protein quality (G), expressed as in vitro protein digestibility corrected amino acid score (PDCAAS). The cultivation was carried out at 30°C for 72 h, including an initial 48 h anaerobic phase, followed by a 24 h aerobic phase. n = 3.

NCC 1177. Lactate itself was not detectable in the mixed cultures which let us conclude that the entire lactate, accumulating in cultures of NCC 156 under anaerobic conditions (**Fig. 21, Appendix, Table S3**), was completely re-consumed by the *Propionibacterium*. Second, NCC 156 provided a rich spectrum of free amino acids that displayed easily accessible carbon for the vitamin B_{12} producer (**Fig. 23E**). NCC 156 furthermore supplied vitamin B_3, potentially stimulating B_{12} production (**Fig. 21, Appendix, Table S3**).

In addition, sunflower seed milk, fermented by the two-strain combination, was largely depleted of the indigestible sugars raffinose and stachyose (**Fig. 23C**, and the overall content of amino acid was substantially enhanced (**Fig. 23F**). The PDCAAS was 0.63, approximately 7% higher than that of all other fermented milks and that of the native plant milk (**Fig. 23G**).

Subsequent experiments aimed at optimizing the performance of this promising two-strain combination. Hereby, the inoculum ratio was identified as a crucial parameter. A 1,000-fold excess of *Propionibacterium* over *Bacillus* cells resulted in a marked improvement of vitamin B_{12} production, whereby growth itself was not affected (**Table 7**).

In contrast, *L. paracasei* subsp. *paracasei* NCC 2511 did not improve vitamin B_{12} production when added. It neither stimulated the *Propionibacterium* directly nor provided an indirect benefit in the triple co-culture.

4.2.8 Scaling the collaboration of co-cultures to pilot scale processed sunflower seed milk

Among all tested combinations, the co-culture of *P. freudenreichii* NCC 1177 and *B. amyloliquefaciens* NCC 156 seemed to work the best. It achieved the highest level of vitamin B_{12}, the lowest level of indigestible sugars, and the highest protein quality score – and thereby required only three things: natural sunflower seed milk and the two microbes. It was therefore selected for further optimization.

Table 7. Growth and vitamin B_{12} production during co-culture fermentation of *P. freudenreichii* NCC 1177 and *B. amyloliquefaciens* NCC 156 in sunflower seed milk: Impact of inoculum level and fermentation sequence. In different set-ups, strain NCC 1177 was inoculated at a 10-fold, 100-fold and 1,000-fold higher level than strain NCC 156. In all cases, the total inoculum of both strains was 2×10^7 cfu mL^{-1}. Regarding the fermentation sequence, one set-up comprised first a 24 h aerobic phase, followed by a 48-h anaerobic phase, whereas the two phases were reverted in a second set-up. All fermentations were carried out at 30°C. The plant milk was pasteurized prior to fermentation. Vitamin level and cell growth display the final values after 72 h. n=3.

Fermentation sequence	Inoculation ratio NCC 1177:NCC 156	Vitamin B_{12} (µg (100 g)$^{-1}$)	*P. freudenreichii* NCC 1177 (log cfu mL^{-1})	*B. amyloliquefaciens* NCC 156 (log cfu mL^{-1})
24 h aerobic + 48 h anaerobic	10:1	2.5 ± 0.2	8.9 ± 0.0	7.6 ± 0.1
	100:1	3.5 ± 0.3	9.1 ± 0.2	7.0 ± 0.0
	1000:1	3.8 ± 0.2	9.2 ± 0.1	6.7 ± 0.4
48 h anaerobic + 24 h aerobic	10:1	6.3 ± 0.8	9.9 ± 0.1	9.3 ± 0.1
	100:1	7.6 ± 0.5	10.0 ± 0.1	9.8 ± 0.1
	1000:1	9.1 ± 0.4	9.7 ± 0.1	9.8 ± 0.1

The fermentation capacity of the co-culture of NCC 1177 and NCC 156 was now compared for the differently pre-treated milks, the LPP and the UHT processed one respectively, again using the workflow of three days with two days anaerobic and one day aerobic incubation. Notably, the UHT milk performed excellent and yielded almost 40% more vitamin B_{12} than the lab-scale LPP milk, i. e. a final level of 13 versus 9 µg $(100\ g)^{-1}$ (**Fig. 24A**).

4.2.9 Fermentation optimization: tuning of aeration and fermentation time

Next, the influence of the dissolved oxygen level (during the second aerobic phase of the fermentation process) was evaluated. It turned out that a balanced oxygen supply was crucial to achieving a high vitamin B_{12} content (**Fig. 25A**). Too little aeration resulted in up to 26% lower titers, but too strong oxygen supply also reduced production. Furthermore, the oxygen supply influenced growth of both microbes, *B. amyloliquefaciens* NCC 156 and *P. freudenreichii* NCC 1177 (**Fig. 25B**), and the production of the vitamins B_2, B_3, and B_6 (**Fig. 25D**), indicating a complex interplay. The specific vitamin B_{12} production per single cell, estimated from the data, revealed that NCC 1177 was most productive without oxygen exposure (**Fig. 25C**). However, also high oxygen supply resulted in a good performance. Under these conditions, the growth of *B. amyloliquefaciens* NCC 156 was strongest. The microbe supplied elevated levels of vitamins B_2 and B_3, known to stimulate B_{12} formation (Hörig and Renz 1980; Roth et al. 1996). Overall, the oxygen influence on vitamin B_{12} production appeared complex. Its impact likely included stimulation of growth of NCC 1177 and NCC 156 but also activation of the B_{12} biosynthetic pathway itself. One piece of the underlying complexity is the response of *P. freudenreichii* to oxygen. The microbe is known to be anaerobic to aerotolerant (Bücher et al. 2021), grows best anaerobically but is capable to generate energy also under microaerobic conditions using the TCA cycle and functional electron transport chains supplying the right amount of oxygen, it appears relevant to resolve

this picture on the metabolic level in more detail in the future. Clearly, oxygen availability emerged as a crucial factor to boost performance.

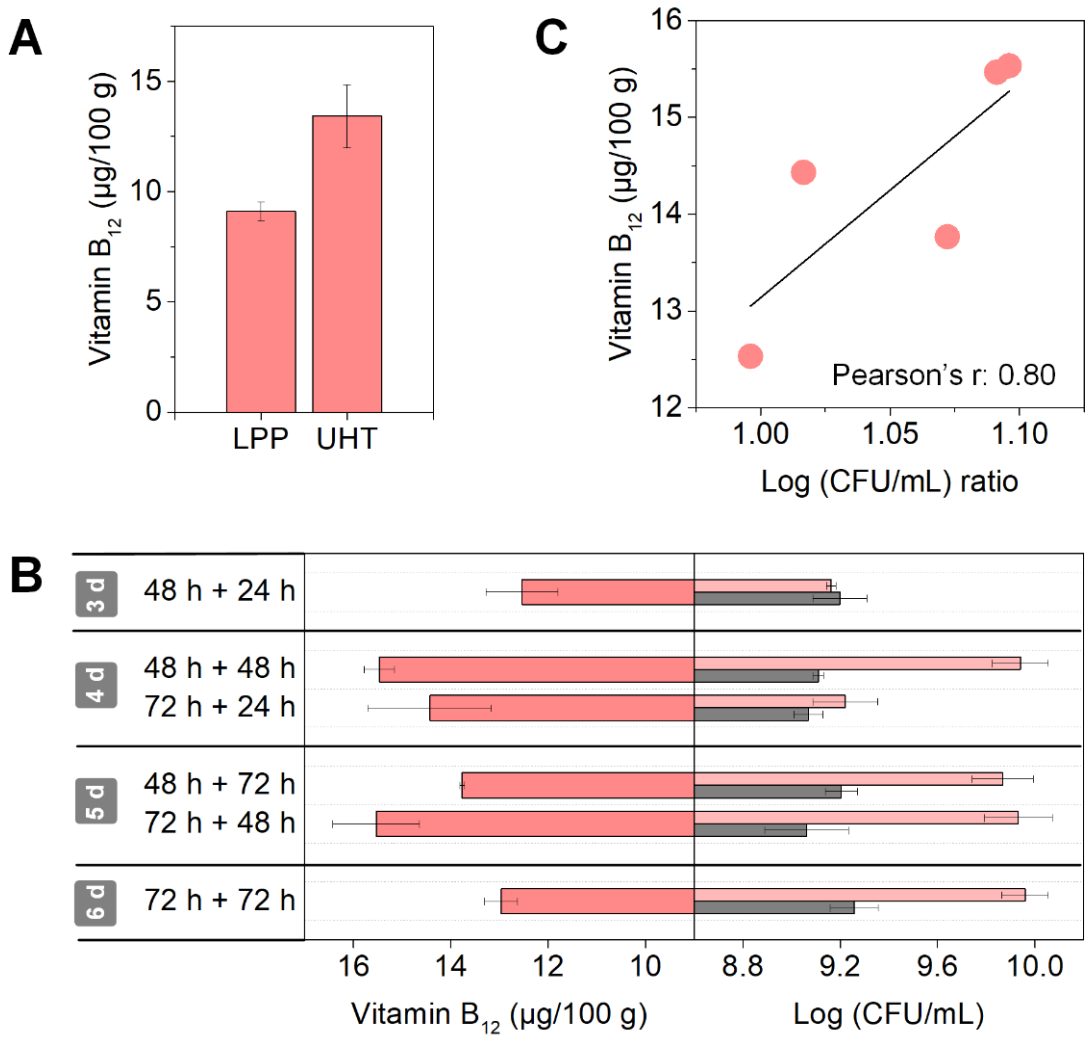

Fig. 24. Impact of raw material pre-processing and inoculation ratio on the fermentation of sunflower seed milk by a co-culture of *P. freudenreichii* NCC 1177 and *B. amyloliquefaciens* NCC 156. Production of vitamin B_{12} on low pressure pasteurized (LPP) and ultra-high temperature (UHT) treated milk (A). Impact of the fermentation set-up with varied aerobic and anaerobic phases on cell growth and vitamin B_{12} production (B). Impact of the ratio between the strains on vitamin B_{12} production (C). For the latter, the numbers reflect the cfu ratio between NCC 1177 and NCC 156 at the end of the process. n = 3

Optimum vitamin B_{12} production was as high as 14.8 µg (100 g)$^{-1}$ and occurred under three different aeration regimes, indicating a robust process window. Finally, we tested different set-ups, regarding the duration of the process (**Fig. 24B**). A slightly prolonged aerobic fermentation phase allowed to increase vitamin B_{12} production even further. Notably, the vitamin B_{12} level of the fermented sunflower seed milk appeared proportional to the final cfu ratio between *P. freudenreichii* NCC 1177 and *B. amyloliquefaciens* NCC 156 (**Fig. 24C**, r = 0.80). A dual-phase fermentation with two days anaerobic and then two days aerobic incubation resulted in almost 16 µg (100 g)$^{-1}$ vitamin B_{12}. Similarly performance was a three-plus-two-day setup.

4.2.10 Functional interactions between *P. freudenreichii* NCC 1177 and *B. amyloliquefaciens* NCC 156

The optimized fermentation process was now studied over 96 hours, regarding the dynamics of strain growth, and the metabolism of sugars, amino acids, organic acids, vitamins, and flavor compounds (**Fig. 26**). Fermented sunflower seed milk appeared best in composition after 80 h (**Fig. 27**). The initial anaerobic phase was the major growth phase of NCC 1177. After inoculation, the *Propionibacterium* immediately started to proliferate, and its cfu number increased about 30-fold over the first 48 h (**Fig. 27A**). Cells of NCC 156 multiplied almost 100-fold during the first 24 h. Notably, the growth of NCC 156 resulted in an increased level of vitamin B_7 (29.4%), which was afterward consumed by NCC 1177 (**Fig. 27C**). In addition, the PDCAAS was slightly improved (14%) (**Fig. 27H**). Propionate (3.9 mM), acetoin (3.5 mM), and (Beck and Schink 1995; Dank et al. 2021; Emde and Schink 1990; Schwartz and Sporkenbach 1975). Due to the huge optimization potential of acetate (3.1 mM) were major fermentation by-products during the anaerobic phase (**Fig. 27G**). An interesting picture resulted for the substrates. Sucrose was consumed from early on (by NCC 156, because NCC 1177 could not use it). After 30 h, the sucrose level

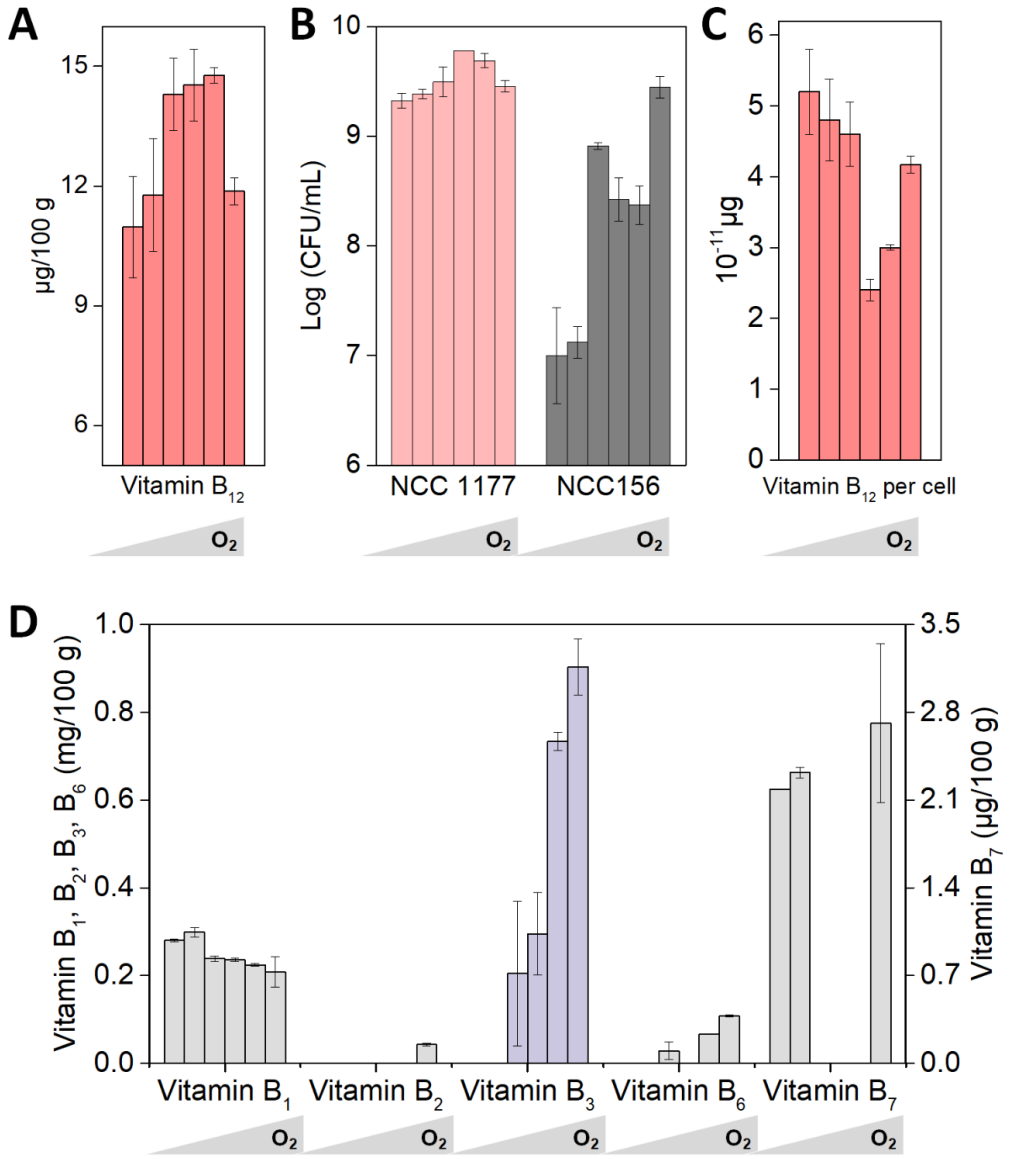

Fig. 25. Impact of the dissolved oxygen level on the fermentation of sunflower seed milk by a co-culture of *P. freudenreichii* NCC 1177 and *B. amyloliquefaciens* NCC 156. Impact of oxygen supply on vitamin B_{12} production (A), growth (B), vitamin B_{12} production per cell (C), and the production of vitamins B_1, B_2, B_3, B_6, and B_7 during fermentation (D). Prior to fermentation, the milk was processed by ultra-high temperature treatment. The different aeration regimes from low to high (shown from left to right) were created by incubation in non-baffled flasks at 80 rpm, baffled flasks at 80 rpm, non-baffled flasks at 130 rpm, baffled flasks at 130 rpm, non-baffled flasks at 180 rpm and baffled flasks at 180 rpm. The inoculum ratio between strains NCC 1177 and NCC 156 was 1000:1. Fermentation was carried out at 30°C for 72 h, including an initial 48 h anaerobic phase, followed by a 24 h aerobic phase. n = 3

remained constant, while raffinose started to be consumed. The metabolization of the trisaccharide formed sucrose (superimposing the probably on-going consumption by NCC 156) plus galactose (Hobbs et al. 2019). Galactose displayed well-accessible carbon (Chen et al. 2007) and seemed directly consumed, as it could not be detected. As shown for the monoculture, the fermentation of sugar by *B. amyloliquefaciens* yielded lactate (**Fig. 21**). However, different from the monoculture, the organic acid was not observed. We conclude that lactate was immediately taken up as the preferred carbon source by the *Propionibacterium*. In addition, the *Propionibacterium* used a range of amino acids during this stage (**Appendix, Table S4**). Moreover, the anaerobic fermentation beneficially changed the flavor profile (**Fig. 27D**). With the onset of aeration after 48 h, the metabolism of the co-culture immediately changed. Growth of NCC 156 was strongly stimulated. NCC 1177 continued to grow, and the number of living cells increased once more about 10-fold (**Fig. 27A**). Notably, the aerobic phase was the major phase of vitamin B_{12} production. After 80 h, the vitamin had reached a high level of 15.2 µg $(100\ g)^{-1}$ (**Fig. 27B**). Notably, also other B vitamins were formed including vitamin B_3 (0.5 mg $(100\ g)^{-1}$) and vitamin B_6 (70 µg $(100\ g)^{-1}$) (**Fig. 27C**). NCC 156 quickly consumed sucrose. After 60 h, when sucrose was almost depleted, co-consumption of the disaccharide together raffinose and stachyose was observed (**Fig. 27E**). With the depletion of sucrose after 66 h, propionate, acetoin, and 2,3-butanediol started to get consumed (**Fig. 27G**), while raffinose and stachyose degradation continued (**Fig. 27E**). The transient increase of the raffinose level seemed due to the onset of stachyose metabolism, which yields raffinose plus galactose during initial cleavage (Hobbs et al. 2019), a similar picture as observed for sucrose and raffinose earlier during the process. Regarding propionate, certain *Bacillus* strains can use it as a carbon source but *B. amyloliquefaciens* cannot (Dworkin et al. 2006). In fact, the *Propionibacterium* re-used its own previously synthetized propionate, as also

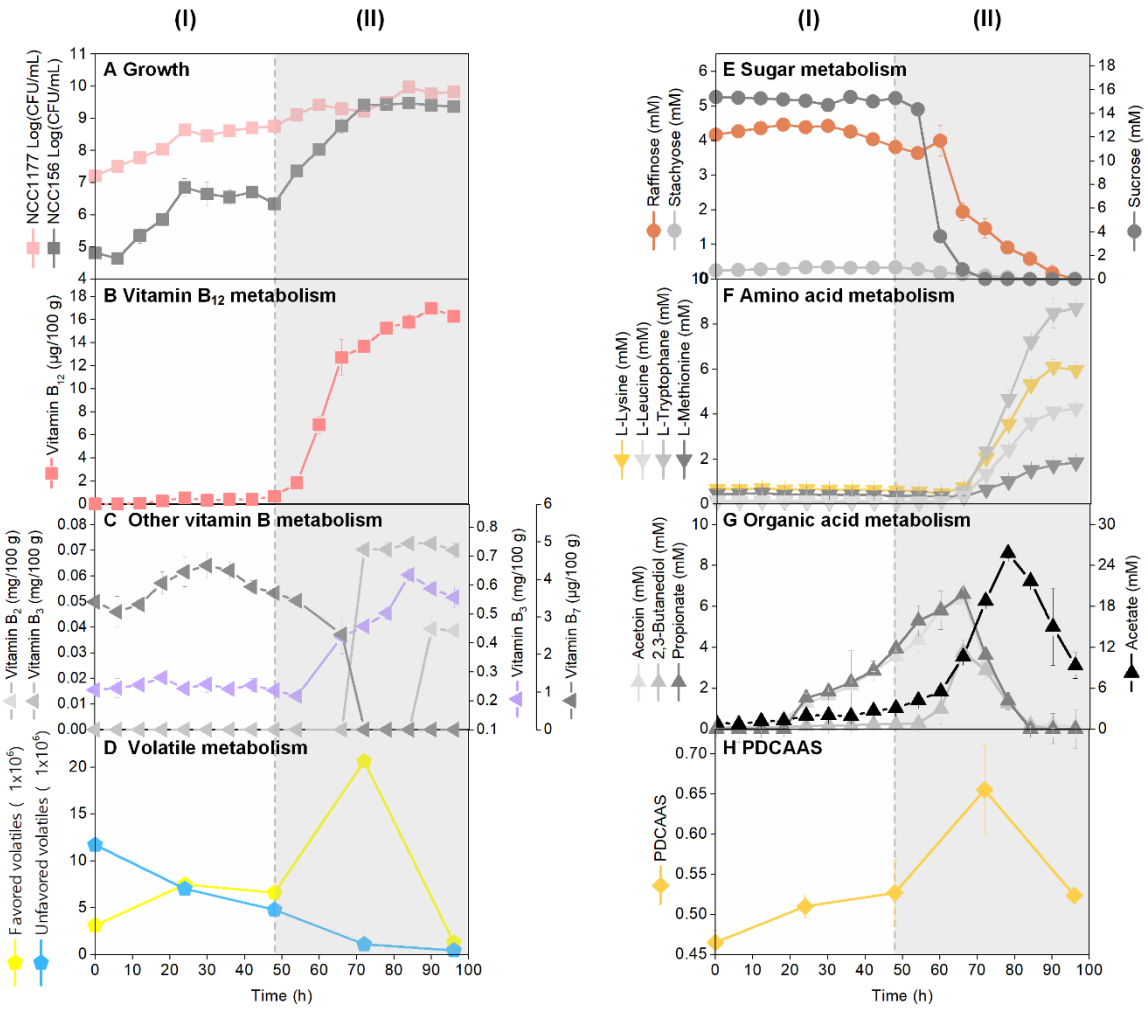

Fig. 26: Time-resolved 96 h co-fermentation of sunflower seed milk by using *P. freudenreichii* NCC 1177 and *B. amyloliquefaciens* NCC 156. The data comprise living cell number (cfu) (A), vitamin B_{12} (B), vitamin B_3, B_6, and B_7 (C), the aera of favored and unfavored volatile (D), sucrose, raffinose, and stachyose (E), extracellular L-lysine, L-leucine, L-tryptophan, and L-methionine (F), acrtoin, 2,3-butanediol, propionate, and acetate (G), PDCAAS (H). The phases of anaerobic (I) and aerobic (II) incubation are indicated in the time profile. The groups of favored and non-favored volatiles were assigned from the flavor properties of the individual compounds (see legend of **Fig. 28**). n=3.

observed in the monoculture (**Fig. 23**). In the absence of other substrates, propionic acid is metabolized by *P. freudenreichii* using the reversed Wood–Werkman cycle (Turgay et al. 2022). This seemed the case here: NCC 1177 could not use the remaining sugars and could no more use lactate, because NCC 156 did not form it

anymore under the aerobic conditions (**Fig. 21**). Therefore, the *Propionibacterium* had to rely on organic acids or amino acids instead.

Overall, the co-fermentation showed an outstanding capacity to degrade indigestible sugars: raffinose (78.2%) and stachyose (78.8%) were largely depleted. Acetate

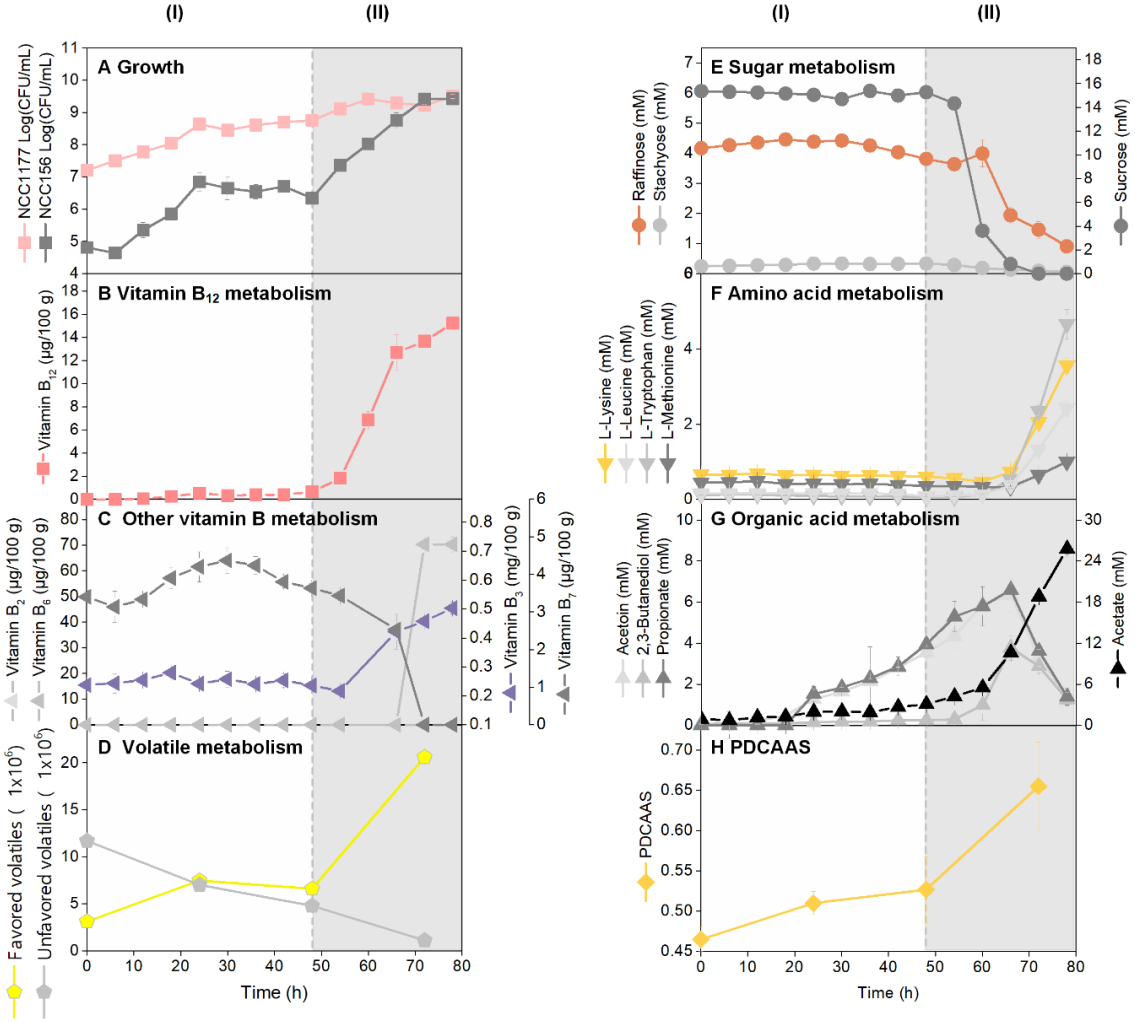

Fig. 27. Dynamics of co-culture fermentation of *P. freudenreichii* NCC 1177 and *B. amyloliquefaciens* NCC 156 on UHT pre-treated sunflower seed milk. The data comprise living cell number (cfu) (A), vitamin B_{12} (B), vitamin B_3, B_6, and B_7 (C), the area of favored and unfavored volatile (D), sucrose, raffinose, and stachyose (E), extracellular L-lysine, L-leucine, L-tryptophan, and, L-methionine (F), acetoin, 2,3 butanediol, propionate, and acetate (G), PDCAAS (H). The phases of anaerobic (I) and aerobic (II) incubation are indicated in the time profile. The groups of favored and non-favored volatiles were assigned from the flavor properties of the individual compounds (see legend of **Fig. 28**). n = 3.

(25.8 mM) was the major organic acid after 80 h. Remarkably, free extracellular amino acids, including L-lysine, L-methionine, and L-tryptophan, essential amino acids which are often limited in plant-based materials, increased during the final cultivation phase (**Fig. 27F**). This accumulation improved the PDCAAS to 0.66 (**Fig. 27H**). The amount of favored flavor compounds (inferred from the total peak area during GC-MS analysis) increased three-fold times during the aerobic phase, while unfavoured volatiles were almost depleted (90.6%) (**Fig. 27D**). A continuation of the fermentation process to finally 96 h, resulted in an even higher vitamin B_{12} level (17.0 µg (100 g)$^{-1}$), and increased levels of vitamin B_2, and extracellular amino acids, however, linked to a somewhat the decreased flavor value and a reduced PDCAAS (**Fig. 26**).

In the optimized co-culture fermentation process, several synergistic effects could be detected: (i) both strains showed better growth; (ii) vitamin B_{12} production of *P. freudenreichii* increased more than 7-fold as compared to mono-fermentation (17.0 µg (100 g)$^{-1}$); (iii) the PDCAAS was improved (29%), (iv) the level of L-lysine, the most limiting essential amino acid, was increased by 43%, (iv) the level of vitamins B_3 (0.5 mg (100 g)$^{-1}$) and B_6 (70 µg (100 g)$^{-1}$) was increased, and (v) the overall flavor profile was improved. Without a doubt, the rich spectrum of benefits originated from microbial collaboration.

From a biochemical perspective, the successful synthesis of vitamin B_{12} relies on several factors. First, and important to note here, vitamin B_{12} production in *P. freudenreichii* (including strain NCC 1177) is growth-associated, making accessible and growth-promoting substrates one of the determinants (Hedayati et al. 2020). Furthermore, cobalt and DMBI drive B_{12} production (Hugenschmidt et al. 2011), while L-glutamate, glycine, L-threonine, and succinyl-CoA display the building blocks of the vitamin (Fang et al. 2017). Earlier studies with *P. freudenreichii* cell homogenates showed that DMBI was derived from vitamin B_2 (riboflavin) (Lingens

et al. 1992) and that the biosynthesis process was stimulated by vitamin B_3 (nicotinamide) (Hörig and Renz 1980). Here, we obtained a complex picture when trying out the effect of supplements on vitamin B_{12} production by NCC 1177 during sunflower seed fermentation. As shown, the addition of lactose, glucose, and riboflavin boosted vitamin B_{12} formation, while cobalt, DMBI, nicotinamide, L-glutamate, glycine, L-threonine, and succinate did not. These findings implied that major limiting factors were weak growth of *Propionibacterium* and the low availability of vitamin B_2. A combination of all supplements resulted in a more than five-fold increased vitamin B_{12} level, indicating the benefits of a more fine-tuned synergy regarding growth and supplementation.

On basis of the elaborated microbe's physiology, growth and vitamin B_{12} biosynthesis were limited by several factors: (i) *P*. *freudenreichii* could only use extracellular amino acids but none of the sugars present in sunflower seed milk (**Fig. 21**); (ii) *P*. *freudenreichii* required micronutrients such as biotin (vitamin B_7) to grow (**Fig. 21**) (Lichstein 1955); (iii) the microbe formed propionic acid causing an inhibiting pH decrease (Furuichi et al. 2006); (iv) vitamin B_{12} biosynthesis was limited by insufficient availability of vitamin B_2. Considering these requirements, *B. amyloliquefaciens* emerged as a perfect partner for *P. freudenreichii*, providing a natural dual culture consortium, co-working for improved growth, and finally yielding a multi-benefit fermentation. Proven interactions during the first phase included the donation of accessible carbon and essential micronutrients by NCC 156, stimulating the growth of NCC 1177. During the second (aerobic) phase of the fermentation, *B. amyloliquefaciens* quickly proliferated. It released free amino acids from the protein and eventually lowered the dissolved oxygen level in a beneficial way, further stimulating the growth of *P. freudenreichii.* In this regard, the ability of the *Bacillus* to form carbohydrates and proteinases and provide accessible carbon appeared crucial. At the same time, the *Bacillus* produced vitamin B_2, vitamin B_3, and vitamin

B_7, directly supporting vitamin B_{12} synthesis. We conclude that microbial cooperation more than compensates for a lack in key nutrients and adverse physicochemical conditions in plant-based materials, given that well-collaborating microbes are put together. Their careful selection and combination, as shown here, appears crucial for the success.

As shown, co-fermentation of NCC 156 and NCC 1177 was strikingly superior to that of the single strains. It stands in line with a range of successful co-fermentations, reported previously for food manufacturing, for example, to produce yogurt, wine, and aroma-rich cocoa beans (Adler et al. 2014; Ciani and Comitini 2015; Sieuwerts et al. 2008). Co-fermentation of *P. freudenreichii* with the fungus *Rhizopus oryzae* on lupin tempeh yielded 20-fold more vitamin B_{12} than *P. freudenreichii* alone. Interestingly, the major interaction between these two microbes was hydrolysis of the seed protein by *R. oryzae*, releasing free amino acids to support growth of *P. freudenreichii* (Signorini et al. 2018). *Propionibacterium* also showed increased capacity to produce vitamin B_{12}, when co-fermented with *Rhizopus oligosporus* on soybean (Krusong et al. 1991), with kefir grains (Van Wyk et al. 2011), and with different lactobacilli in wheat bran (Xie et al. 2019) and whey(Hugenschmidt et al. 2010; Hugenschmidt et al. 2011; Xie et al. 2021). However, no synergistic effects on vitamin B_{12} production could be unraveled, when mixing *Propionibacterium* with lactobacilli (Xie et al. 2021).

4.2.11 Flavor development during co-culture fermentation

GC-MS-based analysis revealed a strong impact of co-fermentation on the spectrum of flavor-related volatiles (**Fig. 28**). In total, 34 volatiles were identified in unferment--ed and fermented sunflower seed milk, including various saturated and unsaturated organic alcohols, aldehydes, ketones, organic acids, terpenoid, lactones, and furans.

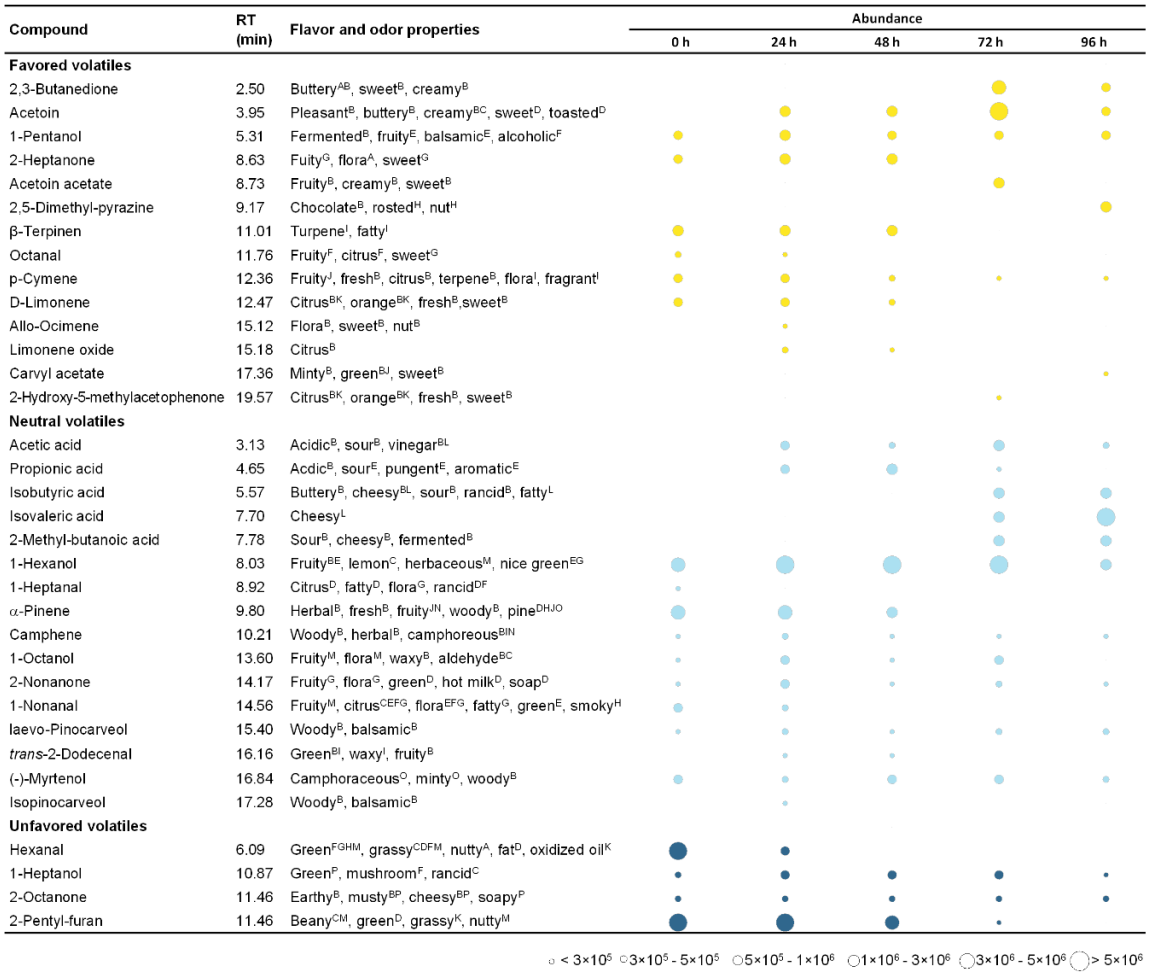

Fig. 28. Flavor formation of food-grade microbes during sunflower seed milk fermentation. The data for flavor volatiles reflect the change in abundance in comparison to non-fermented sunflower seed milk (control). Classification into favored volatiles with flora, fruity, sweet, and creamy aroma properties (yellow), neutral volatiles with concentration dependent desired and non-desired aroma properties (light blue), and unfavored volatiles ,potentially contributing to the beany, green, and mushroom flavor (dark blue) relates to previous dedicated studies and databases on odor and taste (Aznar et al. 2001; Boatright and Lei 1999; Costa et al. 2008; Duar et al. 2017; Fu et al. 2020; Giri et al. 2010; Högnadóttir and Rouseff 2003; Lv et al. 2011; Ounamornas et al. 2017; Sabatini et al. 2008; The Good Scents Company Information System 2021; Van Opstaele et al. 2012; Verginer et al. 2010; Xu et al. 2019; Yang et al. 2008; Zeng et al. 2008). RT = retention time. The flavor properties are taken from previous studies and databases: A (Boatright and Lei 1999), B (The Good Scents Company Information System 2021), C (Xu et al. 2019), D (Verginer et al. 2010), E (Sabatini et al. 2008), F (Lv et al. 2011), G (Yang et al. 2008), H (Fu et al. 2020), I (Ounamornas et al. 2017), J (Högnadóttir and Rouseff 2003), K (Duar et al. 2017), L (Aznar et al. 2001), M (Zeng et al. 2008), N (Van Opstaele et al. 2012), O (Costa et al. 2008), P (Giri et al. 2010). n=3.

In unfermented sunflower seed milk, 1-hexanal and 2-pentyl-furan, which are volatiles with grassy and beany flavor, dominated, while sweet and fruity aroma compounds (e.g. 2-heptanone, β-terpinene, p-cymene, and D-lemonene) were present in a lower amount. Co-fermentation with *P. freudenreichii and B. amyloliquefaciens* changed the flavor profile significantly (**Fig. 28**). Several valuable volatiles were generated after 24 h, such as acetoin, 1-pentanol, allo-ocimene, limonene oxide, acetic acid, propionic acid, 1-octanol, 2-nonanone, trans-2-dodecanal, isopinocarveol, and 1-haptanol. After switching to aerobic conditions (48 h-72 h), acetoin and 2,3-butanediol, yielding buttery and fruity notes, were formed. Notably, the largest area of favored volatiles was detected after 72 h fermentation. Unfavoured compounds such as hexanal and 2-pentyl-furan were completely removed after 48 h and 72 h of fermentation, respectively.

Several fermented foods such as natto and dawadawa rely on the participation of various *Bacillus* species, which generated unique and characteristic aroma and sensory properties. Moreover, the hydrolytic capabilities of *Bacillus* result in a precursor-rich environment, which could be used for flavor production (Beaumont 2002). However, the aromas generated by *Bacillus* are often related to ‘‘meaty”, “fishy”, and “grilled”, which may be recognized as “off-flavor” for plant milk products. A careful selection of the matrix and optimization of fermentation conditions helped to soften the flavor of *B. amyloliquefaciens* and increased consumer acceptance.

4.2.12 Impact of co-fermentation and fermented sunflower seed milk

Industrial impact of naturally fermented sunflower seed milk, rich in vitamin B_{12}

As shown, co-fermentation of two carefully selected food-grade microbes provided sunflower seed milk which contained up to 17 µg $(100\ g)^{-1}$ vitamin B_{12}, accompanied

by concurrently increased levels of vitamins B_3 and B_6, improved protein quality and flavor profile, and strongly reduced amounts of indigestible sugars.

Vitamin B_{12} is one of the most important micronutrients for the human body but is unfavorably absent from plant-derived food (Watanabe et al. 2014), including non-fermented sunflower seed milk (this work). Therefore, vegetarians can face vitamin B_{12} deficiency regardless of their demographic characteristics, place of residency, age, and type of vegetarian diet (Watanabe et al. 2014). Vitamin B_{12} deficiency can have severe consequences. It leads to increased homocysteine levels in the blood, a recognized risk factor for atherothrombotic and neuropsychiatric disorders (Stabler 2013; Watanabe et al. 2014). Of specific impact at this moment, vitamin B_{12} emerges as an active ingredient against COVID-19 symptoms and SARS-CoV-2 infections (Shakoor et al. 2021). A recent study showed that methyl-cobalamin supplements help to reduce COVID-19-related organ damage and other symptoms (dos Santos 2020). A clinical study conducted in Singapore showed reduced COVID-19 symptoms in patients who received a daily supplementation with vitamin B_{12}, vitamin D, and magnesium, significantly reducing the need for oxygen and intensive care support (Tan et al. 2020). Thus, the demand for safe and cost-effective dietary nutrition with elevated vitamin B_{12} levels sharply increases. According to European Union food regulations, the recommended daily uptake (RDA) for vitamin B_{12} is 2.5 µg (European Union 2008). Hence, a single daily serving of 100 mL of our fermented sunflower seed milk would deliver up to 6-fold of the RDA level for vitamin B_{12}, far more than the minimum value required to claim: “high in content” (> 30% RDA). The vitamin B_{12} level (17 µg (100 g)$^{-1}$) is among the highest values, achieved by supplement-free fermentation of plant-based materials. This achievement makes fermented sunflower seed milk a promising alternative to animal-derived foods such as milk (0.4 µg (100 g)$^{-1}$), eggs (1.4 µg (100 g)$^{-1}$), lean

red meat (3 µg (100 g)$^{-1}$), and fish (2 µg (100 g)$^{-1}$) (Watanabe et al. 2014) in providing vitamin B_{12}.

Fermentation provides sunflower seed milk with multiple benefits

Though numerous types of innovative food beverages from plant sources are being exploited for animal milk alternatives, many of these face some/any type of nutritional and organoleptic issues (Jeske et al. 2018; Tangyu et al. 2019) . Above all, the major reasons impeding the consumer's interests and application of plant-based milks are: (i) inferior nutrient value limited by vitamin content and protein quality; (ii) poor digestibility due to the existence of indigestible compounds and anti-nutrients which may cause flatulence, diarrhea, and other symptoms; (iii) off-flavor and taste such as beany, bitter, and earthy flavor associated to the raw materials. In this regard, selection and testing of the most appropriate multi-purpose strains and strain combinations appear crucial. It is therefore an important outcome of this study that *P. freudenreichii* NCC 1177 and *B. amyloliquefaciens* NCC 156 addressed several of the key requirements, partly alone but optimally in combination. Both strains showed robust growth in sunflower seed milk. *P. freudenreichii* NCC 1177 anaerobically produced 0.9 µg (100 g)$^{-1}$ vitamin B_{12} (**Appendix, Table S3**), whereas *B. amyloliquefaciens* NCC 156 aerobically increased the L-lysine level and the overall protein quality, produced vitamins Bs, and decreased indigestible sugars (**Fig. 21**). Notably, the previous study on chickpea milk fermentation revealed that strain NCC 156 provided several benefits at the same time, including increased levels of L-lysine, increased protein quality, decreased levels of indigestible sugars, and improved flavor profile (Tangyu et al. 2021), indicating that this strain works in different plant milks. Process control avoiding nutrition limitation and downstream heat treatment might help to prevent overgrowth and spore formation of this fast-growing strain (Grossman and Losick 1988; Silvetti et al. 2018). The two well-performing isolates stand in a prominent line with related microbes, applied in plant-

based fermentation and recognized as probiotics (Assis et al. 2020; Ngalimat et al. 2021). Strains of *P. freudenreichii* were shown to synthesize vitamin B_{12} on plant-based materials such as barley (0.9-3.7 µg (100 g)$^{-1}$) (Chamlagain et al. 2018), wheat (around 2.6-4.5 µg (100 g)$^{-1}$) (Xie et al. 2018; Xie et al. 2019), durum (1.3 µg (100 g)$^{-1}$) (Xie et al. 2018), lupin (6.0 µg (100 g)$^{-1}$) (Signorini et al. 2018), sauerkraut, and vegetable juice (7.2 µg (100 g)$^{-1}$) (Babuchowski et al. 1999). In addition, strains of *B. amyloliquefaciens* proved value regarding flavor formation and the hydrolysis of plant protein, the release of peptides, and the generation of bioactive compounds and vitamins (WoldemariamYohannes et al. 2020).

It is worth noting that as a monoculture, *Propionibacterium* needs a quite long fermentation time (up to 7 days or even longer) for optimized vitamin B_{12} production (Assis et al. 2020; Chamlagain et al. 2016; Hedayati et al. 2020; Hugenschmidt et al. 2011). In this study, we were able to shorten the fermentation time to 3 or 4 days, which is more efficient for industrial applications. Moreover, our development comes with even more commercial benefits: improved flavor, improved digestibility, improved protein quality, and elevated levels of vitamins B_3 and B_6. The two strains NCC 1177 and NCC 156 are natural isolates, generally recognized as safe to be used in food fermentation (EFSA Panel on Biological Hazards et al. 2017). Furthermore, no additives were used. The fermentation process contained only four ingredients: sunflower seed flour, the two food-grade microbes, and water, enabling “clean labeling” as expected by consumers. Taken together, the developed co-culture fermentation displays a valuable development for human nutrition.

4.3 Food-grade LAB upgrade the volatile aroma spectrum of plant milks towards improved flavor and sensory perception

Flavor and taste have a key impact on selecting plant-based non-dairy beverages (McCarthy et al. 2017). Unfortunately, natural plant milks have only limited acceptance. Their typically beany and grassy flavor, as well as the bitter and seedy taste are received as “off-flavor” by consumers, especially in countries without a tradition for this type of products (Diarra et al. 2005; Sethi et al. 2016; Tangyu et al. 2022).

Natural fermentation appears an appealing option to improve the aroma of plant milks and, moreover, deliver co-benefits such as increased nutritional value, stability, and microbial safety (Ayivi et al. 2020; Cichońska and Ziarno 2021b; Sethi et al. 2016; Tangyu et al. 2019). A rich microbial source to ferment plant milk are lactic acid bacteria (LAB). Many LAB species grow in plant-based materials and have been proven valuable to contribute to improved sensory profiles. As an example, species of *Streptococcus, Lactococcus, Lacticaseibacillus*, *Lactiplantibacillus*, *Lactobacillus, Limosilactobacillus,* and *Leuconostoc* affected the flavor of cereal-based milk (Kumar et al. 2020; Shin and Han 2015), soy milk (Beasley et al. 2003; Blagden and Gilliland 2005), mung bean milk (Liang et al. 2022), cowpea milk (Sanni et al. 1999), pea-based materials (Pei et al. 2022; Yousseef et al. 2016), as well as fruity and vegetable juice (Cui et al. 2019).

Following this promising potential, here we systematically evaluated LAB to ferment different types of plant milk. On the microbial side, we selected 15 LAB that represented a broad range of genera and species. On the plant milk side, we considered four emerging plant milks for the fermentation: oat milk (representing cereal-based milk), sunflower seed milk (representing seed-based milk), and pea and faba milk (representing legume-based milk). Using GC-MS analysis with solid phase micro extraction, flavor changes during the fermentation were studied in detail,

revealing species-related and plant milk-related differences and, notably, highlighting several well-performing strains that delivered a range of beneficial changes. For six of these well-performers, we predicted perceivable sensory impressions of the obtained fermentations on basis of odor activities of the contained volatiles which allowed to bridge compositional changes with consumer response. In this regard, our work provides a comprehensive understanding of LAB-related flavor alteration in various plant-based nutrient environments. Moreover, a few specific strain-milk combinations were identified that promise to deliver flavor by design, opening up further developments towards cheesy products, buttery products, as well as other innovative products in the future.

4.3.1 Screening of a collection of different LAB strains for their plant milk fermentation capacity

The main goals of this work were to (i) improve the naturally undesired flavor of plant milk using microbial fermentation and (ii) discover beneficial microbes that perform well as flavor-enhancer in plant milks of different origins. In a first step, a set of 15 food-grade LAB was selected, considering species that had previously proven value in affecting the flavor of plant-based food in general (Engels et al. 2022; Lee et al. 2016b; Lu et al. 2018; Ricci et al. 2018; Smid and Kleerebezem 2014). The selected strains belonged to two major LAB families, namely *Streptococcaceae* and *Lactobacillaceae*, and covered different 8 genera: *Streptococcus* (4), *Lactococcus* (2), *Lacticaseibacillus* (3), *Lactiplantibacillus* (1), *Lactilactobacillus* (1), *Lactobacillus* (2), *Limosilactobacillus* (1), and *Leuconostoc* (1) (**Table. 2**). From a metabolic viewpoint, the selected strains comprised homolactic LAB, e. g. *Streptococcus* and *Lactococcus*, and also a heterofermentative LAB, *Leuconostoc*.

It could be expected that the flavor metabolism of the isolates would require their growth, linked to the fact that the degradation, formation, and interconversion of aroma molecules requires energy, redox power, and building blocks, which all are provided in actively growing cells (Bartowsky and Pretorius 2009). In this regard, the gross nutrient composition of the four selected milks differed substantially (**Table 8**). Protein represented the largest fraction (2.0-4.3%), followed by carbohydrates (0.3-3.5%), and fat (0.1-2.7%). Oat milk exhibited the highest content of the major nutrients, suggesting it is a suitable source for fermentation (Lee et al. 2016b; Mårtensson et al. 2000). Differently, pea milk and faba milk contained relatively low levels of protein (around 2.0%) and carbohydrates (0.3% and 0.4% respectively), potentially indicating a poorer growth environment. Sunflower seed milk exhibited a medium level of protein (3.6%) and carbohydrates (0.7%), while its fat level was negligible because defatted sunflower seed flour had been used for its preparation (Tangyu et al. 2022).

Table 8: Main nutrient composition of oat milk, sunflower seed milk, pea milk, and faba milk.

	Protein (%)	Carbohydrates (%)	Fat (%)
Oat milk	4.30	3.50	2.7
Sunflower seed milk	3.60	0.70	0.05
Pea milk	2.00	0.29	0.14
Faba milk	2.20	0.41	0.12

The strains were now evaluated for their capacity to anaerobically grow in the different plant milks. Towards clean label products, plain plant milks were used, i.e. aqueous suspensions of the plant material without additional supplements (Cichońska and Ziarno 2021a). The extent of growth strongly varied with strain and plant milk, whereby 14 strains grew in more than one plant milk. As an exception, *L. helveticus* NCC 1276 could not grow in any of the four milks within the test range of

24 hours. Among the growing strains, the increase in living cells ranged from 0.05 to 2.43 log cfu mL^{-1} (**Table 9**), reflecting a 1.1-fold to 269.2-fold increase in living cells. Most *Lactobacillaceae* grew well, while *Streptococcaceae* showed weaker growth. *L. mesenteroides* NCC 2832 revealed strong growth in all plant milks, whereas the other strains showed preferences for specific types of milk. As an example, *L. fermentum* NCC 660, *S. thermophilus* NCC 2019, and *S. thermophilus* NCC 2059 grew well in oat and sunflower seed milk but did not, or only weakly, grow in legume-based milks. Differently, *L. rhamnosus* NCC 2891 and NCC 4007*, L. lactis* NCC 2180 and NCC 2242, and *L. paracasei* subsp. *paracasei* NCC 2511 grew well only in oat milk and pea milk. Four strains of *S. thermophilus*, *L. johnsonii* NCC 533, and *L. sakei* NCC 1692 preferred sunflower seed milk, while *L. plantarum* NCC 2988 was the second-best grower in sunflower seed, pea, and faba milk among all strains but grew poorly in oat milk.

Table 9: Growth of LAB strains in oat milk, sunflower seed milk, pea milk, and faba milk, expressed as increase in log (cfu mL^{-1}). Light green, slight growth (0 < log increase < 0.5); green (0.5 < log increase < 1); dark green (log increase > 1); -, no growth. n=3.

Strains	Oat milk	Sunflower seed milk	Pea milk	Faba milk
Streptococcaceae				
S. thermophilus NCC 1326	0.47 ± 0.06	1.16 ± 0.05	0.69 ± 0.03	0.19 ± 0.03
S. thermophilus NCC 1988	0.43 ± 0.20	0.63 ± 0.08	0.34 ± 0.04	0.08 ± 0.05
S. thermophilus NCC 2019	0.23 ± 0.05	0.69 ± 0.04	-	0.13 ± 0.02
S. thermophilus NCC 2059	0.05 ± 0.05	0.39 ± 0.07	-	-
L. lactis NCC 2180	0.94 ± 0.03	0.73 ± 0.06	1.02 ± 0.03	0.59 ± 0.03
L. lactis NCC 2242	1.12 ± 0.05	0.49 ± 0.10	0.67 ± 0.09	0.42 ± 0.03
Lactobacillaceae				
L. rhamnosus NCC 2891	1.66 ± 0.09	0.88 ± 0.06	1.01 ± 0.06	0.71 ± 0.04
L. rhamnosus NCC 4007	0.89 ± 0.06	0.25 ± 0.09	0.62 ± 0.01	0.45 ± 0.05
L. paracasei NCC 2511	1.32 ± 0.10	0.88 ± 0.02	1.17 ± 0.10	0.86 ± 0.35
L. plantarum NCC 2988	0.30 ± 0.07	1.79 ± 0.07	1.52 ± 0.07	1.78 ± 0.44
L. sakei NCC 1692	0.48 ± 0.09	1.29 ± 0.05	0.82 ± 0.01	0.57 ± 0.02
L. johnsonii NCC 553	0.90 ± 0.06	1.19± 0..36	0.81 ± 0.40	0.66 ± 0.37
L. fermentum NCC 660	0.54 ± 0.07	0.48 ± 0.10	-	-
L. mesenteroides NCC 2832	1.77 ± 0.04	2.43 ± 0.01	2.07 ± 0.03	1.59 ± 0.02

Altogether, three conclusions could be drawn from the growth data. First, the nutrient composition of the milk had a strong impact on growth. Generally, oat milk enabled better growth than legume-based milks. Second, the *Lactobacillaceae* grew better than the *Streptococcaceae,* eventually because lactobacilli appear to have an evolutionary history on plant materials (Duar et al. 2017). Third, a few isolates stood out, spearheaded by *L. mesenteroides* NCC 2832, the only strain with a heterofermentative metabolism (Koduru et al. 2017), as the best grower. Overall, a range of promising combinations of strains and plant milks could be identified that were different from previous work and did not require fortification with other nutrients (such as sugars or animal milk) (Cichońska and Ziarno 2021b; Emkani et al. 2021; Mårtensson et al. 2000; Xu et al. 2017b). or use of mixed cultures to support growth (Cichońska and Ziarno 2021b; Herrera‐Ponce et al. 2014; Mårtensson et al. 2000).

4.3.2 Spectrum of volatile aroma compounds in unfermented plant-based milks assessed by solid-phase microextraction and GS-MS analysis

Following several rounds of development and improvement, SPME using a DVB/CAR/PDMS fiber plus GC-MS allowed sensitive, reproducible, and robust volatile analysis of the different plant milks. (**Appendix, Table S5**).

Next, the volatile flavor profile of the four raw materials was evaluated using the established approach (**Fig. 29**). Generally, the measurement showed high reproducibility (standard deviation < 20%). Overall, 82 volatiles were detected and identified, most of them in sunflower seed milk, and least in the legume-based milks. Many of the volatiles matched previously described profiles (Akkad et al. 2021; Akkad et al. 2019; Fischer et al. 2021; Guo et al. 2019; Lee et al. 2016b; Wronkowska et al. 2022; Xu et al. 2019). From a chemical perspective, oat milk contained volatiles from all nine groups, whereby alcohols (16% of all detected volatiles, 53% of total peak area), aldehydes (27% of all volatiles, 11% of total peak area), and ketones (14% of all volatiles, 10% of total peak area) were dominant. On

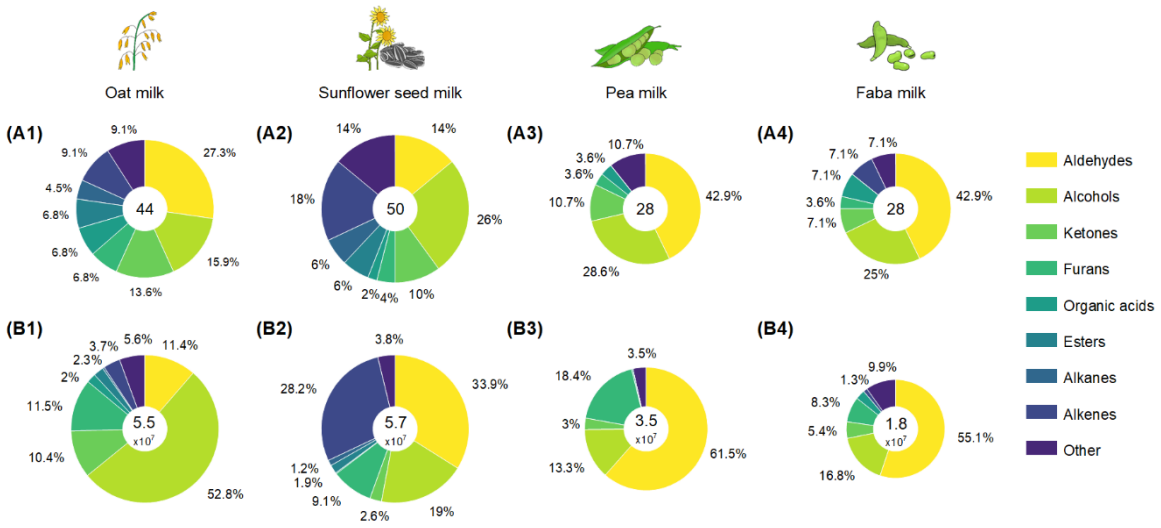

Fig. 29: Volatile analysis of non-fermented oat milk (1), sunflower seed milk (2), pea milk (3), and faba milk (4) using GC-MS. The data comprise the number of known detected volatile compounds (A) and the relative peak area (B) associated to specific chemical groups. The relative diameter of each pie graph represents the corresponding number (A) and the total peak area of all volatiles detected (B), respectively. The values are additionally shown in the middle of each chart. n=3.

the level of single compounds, 1-hexanol, 2-pentylfuran, and dimethyl ether were most abundant. These three molecules accounted for 40%, 11%, and 5% of the total detected peak area, respectively. Sunflower seed milk was found rich in alkenes, especially terpinene-based volatiles such as α-pinene and β-terpinene which accounted for 23% of the total peak area. Besides, aldehydes and alcohols, represented by 1-hexanal (23% of total peak area) and 1-hexanol (10% of total peak area), were predominant. The two legume-based milks from pea and faba showed less volatiles. Aldehydes (43% of all volatiles) were detected in both plant milks, followed by alcohols (25-29%), whereas, different from other plant milks, no esters and alkanes were present.

For increased interpretability, the flavor data were now subjected to PCA. The difference in the abundance of volatiles led to a clear separation of the data in the obtained PCA plot (**Fig. 30**)**.** The unfermented plant milks clustered into three

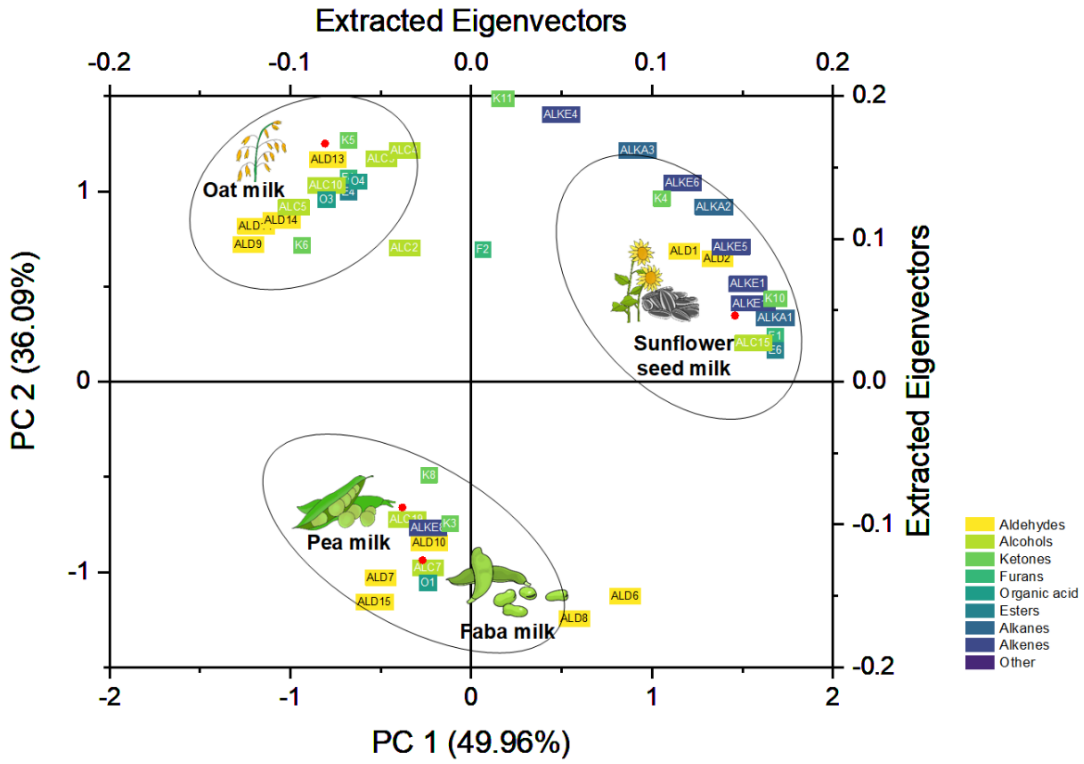

Fig. 30: Principal component analysis of the volatiles detected by GC-MS in unfermented oat milk, sunflower seed milk, pea milk, and faba milk. The data are shown as bi-plots, including a few signature loading points. ALC, alcohols; ALD, aldehydes; ALKA, alkanes; ALKE, alkenes; E, esters; F, furans; K, ketones; O, organic acids. The given number of each compound corresponds to **Table S6**. n=3.

groups: (i) cereal-based oat milk, (ii) seed-based sunflower seed milk, and (iii) the two legume-based milks (pea and faba). Alkenes such as α-pinene, β-terpinene, and camphene appeared as signature volatiles of sunflower seed milk, largely contributing to its unique profile. Oat milk and legume-based milks were well separated by differences in the level of alcohols, ketones, and esters (e. g. 1-heptanol, 1-hexanol, 1-(4-ethylphenyl)-ethanone, 2, 3-butanedione, ethyl-2-methyl-butanoic acid, and others), and aldehydes (such as benzene acetaldehyde, decanal, benzaldehyde, 2-decenal, and 1-hexanal) and terpinene-based alcohols (linalool and phytol). Oat milk was rich in alcohols, ketones, and esters, while pea milk and faba milk were characterized by a high aldehyde content.

4.3.3 Impact of LAB-based plant milk fermentation on the profile of flavor-associated volatiles

In the next step, the plant milk fermentations were analyzed for potential changes in the spectrum of aroma compounds volatiles. For this purpose, volatiles were extracted after 24 h of fermentation using SPME, analyzed by GC-MS, and processed. It turned out that all fermentations strongly affected the volatile spectrum (**Fig. 31, Appendix, Table S6**).

Most of the aldehydes and some ketones, originally contained in the non-fermented plant milks, decreased, including 1-heptanal, 1-hexanal, and 1-nonanal. In turn, the corresponding alcohols (1-heptanol, 1-hexanol, 1-nonanol) and carboxylic acids (heptanoic acid, hexanoic acid, nonanoic acid) increased pointing to a range of biocatalytic conversions. In addition, esters, specific ketones, and ethers increased notably, matching at least the trend observed in other LAB-based plant fermentations (Goswami et al. 2018; Lee et al. 2016b; Smid and Kleerebezem 2014; Tangyu et al. 2021). Among the fermented samples, the two legume-based milks were rich in aldehydes, acids, and alcohols but rather poor in other aroma compounds. In contrast, the other two plant milks were lower in aldehydes and richer in e. g. ketones, alcohols, acids, and alkenes. In addition, 43 different volatiles newly emerged during the fermentation: 15 novel compounds were detected in oat and faba milk, 17 in pea milk, and even 21 in sunflower seed milk (**Fig. 31**). The latter plant milk finally exhibited the most diverse aroma profile. Most of the newly formed molecules were small. As an example, newly arising alcohols and ketones comprised four to nine carbon atoms. As an exception, alcohols eugenol and 3-ethyl-4-nonanol, esters bornyl acetate and linalyl propionate, and the alkenes α-thujene and alloocimene, formed by selected microbes, exhibited 10-13 carbon atoms. As many aldehydes were presumed to contribute to undesired beany and grassy off-flavor (Fischer et al. 2022), while many alcohols were often related to

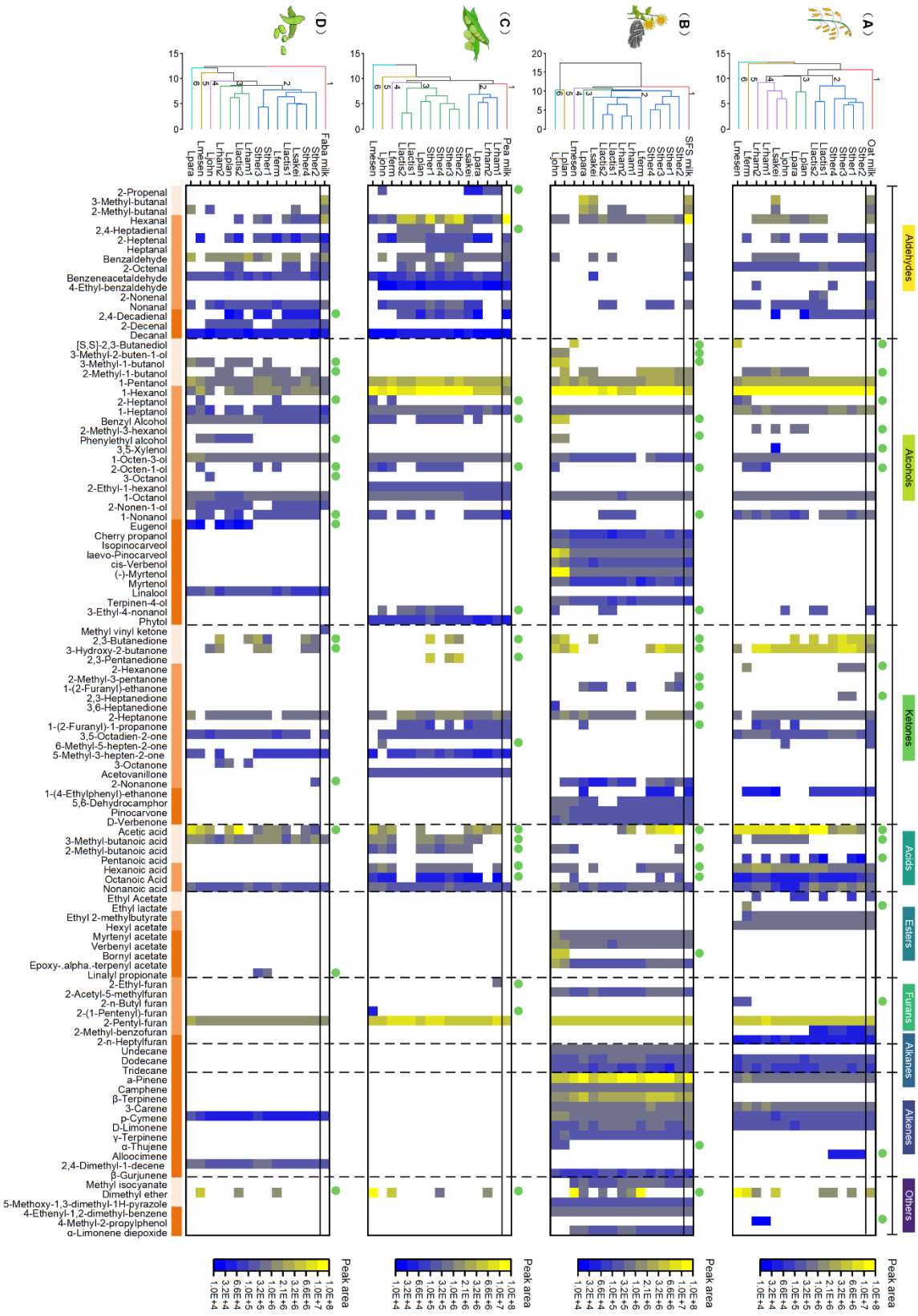

Fig. 31: Changes of individual flavor compounds during the fermentation of oat milk (A), sunflower seed milk (B), pea milk (C), and faba milk (D). The abundance of each flavor compound is shown by color boxes which indicate the observed average peak area:

yellow, high abundance; blue, low abundance; and white, not detectable. The flavor compounds are grouped into aldehydes, alcohols, ketones, acids, esters, furans, alkanes, alkenes, and others with the separation of dotted lines. Within each chemical group, the compounds are sorted from small to large: light orange, < C_5; orange, C_6-C_9; strong orange, > C_{10}. Newly formed flavor compounds, detected after fermentation, are labeled with green dots. For each milk, the columns are sorted based on hierarchical clustering analysis. Hereby for each plant milk, the 14 fermented samples and the non-fermented sample clustered in 6 groups with a distance of 9.2 (oat milk), 10.3 (sunflower seed milk), 9.0 (pea milk), and 8.6 (faba milk). SFS milk, sunflower seed milk. n=3.

favored sweet and fruity notes (Lu et al. 2018), the observed changes appeared as a first sign of a beneficially affected flavor profile by the fermentation process. Overall, many changes were strain-specific, leading to considerable differences in the volatile profiles after the fermentation. Hierarchical clustering of the samples revealed that unfermented plant milk was grouped out from all fermented plant milks, underlying the huge impact of the microbes. For most plant milks, taxonomically related strains changed the aroma similarly, as found, for example for different species of *S. thermophilus* and *L. lactis*. As an exception, faba milk resulted in larger differences even for closely related strains, which might be related to the different capabilities of the strains to grow in this raw material.

Overall, two major classes of conversions could be observed: (i) reducing and (ii) oxidizing ones. Notably, the different strains exhibited quite different activities across these two routes. Many strains showed a remarkable capacity for reduction. *S. thermophilus* isolates formed e. g. 1-heptanol, 1-hexanol, 1-octanol, 2-methyl-1-butanol, and 1-pentanol) and specific ketones, such as 2,3-butanedione, 3-hydroxy-2-butanone, 2-hexanone, 2,3-pentanedione, 2-heptanone, 5-methyl-3-hepten-2-one, 1-(4-ethylphenyl)-ethanone and 2-nonanone). Similarly, *L. fermentum* formed a high amount of several alcohols, including 2-methyl-1-butanol, 1-heptanol, 1-hexanol, 2-heptanol, 1-heptanol, 2-octen-1-ol, 1-octanol. In contrast, *L.*

mesenteroides formed a greater level of oxidized products such as carboxylic acids (nonanoic acid, hexanoic acid, acetic acid). Some strains exhibited a more mixed behavior and catalyzed reductions as well as oxidations. As an example, oat milk, fermented by *L. rhamnosus,* contained specific alcohols (2-octen-1-ol, 1-octanol, 4-methyl-2-propylphenol, and 2-methyl-1-butanol), ketones (3-hydroxy-2-butanone and 2-heptanone), but also organic acids (acetic acid, 3-methyl-butanoic acid, 2-methyl-butanoic acid, hexanoic acid, and pentatonic acid). For some strains, the catalyzed chemistry depended on the plant material. *L. johnsonii* actively formed several alcohols in sunflower seed milk and faba milk, revealing a strong reduction capacity, while it produced more organic acids in pea milk, representing a stronger oxidizing capacity, showing that this strain may modify the volatile profiles differently in different plant milks.

4.3.4 Flavor metabolism during fermentation

On the level of single compounds, a few prominent types could be distinguished, pointing to the impact of specific nutrients in the raw materials on flavor formation: (i) alcohols and acids from the reduction and oxidation of aldehyde precursors, respectively, (ii) terpenoid-based compounds and catabolic intermediates from (iii) the degradation of carbohydrates via pyruvate, (iv) of proteins via amino acids, and (v) of lipids via unsaturated fatty acids, matching the nutrient and flavor composition of the raw materials (**Table 8**). The reduction/oxidation of aldehydes into the corresponding alcohols and organic acids was detected in all fermentations. The aldehyde 1-hexanal, most abundant in all four plant milks and linked to an undesired beany and grassy note already at a low threshold (Chemical Book Group 2016; Yang et al. 2008), decreased by 14% to even 100% due to the fermentation, while 1-hexanol and hexanoic acid were formed. Terpenoid-related flavor changes were specifically observed during sunflower seed milk and faba milk fermentation, linked to the presence of terpene alkenes, such as α-pinene, camphene, α-, β-terpinene,

3-carene, p-cymene, and D-limonene in these two plant milks, found here and before (Guo et al. 2019; Ivanova-Petropulos et al. 2015). These terpenes contribute to a fruity, woody, terpenic, and nutty and flavor. Their alcoholic derivatives have a lower odor threshold, resulting in an even stronger aroma (Savithiry et al. 1998). In this regard, it was interesting to see that pinene and limonene were reduced to myrtenol, pinocarveol, carveol, and further converted to other related ketones and esters (e. g. pinocarvone, carvone). As an example, *S. thermophilus*, *L. lactis*, *L. plantarum*, *L. johnsonii*, and *L. sakei* fermented sunflower seed milk to increased levels of pinocavenol, isopinocarvenol, mytenol, (-)-mytenol, pinocarvone, and myrtenol acetate. Notably, *L. johnsonii* and *L. plantarum* increased the relative amount of (-)-mytenol, related to a nice minty and woody odor, about 50-fold. Considered as one of the most preferred alcoholic terpinenes with regard to its citrus and floral aroma (Cai et al. 2014; Chigo-Hernandez et al. 2022; Howe 2020; Lu et al. 2018), as well as its antioxidant and antimicrobial activity (Herman et al. 2016; Jabir et al. 2018; Sasaki et al. 2016), substantial linalool formation was observed in faba milk. It was present only in a relatively low amount in unfermented faba milk but increased 1.2- to 13.5-fold by fermentation. Notably, *L. johnsonii* achieved the highest linalool level among all strains. The increase of linalool in plant materials has been previously reported for *L. paracasei*, *L. plantarum*, and *L. rhamnosus* (Cheng et al. 2021; Lu et al. 2018) and is regarded as beneficial with regard to the overall aroma.

Volatiles with strong buttery and creamy notes (3-hydroxy-2-butanone, 2,3-butanedione, 2,3-butanediol, 2,3-pentanedione, and their ester derivatives) obviously originated from sugars, organic acids, or amino acids through the pyruvate pathway (Tangyu et al. 2021). Protein and amino acid-derived flavors resulted from transamination to α-keto acids and further to aldehydes, alcohols, and acids (Engels et al. 2022). Almost all samples contained 2-methylbutanal, and 3-methylbutanal (malty, cocoa-like flavor) (Engels et al. 2022), and/or 2-methyl butanol, 3-methyl

butanol, and 2-methyl butanoic acid and 3-methyl butanoic acid that exhibit a fruity, ethereal, nice alcoholic and or cheesy note (Osorio et al. 2006; Pogačić et al. 2016; Xu et al. 2019), and are known to be derived from the degradation of the branched-chain amino acids valine, leucine, and isoleucine (Engels et al. 2022). Finally, the formation of unsaturated aldehydes (e.g. 2,4-decadienal, 2-octenal), observed during fermentation of oat, pea, and faba milk, seemed related to lipid/fatty acid oxidation (Fischer et al. 2022). This was not the case for sunflower seed milk, related to its negligible fat level (**Table 8**).

4.3.5 PCA extracts strain-specific flavor phenotypes

Additional information on similarities and differences in the complex patterns was assessed by PCA using GC-MS and growth data as input (**Supplementary Material 2**). The latter allowed to consider growth effects on flavor changes, a trend that seemed important based on the hierarchical clustering results for faba milk (see above). Altogether, 60 unfermented and fermented plant milk samples were analyzed. It turned out that the first (PC1), the second (PC2), and the third principal component (PC3) together explained up to 65% of the total variance, so their inspection allowed to extract important features in the data set (**Fig. 32, Appendix, Table S7, Table S8, Table S9, Table S10**).

In all cases, unfermented plant milk clustered separately from fermented plant milk, independent of the strain used, matching with the hierarchical clustering result. The highest distance was observed for sunflower seed, pea, and faba milk (**Fig. 32BCD**), e. g. on the levels of PC2 and PC3. From the flavor compound view, the negative PC2 correlation of unfermented plant milk co-clustered with aldehydes, followed by furans and certain organic acids, alcohols, and ketones, meaning that its unique position was due to a high abundance in these compounds. Opposite, fermented plant milks positively correlated with PC2, represented by esters, alkenes, and ethers, as well as other organic acids, alcohols, and ketones, which were all less

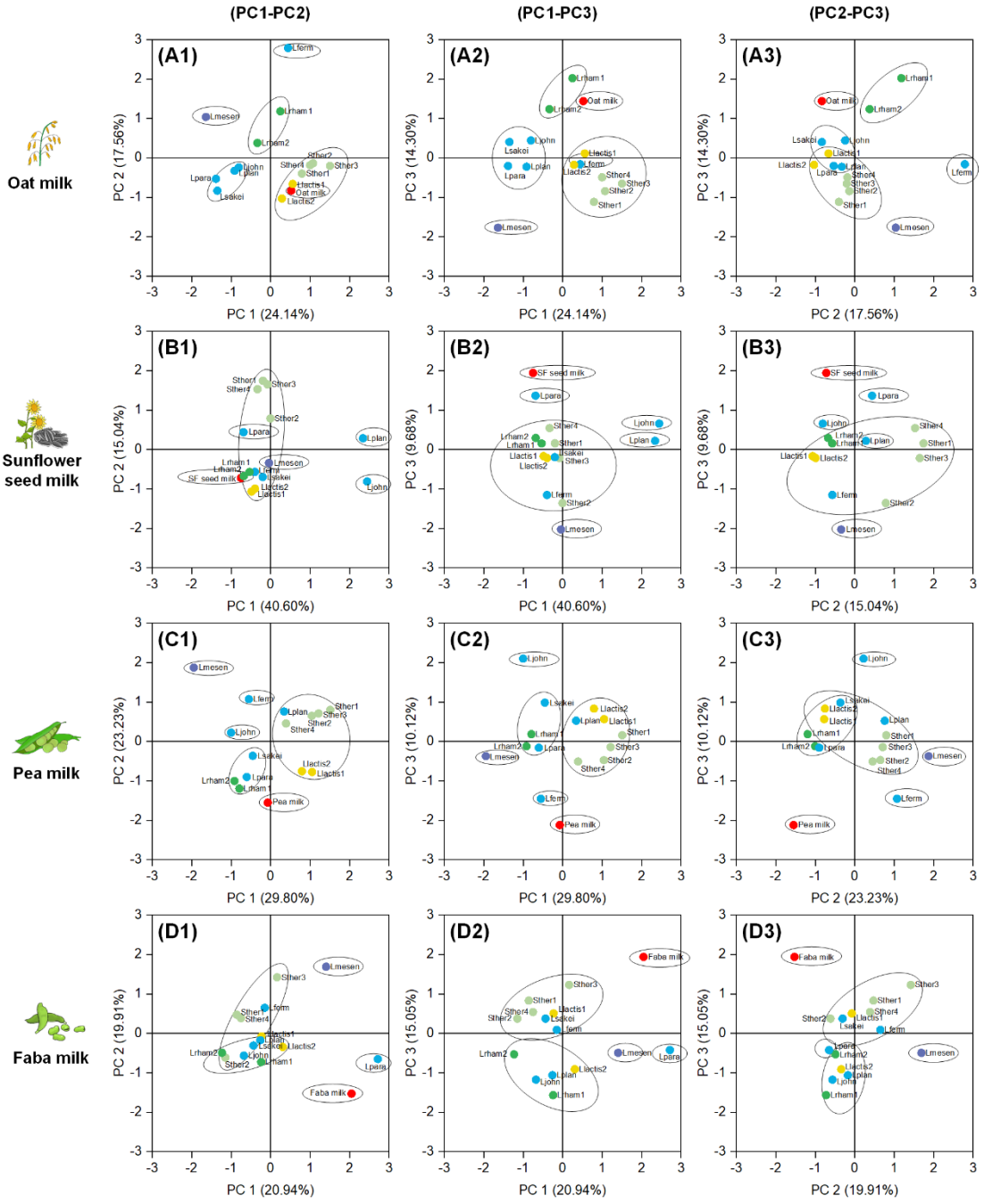

Fig. 32: Impact of plant milk fermentation on the volatile spectrum assessed by PCA. The data comprise the results for oat milk (A), sunflower seed milk (B), pea milk (C), and faba milk (D) detected by HS-SPME-GC-MS. (1), Score plot of principal components 1 and 2; (2), Score plot of principal components 1 and 3; (3), Score plot of principal components 2 and 3. The color was given to unfermented plant milk and different species: red, unfermented plant milk; light green, *S. thermophilus*; dark green, *L. rhamnosus*; yellow, *L. lactis*, purple, *L. mesenteroides*; blue, others. n=3.

present in the pure milk. In addition, sunflower seed milk clustered separately on the level of PC3 mainly characterized by differences in aldehydes, alcohols, and alkenes. Unfermented and fermented oat milk did not differ much for several strains. However, specific strains seemed to result in larger changes, as visualized on the level of PC3 (**Fig. 32B**).

On the strain level, different isolates from the same species revealed a similar activity to affect the spectrum of volatiles, at least in some of the principal components. Typically, they clustered together (**Fig. 32**). As an example, the four *S. thermophilus* isolates grouped together in all plant milks. Differently, the two strains of *L. rhamnosus* clustered together in all plant milks, except faba milk. Likewise, both *L. lactis* strains clustered together in oat, sunflower seed, and pea milk, but were separated in faba milk. Interestingly, the aroma effects caused by *L. lactis* highly were very similar to the ones of *S. thermophilus*. Overall, these strains responded similarly, when fermenting on different plant milks so creating a rather robust flavor profile. In contrast, rather pronounced plant milk-based differences were observed for strains *L. fermentum, L. johnsonii, L. paracasei, and L plantarum*.

These strains exhibited a specific response to each nutrient environment, indicating a more individual flavor metabolism and offering more flexibility with regard to the desired profile. *L. sakei*, although taxonomically more distant, seems did not provide a significanty different type of flavor. The strain always clustered with other strains so that it appeared replaceable to some extent. Notably, *L. mesenteroides* behaved quite differently. It was located far away from all other microbes, indicating that this microbe created a unique flavor profile among all isolates.

4.3.6 Odor implications of plant-based milk alternatives fermented by representative strains

As shown, fermentation strongly affected the flavor profile of the plant milks. A set of signature molecules could be identified, and strains could be classified in terms of similarity and uniqueness with regard to their effects. Hereby, the high reproducibility of the experimental workflow enabled clear conclusions.

Admittedly, the observed changes were complex, posing a challenge to the evaluation of the overall impact on the perceivable flavor. We tackled this question by weighing the measured compositional volatile data on basis of their impact on perceivable flavor, considering their relative abundance plus their odor threshold. This approach mimicked the concept of odor activity, which is based on ratios between concentration and odor threshold and is frequently used to infer the impact of compounds on overall flavor (Zhu et al. 2020). A test set of six representative strains that yielded quite different flavor profiles, based on PCA and HCA analysis, was used (**Fig. 33**).

Our approach allowed to evaluate the flavor impact of single compounds within a given mixture based on relative peak areas, which can be obtained far more easily than absolute concentrations. In short, the relative peak areas of the detected volatiles were normalized, given differences between them in odor threshold. Hereby, the least perceivable volatile within the sample was used as a reference and the other (more perceivable) volatiles were normalized to this reference, based on the threshold ratio. This yielded the relative odor activity for each compound within the mixture, i. e. its impact on the overall flavor. In total, 75 out 99 detected volatiles with known flavor attributes, for which a threshold value was available, could be included in the evaluation. Then, we went one step further towards predicting overall flavor notes for entire samples and not only for single compounds. For this purpose, we summed up all relative odor activities that belonged to a certain

flavor group which allowed to infer the relative impact of this flavor note (**Fig. 33A**). Doing so for various flavor groups finally aimed to provide a rough estimate on the resulting overall flavor that we designate “predicted flavor” here. Sunflower seed milk, pea milk, and faba milk are well known to have a strong grassy and beany flavor (Akkad et al. 2019; El Youssef et al. 2020; Tangyu et al. 2022), which is most pronounced in pea milk (Fischer et al. 2022). It was therefore nice to see that our approach exactly predicted this attribute. The three unfermented plant milks were dominated by a grassy and beany note: 51% for faba milk, 87% for sunflower seed milk, and 95% for pea milk. The predicted undesired flavor note, related to the presence of high-abundance-low-threshold aldehydes and furans, e.g. 1-hexanal, 1-heptanal, and 2-pentylfuran, was consistent with previous studies (Bott and Chambers IV 2006; Roland et al. 2017; Wang et al. 2021). As shown, fruity notes made only a minor contribution to the flavor of these plant milks. Differently, unfermented oat milk was classified to be more fruity-sweet and buttery-fatty (87% of total odor impact), and also this attribute well matched previous studies that many consumers highly value oat milk with good taste (Pham Thi Thu 2019).

Several interesting findings could be now extracted from the fermentation processes. For pea, faba, and sunflower seed milk, fermentation with most strains resulted in drastic alterations of the predicted flavor profile, whereas for oat milk only one strain (*L. mesenteroides*) caused an apparently strong change (**Fig. 33**). Within the flavor fingerprints, we recognized notable trends among (i) favored plant milk-flavors such as sweet, flora, and buttery (shown green), (ii) functional flavors that add complexity and uniqueness to the food (Liu 2012), such as minty, cheesy, and nutty (shown yellow), (iii) and generally unfavored flavors such as grassy and beany (shown in blue).

Most strains could substantially increase favored flavors (fruity, sweet, floral, buttery, and fatty) during fermentation. Strikingly, *S. thermophilus* NCC 1326 was able to

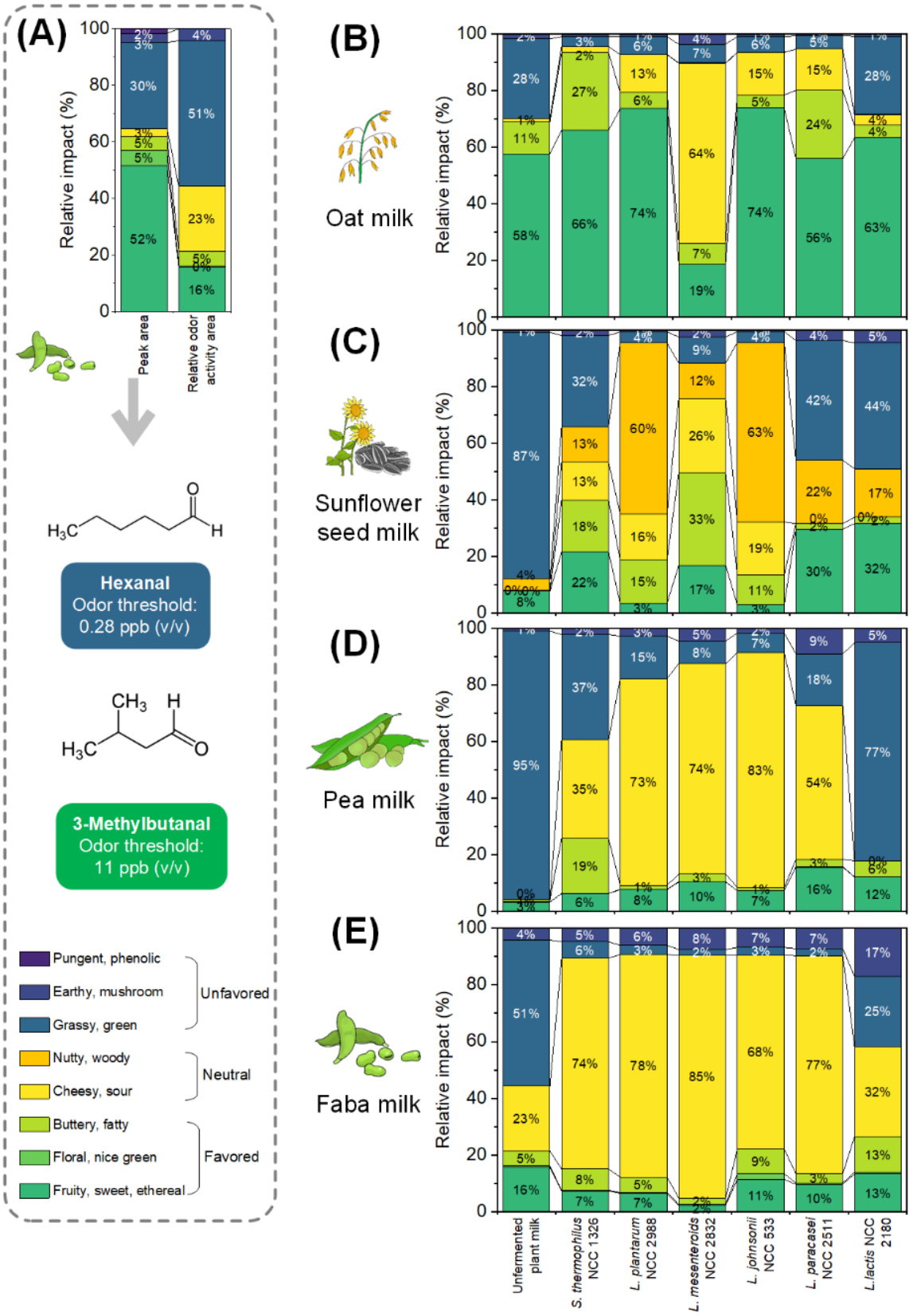

Fig. 33: Prediction of perceivable flavor from non-fermented and fermented plant milks based on GC-MS volatile analysis. Illustration of the concept to infer relative odor activity from relative abundance using unfermented faba milk as an example (A). Prediction of the flavor profile and the overall sensory profile for oat milk (B), sunflower seed milk (C),

pea milk (D), and faba milk (E). Prior to estimation, the detected volatiles were classified into eight odor groups: (i) Fruity, citrus, sweet, melty, ethereal (designated fruity, sweet), (ii) floral, nice green (floral, nice green), (iii) buttery, fatty, waxy, creamy (designated buttery, fatty), (iv) cheesy, sour (designated cheesy, sour), (v) nutty, woody, minty, toasted, turpentine, balsamic, camphoraceous (designated nutty, woody), (vi) green, grassy, beany, herbal (designated grassy, green), (vii) earthy, mushroom (designated earthy, mushroom), and (viii) pungent, spicy, sharp, phenolic earthy, mushroom pungent, phenolic). The colors highlight desired flavors (green), functional flavors (yellow), and off-flavors (blue). n=3.

increase the buttery note in all four plant milks. This is regarded as very attractive, given the fact that this note is generally preferred in fermented plant milk products. The highest increase of favored flavors was observed for fermented oat and sunflower seed milk but also fermented pea and faba milk contained elevated amounts of these flavors. Eventually, the weaker formation of favored flavors in the legume-based milks might relate to the weaker growth of the used microbes in these raw materials, because important flavor compounds of this group are formed from carbohydrates and/or protein through the pyruvate route (Tangyu et al. 2021), a growth-associated pathway (Cocaign-Bousquet et al. 1996), examples being 2,3-butanediol and 3-hydroxy butanone. Notably, pea and faba milk contained less carbohydrates and protein (**Table 8**).

Cheesy and sour odor was increased in fermented pea milk and faba milk. In particular, *L. mesenteroides*, *L. plantarum*, and *L. johnsonii* were most effective in increasing these notes (from 0% up to 85%). The formation of cheese-like flavors is challenging in plant-based materials since these materials lack the precursor casein (Engels et al. 2022). Therefore, a fermentation with the mentioned microbes might support the development of non-dairy cheese alternatives from legume-based plant materials. Such products currently gain huge attention based on increased consumer demand (Engels et al. 2022; Grossmann and McClements 2021).

Nutty, woody, and minty notes increased in fermented sunflower seed milk, driven by the availability of corresponding flavor precursors and the capacity of the used strains. These flavors offer promising applications by adding complexity and uniqueness to the food (Liu 2012), while nutty and minty flavor is supposed to upgrade the taste of plant milks, e.g. in the Asian market (Ju et al. 2021; Rozhkova and Olentsova 2020). Regarding these notes, the most obvious changes were observed using strains of *L. plantarum* and *L. johnsonii*, which increased this flavor type from 4% up to 63%. These fermented plant milks could open up the creation of new products and contribute to the innovation and diversity of plant milk products.

Most beneficially, all strains were able to remove unfavored beany and grassy volatiles, in some combinations even drastically. As an example, *L. johnsonii* reduced the faction of these notes from 95% to 7% in pea milk. Among all strains, *L. plantarum*, *L. mesenteroides*, and *L. johnsonii* were found most efficient in decreasing this off-flavor, providing a valuable trait (Fischer et al. 2022). In contrast, *L. lactis* was weaker in lowering the beany and grassy notes, likely linked to its rather poor capacity to metabolize aldehydes (Engels et al. 2022).

Given the complex nature of food, developing instrumental methods using GC-MS and analysis approaches to describe sensory profile could be very beneficial, since they are nonsubjective, highly repeatable and reproducible, and do not suffer from fatigue or adaptation (Smyth and Cozzolino 2013). However, predicting human perception by instrumental measurements is quite challenging as it is a multisensory experience evoking smell and taste (Smyth and Cozzolino 2013). In this scenario, new technologies such as reverse engineering combined with machine learning have been developed and applied in the flavor field (Bi et al. 2019; Queiroz et al. 2022). It takes experimental data and automates flavor development itself, thereby creating a more rapid, repeatable cyclical workflow for the discovery and prediction of flavor formation (Queiroz et al. 2022). In the future, it is promising to combine

different instrument approaches such as GC-MS, electronic noses, and electronic tongues with machine learning to build a comprehensive approach to developing and predicting flavors based on multi factors including quantitive volatile fingerprint, simulative sensory sensors, and generative neural network models.

5 Conclusions and Outlook

The market of plant-based milk alternatives is quickly increasing. However, unbalanced nutrition and unwanted organoleptic characteristics still limit consumption. In particular, the use of mono- and mixed-culture fermentation holds great potential in improving the nutritional quality and the sensory profile of plant materials.

In this work, a genome-based approach supported the selection of two well-performing strains, *B. amyloliquefaciens* NCC 156 and *L. paracasei* subsp*. paracasei* NCC 2511, for the fermentation of chickpea milk. As shown, each of the microbes exhibited multiple benefits: an improved L-lysine content due to *de novo* synthesis of the amino acid, which was proven in ^{13}C experiments; an improved digestibility due to the removal of raffinose and stachyose; and an improved flavor profile due the elimination of off-flavor aldehydes and the generation of sweet and fruity aromas. Notably, each of the two microbes exhibited a unique signature. *B. amyloliquefaciens* NCC 156 showed a greater capacity to grow, likely enabled by its stronger portfolio of hydrolytic enzymes, whereas *L. paracasei* subsp*. paracasei* NCC 2511 exhibited pronounced synthesis of selected beneficial molecules. In this regard, each of the two strains has its own benefits for use in the fermentation of chickpea milk and appears promising for other plant-based milks.

Subsequently, a novel co-culture fermentation process was developed by a knowledge-based assembly of vitamin B_{12} production strain, *P. freudenreichii* NCC 1177 and above selected strain *B. amyloliquefaciens* NCC 156 for upgrading of sunflower seed milk, derived from sunflower press cakes as a waste product during sunflower oil production. This plant-based material offers the concept of sustainability and circular economy, when used for human consumption, and is therefore considered particularly eco-friendly in the strongly developing market of plant-based food (Petraru et al. 2021). As shown, the interactions between *P.*

freudenreichii NCC 1177 and *B. amyloliquefaciens* enabled a cooperative process with remarkable benefits: (i) enriched content of vitamin B_{12}, the key micronutrient for all vegans to be aware of, (ii) improved digestibility due to the removal of raffinose and stachyose, (iii) increased protein quality with increased levels of the most limiting essential amino acid L-lysine, and (iv) an improved flavor profile due the elimination of bitter notes and the generation of sweet and fruity aromas. Notably, the key to the successful process was microbial collaboration. The excellent co-working of the strains even enabled a completely natural process without any supplementation. Inferred from the process data of the co-culture between 48 and 65 hours, a single cell of *Propionibacterium* synthesized up to 100 molecules of B_{12} per second which displays a remarkable synthetic power, considering the complexity of the vitamin.

Additionally, we investigated a selection of 15 LAB strains from various genera and species regarding their capacity to affect the flavor profile of 4 types of plant milks of high commercial interest, a cereal-based (oat) (Fernandesa et al. 2021), a seed-based (sunflower seed) (Tangyu et al. 2022), and two legume-based ones (pea and faba) (Nawaz et al. 2020). Fermentation strongly improved the flavor of the fermented plant milks. The observed flavor changes were strongly associated with the nutrient composition of the used plant milk. Given this space, a careful selection of specific combinations of milk and strain allowed to design the flavor. Our tested flavor box of 60 possible combinations offered e. g. specific selections that yielded a strong buttery or cheesy or woody flavor, opening new options towards more diverse flavor-tailored plant milk-based products. Notably, a few strains revealed generally beneficial properties. As an example, *S. thermophilus* NCC 1326 appeared as “butter-maker” in strongly enhancing buttery notes in all plant milks, while *L. mesenteroides* emerged as a “cheese-maker” microbe that widely increased cheesy notes and *L. johnsonii* proved to be a good “minty-maker”, given

its active terpenoid-based flavor metabolism. Their identification was much facilitated or even enabled by the newly introduced approach that used the GC-MS-based relative abundance of flavor volatiles, together with their odor thresholds, to predict flavor notes and overall flavor profiles. Because relative amounts of flavor volatiles, which are much easier to assess than absolute values, could be used to predict overall flavor notes, the new workflow appears interesting to be used further.

Strikingly, the fermentations did here contained only microbes and plant milk without any further additives, different from many previous studies (Cichońska and Ziarno 2021b; Emkani et al. 2021; Herrera‐Ponce et al. 2014; Mårtensson et al. 2000; Xu et al. 2017b), matching the clean label expectation of consumers (Cichońska and Ziarno 2021a).

Nature demonstrates the power of successful collaboration for complex tasks. Notably, the degradation of plant-based matter is mediated by microbial consortia with complementary features which even apply “task division strategies” (Weiland et al. 2022). This global principle, together with the outcome of this work, suggest looking more into microbial consortia for superior microbial food processing. Hereby, the understanding of the needs and capabilities of the microbes involved appears crucial delivering food observed benefits as the results of natural processes catalyzed by safe microbes (Kohlstedt et al. 2014; Schwechheimer et al. 2018a; Schwechheimer et al. 2018b; Schwechheimer et al. 2018c).

Meanwhile, systems biology approaches have greatly advanced and open up novel possibilities to study even complex systems to a great level of detail. Due to the enormous progress in the field, quantitative systems biology studies of mixed culture fermentations of plant-based milk alternatives could become the next level of research, in order to better understand the underlying physiological, cellular, and molecular processes. The system to be studied is admittedly complex, but seminal studies on similarly complex fermentation processes involving cocoa fermentation

(Adler et al. 2013) oil-based riboflavin production (Schwechheimer et al. 2018b), and salt rich environments (Hou et al. 2000; Kohajdová et al. 2006; Kohlstedt et al. 2014) are encouraging success stories, which demonstrate the power of systems biology to shed more light into the dark and provide valuable guidance for improvement. It can be expected that similar systems level studies, which unravel genomic, transcriptomics, proteomics, metabolomics, and fluxomics in multi-omics approaches, will significantly contribute to a better understanding of plant material fermentation and upgrade the field to a level of more rational design and improvement.

Fermentation provides multi-benefits such as enhancing the gustatory and nutritional value, imparting appetizing flavor and texture of foods, and prolonging their shelf life (Asgher et al. 2020). Nowadays, food-grade microbes are used as commercial starter cultures virtue of acidification, proteolytic, possess antagonistic, antioxidant, antimicrobial, and immunomodulatory properties (Meade et al. 2020). The wide spectrum of food-grade bacteria-based probiotics, bacteriocins, exopolysaccharides, bio preservative, and their relevant benefits towards human health has been involved in more studies (Raj et al. 2021). Fermentation is a promising solution not only for high-quality plant milk alternatives but also for other plant-based applications such as plant-based meat, fish, and cheese. As the market becomes increasingly diverse, the fermentation of novel types of plant materials will become another important trend in the future.

6 Appendix

6.1 Abbreviations

AAS	Amino acid score
Ala	Alanine
ALC	Alcohols
ALD	Aldehydes
ALKA	Alkanes
ALKE	Alkenes
AP	Acetylase pathway
Arg	Arginine
Asn	Asparagine
Asp	Aspartic acid
ATCC	American type culture collection
BHI	Brain heart infusion
CadA	Lysine decarboxylase
cfu	Colony forming unit
Cys	Cysteine
DapA	4-Hydroxy-tetrahydrodipicolinate synthase
DapB	4-Hydroxytetrahydrodipicolinate reductase
DapC	Succinyl-amino-ketopimelate transaminase
DapD	Tetrahydrodipicolinate succinylase
DapE	*N*-succinyl-diaminopimelate desuccinylase
dapF	Diaminopimelate epimerase
DapF	Diaminopimelate epimerase
DavB	Lysine 2-monooxygenase
ddh	Diaminopimelate dehydrogenase
Ddh	Diaminopimelate dehydrogenase
DMBI	Dimethylbenzimidazole
DO	Dissolved oxygen
DP	Dehydrogenase pathway
DSMZ	German collection of microorganisms and cell cultures
E	Esters
EC 1.4.3.14	L-Lysine oxidase
EC 5.1.1.5	Lysine racemase
F	Furans
FA	Fatty acid
Funct. Peptides	Functional peptides
GC-MS	Gas chromatography/mass spectrometry
Gln	Glutamine

Glu	Glutamic acid
Gly	Glycine
HCA	Hierarchical cluster analysis
His	Histidine
Hom	Homoserine dehydrogenase
HPLC	High performance liquid chromatography
HS-SPME	Headspace solid-phase micro extraction
Ile	Isoleucine
IVPD	In vitro protein digestibility
K	Ketones
KamA	L-Lysine 2,3-aminomutase
LAB	Lactic acid bacteria
LBD	Lactic acid bacteria differential
Leu	Leucine
LPP	Low-pressure-pasteurized
LucD	Lysine N6-hydroxylase
Lys	Lysine
LysA	Diaminopimelate decarboxylase
MBDSTFA	*N*-methyl-t-butyldimethylsilyl-trifluoroacetamide
Met	Methionine
MRS	de Mann - Rogosa - Sharpe
MurF	UDP-*N*-acetylmuramoylalanyl-d-glutamyl-2,6-diamino-pimelate-d-alanyl-d-alanyl ligase
NAD-DAC	*N*-acetyl-diaminopimelate deacetylase
NCC	Nestlé culture collection
NDB	National nutrient database for standard reference release
O	Organic acids
OD	Optical density
PatA	*N*-acetyl-amino-ketopimelate aminotransferase
PCA	Principal component analysis
PDCAAS	In vitro protein digestibility corrected amino acid score
Phe	Phenylalanine
Pro	Proline
Ser	Serine
SFS	Sunflower seed
SP	Succinylase pathway
THDP-NAT	Tetrahydrodipicolinate acetylase
Thr	Threonine
Trp	Tryptophan
TSB	Tryptic soy broth
Tyr	Tyrosine

UHT	Ultra-high-temperature
UV	Ultraviolet
Val	Valine
Vitamin B_{12}	Cobalamin
Vitamin B_2	Riboflavin
Vitamin B_3	Niacin
Vitamin B_7	Biotin

6.2 Symbols

A_g	Peak area of an odor group	[/]
a_g	Relative abundance of an odor group	[%]
A_i	Peak area	[/]
a_i	Relative abundance of	[%]
A_{total}	Total peak area	[/]
$C^*_{2\text{-ethyl-furan, air}}$	Odor threshold of 2-ethyl-furan in air	[µg L^{-1}]
$C^*_{i, air}$	Odor threshold of analyte i in air	[µg L^{-1} / ppbv]
$c^*_{i, air}$	Relative odor threshold of analyte i	[/]
$C^*_{i, aqueous}$	Odor threshold of analyte i in water	[µg kg^{-1}]
K_i	The air/aqueous partition coefficient	[/]
M_i	The molecular weight of the compound i	[g mol^{-1}]
o_g	Relative odor activity of an odor group	[/]
o_i	Relative odor activity of analyte i	[/]
V_m	The ideal gas volume	[L mol^{-1}]
ρ	The density of water	[kg L^{-1}]

6.3 Supplementary data

6.3.1 Odor threshold

Table S1. The calculation of odor threthold (OT) in air (ppbv) of volatiles.

	OT1	OT2	OT3	OT4	OT5	OT6	OT7	OT8	OT9	OT10	OT11	OT12	OT13	OT14	Used
2-Propenal	3.6	3.6	210.0	160.0											3.6
3-Methyl-butanal		0.1	11.0												11.0
2-Methyl-butanal			11.0												11.0
Hexanal		0.3				9.7							12.6	0.3	0.3
2,4-Heptadienal		.	8.0										8.5		8.0
2-Heptenal		.	19.0										19.4		19.4
Heptanal		0.2	0.2										10.0	0.8	0.2
Benzaldehyde		100.0											43.4	19.8	19.8
2-Octenal		.	1.0											0.5	0.5
Benzeneacetaldehyde		4.0													4.0
4-Ethyl-benzaldehyde		13.0													13.0
2-Nonenal		0.1	0.0											0.0	0.0
Nonanal		0.3	0.5										2.1	0.5	0.3
2,4-Decadienal		0.1	0.4											0.4	0.1
2-Decenal		1.0	0.4										0.5	0.4	0.4
Decanal		0.	0.4										0.7		0.1
[S,S]-2,3-Butanediol		.			48.9										48.9
3-Methyl-2-buten-1-ol		.						172.5							172.5
3-Methyl-1-butanol						67.8									67.8
2-Methyl-1-butanol						67.8									67.8
1-Pentanol		100.0			9673	604.6							1009	43.0	43.0
1-Hexanol	6.0	6.0			188.7	429.0							87.3		6.0
2-Heptanol		41.0													41.0
1-Heptanol		3.0													3.0
Benzyl Alcohol		10000			3.0E5	14903									10000
2-Methyl-3-hexanol															
Phenylethyl alcohol							173.5								173.0
3,5-Xylenol		1000													1000

1-Octen-3-ol		14.0									0.5	0.5
2-Octen-1-ol		40.0										40.0
3-Octanol	27.0											27.0
2-Ethyl-1-hexanol		130.0										130.0
1-Octanol	27.0	2.7								13.9	4.2	2.7
2-Nonen-1-ol		130.0										130.0
1-Nonanol	0.9	0.9									3.1	0.9
Eugenol		6.0										6.0
Cherry propanol		.										
Isopinocarveol												
leave-Pinocarveol												
cis-Verbenol												
(-)-Myrtenol									0.3			7.0
Myrtenol									0.3			7.0
Linalool		4.0		6.5								4.0
Terpinen-4-ol							150.3					150.3
3-Ethyl-4-nonanol												
Phytol												
Methyl vinyl ketone												
2,3-Butanedione		0.3				0.0						0.3
3-Hydroxy-2-butanone				17183	91.6			1.6				1.6
2,3-Pentanedione		20.0				11.6						11.6
2-Hexanone		24-80										24.0
2-Methyl-3-pentanone												
1-(2-Furanyl)-ethanone		10.0										10.0
2,3-Heptanedione												
3,6-Heptanedione												
2-Heptanone		1.0	1.0							0.8		0.8
1-(2-Furanyl)-1-propanone												

3,5-Octadien-2-one		5.1									5.1
6-Methyl-5-hepten-2-one		50.0	1.0								1.0
5-Methyl-3-hepten-2-one											
3-Octanone		21-50									21.0
Acetovanillone											
2-Nonanone4		5.0								5.4	5.0
1-(4-Ethylphenyl)-ethanone											
5,6-Dehydrocamphor											
Pinocarvone											
D-Verbenone											
Acetic acid	6.0	6.0	162.0	480.0						570.9	6.0
3-Methyl-butanoic acid	0.1	160.0		19.0	183.4						0.1
2-Methyl-butanoic acid	0.0	10.0									0.0
Pentanoic acid		0.0	33.0	9.0		8.9					0.0
Hexanoic acid	0.6	0.6			15.1					639.8	0.6
Octanoic Acid		910.0			26.6						26.6
Nonanoic acid		3.0								1.9	1.9
Ethyl Acetate	870.0	870.0	1000	3900		3844	14416				870.0
Ethyl lactate		50000									50000.0
Ethyl 2-methylbutyrate		0.0						0.5			0.0
Hexyl acetate	1.8		307.0		8224	11.0					1.8
Myrtenyl acetate											
Verbenyl acetate											
Bornyl acetate		75.0									75.0
Epoxy-.alpha.-terpenyl acetate											
Linalyl propionate											
2-Ethyl-furan									12656		12656
									84.4		84.4
2-Acetyl-5-methylfuran											
2-n-Butyl furan									1.0E5		1.0E5

2-(1-Pentenyl)-furan										
2-Pentyl-furan		6.0				178.0		32.4	3.4	3.4
2-Methyl-benzofuran										
2-n-Heptylfuran										
Undecane	870.0	620.0								620.0
Dodecane	110.0	110.0						770.7		110.0
Tridecane										
a-Pinene	18.0		130.0		4.8E4					18.0
Camphene		880.0	130.0							130.0
β-Terpinene			130.0							130.0
3-Carene			140.0							140.0
p-Cymene		57.0								57.0
D-Limonene	38.0	38.0	130.0					130.6		38.0
γ-Terpinene							50185			50185
α-Thujene										
Alloocimene							17787			17787
2,4-Dimethyl-1-decene										
β-Gurjunene										
Methyl isocyanate										
Dimethyl ether		5.0E5								5.0E5
4-Ethenyl-1,2-dimethyl-benzene										
4-Methyl-2-propylphenol										
α-Limonene diepoxide										
5-Methoxy-1,3-dimethyl-1H-pyrazole										
Benzothiazole										
1,2-Benzisothiazole										
Benzene, 1,3-bis(1,1-dimethylethyl)-										

OT1-14 were collected or calculated from following literature: OT1 (Nagata and Takeuchi 2003), OT2 (Chemical Book Group 2016), OT3 (Yan et al. 2020), OT4 (Amoore and Hautala 1983), OT5 (Cai et al. 2014), OT6 (van Gemert 2011), OT7 (Ouyang et al. 2017), OT8 (Liu et al. 2022), OT9 (Tamura et al. 2001), OT10 (Maga and Katz 1979), OT11 (Yang et al. 2019), OT12 (Wu et al. 2016), OT13 (Xu et al. 2017a), OT14 (Yang et al. 2008).

6.3.2 Microbial growth during chickpea milk fermentation

Table S2. Microbial growth during chickpea milk fermentation. The data are given as (colony forming units) mL^{-1}. n=3.

Strains	0 h	24 h	48 h
F. sanfranciscensis NCC 463	7.65 ± 0.03	7.08 ± 0.07	6.14 ± 0.14
F. sanfranciscensis NCC 2572	7.63 ± 0.01	6.80 ± 0.02	6.24 ± 0.20
F. sanfranciscensis NCC 2629	7.56 ± 0.10	6.29 ± 0.00	6.50 ± 0.12
L. pontis NCC 380	7.72 ± 0.03	7.08 ± 0.09	7.72 ± 0.03
B. infantis NCC 365	6.51 ± 0.02	7.07 ± 0.04	6.50 ± 0.11
B. longum subsp. infantis NCC 283	6.08 ± 0.01	6.61 ± 0.25	6.71 ± 0.28
L. helveticus NCC 1182	6.64 ± 0.03	7.02 ± 0.07	6.03 ± 0.02
L. helveticus NCC 1104	6.40 ± 0.22	6.28 ± 0.12	5.86 ± 0.06
L. helveticus NCC 158	6.64 ± 0.02	0.00± 0.00	0.00 ± 0.00
L. hilgardii NCC 1497	7.50 ± 0.08	7.71 ± 0.08	7.72 ± 0.20
L. delbrueckii subsp. bulgaricus NCC 621	7.03 ± 0.00	5.57 ± 0.24	0.00 ± 0.00
L. plantarum NCC 1385	7.66 ± 0.02	8.68 ± 0.12	8.67 ± 0.06
L. brevis NCC 372	7.30 ± 0.03	7.84 ± 0.12	7.82 ± 0.06
L. johnsonii NCC 2774	7.32 ± 0.06	0.00 ± 0.00	0.00 ± 0.00
L. johnsonii NCC 2822	7.19 ± 0.01	6.01 ± 0.03	6.04 ± 0.13
L. johnsonii NCC 2767	8.47 ± 0.94	8.08 ± 0.41	6.18 ± 0.00
L. reuteri NCC 1945	7.19 ± 0.04	8.62 ± 0.12	8.77 ± 0.45
L. reuteri NCC 2823	5.16 ± 0.11	0.00 ± 0.00	0.00 ± 0.00
L. reuteri NCC 2613	6.48 ± 0.06	7.91 ± 0.38	8.52 ± 0.44
L. paracasei subsp. paracasei NCC 2511	7.39 ± 0.03	8.44 ± 0.04	8.48 ± 0.01
L. paracasei NCC 2537	7.46 ± 0.04	8.51 ± 0.05	8.63 ± 0.14
L. acidophilus NCC 2766	5.43 ± 0.01	0.00 ± 0.00	0.00 ± 0.00
L. jensenii NCC 2867	7.00± 0.00	0.00 ± 0.00	0.00 ± 0.00
B. amyloliquefaciens NCC 156	7.19 ± 0.12	8.93 ± 0.00	8.56 ± 0.14
B. amyloliquefaciens NCC 2770	5.57 ± 0.03	8.77 ± 0.07	7.40 ± 0.00
B. subtilis NCC 199	6.09 ± 0.09	7.89 ± 0.01	0.10 ± 0.00
B. licheniformis NCC 2940	6.62 ± 0.08	8.78 ± 0.04	0.10 ± 0.00
B. flexus NCC 2902	6.60 ± 0.11	9.04 ± 0.04	9.64 ± 0.12
B. flexus NCC 2903	7.01 ± 0.05	9.74 ± 0.04	8.51 ± 0.03
B. pumilus NCC 2962	7.01 ± 0.03	8.90 ± 0.13	9.02 ± 0.02
C. stationis NCC 3013	7.25 ± 0.07	9.25 ± 0.03	9.32 ± 0.03
C. stationis NCC 3016	6.85 ± 0.07	9.17 ± 0.00	9.33 ± 0.06

6.3.3 Metabolic signature profile of mono-strain during pasteurized sunflower seed milk fermentation

Table S3. Metabolic signature profile of *P. freudenreichii* NCC 1177, *B. amyloliquefaciens* NCC 156, and *L. paracasei* subsp. *paracasei* NCC 2511 during aerobic and anaerobic fermentation of pasteurized sunflower seed milk. The fermentation was carried out at 30°C either anaerobically (48 hours) or aerobically (24 hours) C. n=3.

	Sunflower seed milk	***P. freudenreichii* NCC 1177**		***B. amyloliquefaciens* NCC 156**		***L. paracasei* subsp. *paracasei* NCC 2511**	
	Non fermented	Aerobic	Anaerobic	Aerobic	Anaerobic	Aerobic	Anaerc
Growth							
Cell count (log cfu mL^{-1})	7.07 ± 0.30	8.18 ± 0.03	8.69 ± 0.04	9.04 ± 0.20	7.15 ± 0.15	7.82 ± 0.22	8.15 ±
Vitamin metabolism							
Vitamin B_{12} (µg 100 g^{-1})	n.d.	0.11 ± 0.01	0.90 ± 0.10	n.d.	n.d.	n.d.	n.d.
Vitamin B_3 (mg 100 g^{-1})	0.41 ± 0.01	0.42 ± 0.00	0.42 ± 0.01	0.64 ± 0.02	0.41 ± 0.01	0.41 ± 0.01	0.43 ±
Vitamin B_7 (µg 100 g^{-1})	3.85 ± 0.25	3.50 ± 0.22	3.50 ± 0.00	4.23 ± 0.19	4.00 ± 0.00	3.80 ± 0.00	3.60 ±
Sugar metabolism							
Sucrose (mM)	13.51 ± 1.13	13.65 ± 0.55	13.33 ± 0.51	n.d.	11.55 ± 0.44	12.36 ± 0.93	12.25 ± 0.44
Raffinose (mM)	3.74 ± 0.32	3.54 ± 0.27	3.49 ± 0.11	1.38 ± 0.08	3.21± 0.10	3.29± 0.21	3.42 ±
Stachyose (mM)	0.39 ± 0.03	0.29 ± 0.01	0.29 ± 0.02	0.07 ± 0.01	0.29 ± 0.01	0.29 ± 0.02	0.34 ±
Organic acid metabolism							
Lactate (mM)	n.d.	n.d.	n.d.	n.d.	1.64 ± 0.20	1.39 ± 0.11	1.26 ±
Propionate (mM)	n.d.	0.48 ± 0.05	1.34 ± 0.16	n.d.	n.d.	n.d.	n.d.
Acetate (mM)	0.94 ± 0.00	1.45 ± 0.66	2.43 ± 0.28	19.83 ± 0.24	1.17 ± 0.03	2.38 ± 0.20	2.22 ±
Amino acid metabolism							
L-Lysine (mM)	0.13 ± 0.00	0.15 ± 0.01	0.13 ± 0.00	0.39 ± 0.04	0.14 ± 0.01	0.12 ± 0.01	0.11 ±
L-Valine (mM)	0.19 ± 0.00	0.17 ± 0.01	0.14 ± 0.01	0.74 ± 0.01	0.20 ± 0.02	0.17 ± 0.01	0.18 ±
L-Leucine (mM)	0.07 ± 0.00	0.06 ± 0.00	0.04 ± 0.00	0.76 ± 0.04	0.09 ± 0.01	0.07 ± 0.00	0.06 ±

6.3.4 Amino acids during 96 h co-fermentation of sunflower seed milk

Table S4. Amino acids during 96 h co-fermentation of sunflower seed milk by using *P. freudenreichii* NCC 1177 and *B. amyloliquefaciens* NCC 156. n=3.

	0 h	6 h	12 h	18 h	24 h	30 h	36 h	42 h	48 h
Aspartate	2.03±0.05	2.06±0.05	2.06±0.07	1.88±0.04	1.58±0.07	1.29±0.02	0.83±0.09	0.64±0.09	0.16±0.18
Glutamate	0.46±0.01	0.49±0.02	0.51±0.02	0.47±0.01	0.46±0.01	0.46±0.01	0.47±0.04	0.45±0.02	0.43±0.01
Cysteine	0.40±0.01	0.41±0.00	0.41±0.01	0.38±0.03	0.37±0.01	0.37±0.00	0.36±0.00	0.36±0.01	0.37±0.01
Serine	0.18±0.01	0.17±0.00	0.18±0.01	0.17±0.01	0.15±0.02	0.14±0.01	0.14±0.01	0.12±0.01	0.10±0.00
Histidine	0.11±0.00	0.10±0.00	0.11±0.00	0.10±0.00	0.09±0.01	0.09±0.00	0.09±0.01	0.05±0.04	0.00±0.00
Glycine	0.34±0.00	0.34±0.01	0.33±0.01	0.29±0.01	0.23±0.02	0.19±0.02	0.12±0.01	0.13±0.01	0.11±0.00
Threonine	0.09±0.00	0.09±0.00	0.09±0.00	0.08±0.00	0.08±0.00	0.08±0.00	0.08±0.00	0.08±0.00	0.08±0.00
Arginine	0.42±0.01	0.42±0.02	0.44±0.02	0.40±0.01	0.39±0.04	0.38±0.01	0.38±0.03	0.36±0.01	0.33±0.01
Alanine	0.66±0.01	0.67±0.02	0.68±0.03	0.63±0.01	0.59±0.02	0.53±0.01	0.43±0.01	0.33±0.02	0.14±0.09
Tyrosine	0.06±0.00	0.06±0.00	0.07±0.00	0.06±0.00	0.06±0.00	0.06±0.00	0.05±0.00	0.05±0.00	0.04±0.00
Valine	0.27±0.01	0.28±0.01	0.28±0.01	0.26±0.00	0.25±0.02	0.24±0.00	0.22±0.02	0.19±0.00	0.15±0.01
Methionine	0.16±0.00	0.16±0.00	0.17±0.00	0.17±0.01	0.16±0.01	0.16±0.00	0.16±0.01	0.15±0.00	0.09±0.06
Tryptophan	0.45±0.02	0.45±0.03	0.49±0.03	0.41±0.02	0.42±0.01	0.40±0.02	0.42±0.05	0.39±0.02	0.36±0.01
Phenylalanine	0.15±0.00	0.15±0.00	0.16±0.01	0.14±0.01	0.13±0.01	0.12±0.00	0.12±0.01	0.11±0.00	0.10±0.00
Isoleucine	0.29±0.01	0.28±0.01	0.28±0.01	0.24±0.01	0.22±0.02	0.21±0.00	0.17±0.01	0.16±0.00	0.04±0.00
Leucine	0.12±0.00	0.13±0.00	0.13±0.00	0.11±0.01	0.09±0.01	0.09±0.00	0.08±0.00	0.08±0.00	0.07±0.00
Lysine	0.67±0.03	0.66±0.03	0.70±0.05	0.64±0.01	0.66±0.03	0.63±0.01	0.64±0.05	0.62±0.02	0.60±0.00
Proline	0.51±0.02	0.53±0.03	0.30±0.16	0.15±0.01	0.14±0.01	0.14±0.01	0.13±0.02	0.10±0.02	0.11±0.01

	54 h	60 h	66 h	72 h	78 h	84 h	90 h	96 h
Aspartate	0.03±0.00	0.03±0.00	0.19±0.20	1.28±0.14	0.93±0.07	1.21±0.05	1.18±0.07	1.09±0.07
Glutamate	0.04±0.00	0.10±0.03	0.52±0.09	2.11±0.23	3.54±0.30	4.46±0.30	4.60±0.19	4.49±0.29
Cysteine	0.37±0.01	0.36±0.01	0.36±0.00	0.38±0.01	0.40±0.01	0.40±0.00	0.40±0.01	0.41±0.01
Serine	0.08±0.00	0.10±0.00	0.22±0.02	0.68±0.07	1.19±0.11	1.58±0.09	1.59±0.07	1.55±0.06
Histidine	0.00±0.00	0.11±0.00	0.30±0.05	1.05±0.08	1.91±0.18	3.04±0.11	3.52±0.18	3.36±0.06
Glycine	0.10±0.00	0.11±0.00	0.15±0.02	0.37±0.04	0.65±0.08	0.89±0.04	0.96±0.05	1.08±0.09
Threonine	0.05±0.00	0.06±0.01	0.17±0.02	0.56±0.08	0.83±0.09	1.08±0.07	1.11±0.06	1.10±0.05
Arginine	0.23±0.03	0.13±0.01	0.51±0.07	1.65±0.17	2.98±0.34	3.90±0.27	3.14±0.20	1.89±0.28
Alanine	0.12±0.01	0.16±0.01	0.31±0.02	0.78±0.08	1.25±0.09	1.56±0.11	1.59±0.04	1.53±0.07
Tyrosine	0.03±0.00	0.03±0.00	0.17±0.03	0.88±0.07	1.70±0.16	2.75±0.10	3.24±0.16	3.54±0.07
Valine	0.08±0.01	0.12±0.01	0.44±0.05	1.54±0.10	3.09±0.24	4.83±0.18	5.80±0.46	6.72±0.16
Methionine	0.11±0.00	0.13±0.00	0.37±0.04	1.32±0.10	2.43±0.17	3.63±0.13	4.09±0.22	4.25±0.06
Tryptophan	0.36±0.02	0.33±0.03	0.33±0.03	0.65±0.05	1.00±0.06	1.50±0.06	1.73±0.09	1.85±0.05
Phenylalanine	0.07±0.00	0.10±0.01	0.52±0.07	2.26±0.16	4.27±0.33	6.51±0.21	7.57±0.37	7.95±0.13
Isoleucine	0.10±0.00	0.11±0.01	0.29±0.03	1.01±0.07	1.96±0.17	2.91±0.09	3.32±0.22	3.59±0.09
Leucine	0.07±0.00	0.13±0.02	0.53±0.07	2.35±0.14	4.66±0.39	7.22±0.27	8.49±0.66	8.71±0.08
Lysine	0.56±0.03	0.47±0.02	0.73±0.06	2.05±0.17	3.56±0.20	5.30±0.23	6.09±0.34	5.95±0.16
Proline	0.10±0.02	0.08±0.01	0.21±0.02	0.85±0.02	1.74±0.20	2.82±0.12	3.29±0.41	4.36±0.40

6.3.5 Fiber Selection for flavor analysis

Solid-phase microextraction (SPME) has been widely used for analysis of plant-based materials due to its fast and simple operation and a high sensitivity, which enables qualitative and quantitative analysis of flavors even at low concentration (Jeleń et al. 2012; Merkle et al. 2015). Notably, the efficiency of SPME strongly depends on the geometry and the material of the fibre coating (Merkle et al. 2015). Fibers, coated with bi-polar materials such as DVB/PDMS and DVB/CAR/PDMS are types often used for the application of plant-based milks and other plant-based foods (Fischer et al. 2021; Lee et al. 2016b; Tangyu et al. 2022; Wronkowska et al. 2022; Xu et al. 2019). Therefore, to evaluate volatile compound extraction, those materials were tested for unfermented plant milks. The impact of the different fibers on the number and the extracted amount of volatile compounds is shown in **Table S5**.

In general, both fibers well extracted aldehydes, alcohols, ketones, furans, organic acids, esters, alkanes, and alkenes from the tested plant milks. However, the fibers behaved differently in their ability to adsorb key flavor compounds. The DVB/CAR/PDMS fiber absorbed between 20 and 50 volatiles from oat milk, sunflower seed milk, pea milk, and faba milk respectively, while the DVB/PDMS fibre trapped 25-34 volatiles, respectively. The triple DVB/CAR/PDMS fiber was more efficient in extracting key flavor compounds, especially aldehydes, alcohols, and ketones, accounting for the most important flavor groups of unfermented plant milks (Lee et al. 2016b; Schindler et al. 2012; Tangyu et al. 2022; Xu et al. 2019). As the main aim of this study was to qualitatively identify volatile compounds during the fermentation, the DVB/CAR/PDMS fiber was chosen for further studies.

Table. S5: Effect of DVB/PDMS and DVB/CWR/PDMS fibers on volatile compounds extraction of oat milk, sunflower seed milk, pea milk, and faba milk.

	Oat milk		Sunflower seed milk		Pea milk		Faba milk	
	PDMS/DVB	DVB/CAR/PDMS	PDMS/DVB	DVB/CAR/PDMS	PDMS/DVB	DVB/CAR/PDMS	PDMS/DVB	DVB/CAR/PDMS
Aldehydes	10	12	5	7	10	12	10	12
Alcohols	6	7	11	13	5	8	4	7
Ketones	4	6	3	5	3	3	2	2
Organic acids	2	3	1	1	1	1	1	2
Esters	1	3	1	3	0	0	0	0
Furans	2	3	1	2	1	1	1	1
Alkanes	2	2	5	3	3	0	3	0
Alkenes	3	4	7	9	1	0	0	2
Others	3	4	3	7	3	3	2	2
Total peak number	33	44	37	50	27	28	23	28
Total peas area (1×10^7)	5.1 ± 0.8	5.3 ± 0.7	5.0 ± 0.4	5.6 ± 1.7	3.2 ± 0.4	3.4 ± 0.6	1.6 ± 0.2	1.6 ± 0.4

6.3.6 Volatile compounds identification

Table S6: Identified volatile compounds in unfermented and fermented plant-based milks, aroma attributes, odor groups, and the odor threshold in air.

Nr	Compound name*	RI cal (min)	Aroma description	Odor group **	Odor threshold (ppbv)	Identification
Aldehydes						
1	2-Propenal[3]	4.62	Fruity [1]	1	3.6 [2]	MS
2	3-Methyl-butanal	3.15	Malty [3], fatty[4], Cocoa [1], fruity [1],	1	11 [5]	MS
3	2-Methyl-butanal	3.23	Malty [3], Cocoa [1]	1	11 [5]	MS
4	Hexanal	6.09	Green [3, 6-10], grassy [3, 7, 9-11], nutty [12], fat [11], oxidized oil [13]	6	0.28 [14]	MS, STD
5	2,4-Heptadienal[3]	11.97	Fatty [1], creamy [1], green [1]	3	8 [5]	MS
6	2-Heptenal	10.48	Green [1, 15], fatty [15]	6	19 [5]	MS
7	Heptanal	8.99	Green [1]. citrus[11], fatty[11], flora[8], rancid[7, 11]	6	0.18 [14]	MS, STD
8	Benzaldehyde	10.55	Sweet [3, 10], fruity [1], almond [3, 10]	1	20 [8]	MS, STD
9	2-Octenal	13.26	Fatty [1]	3	0.53 [8]	MS
10	Benzeneacetaldehyde	12.88	Flora [3], sweet [3, 10], honey [3]	2	4 [14]	MS, STD
11	4-Ethyl-benzaldehyde	15.97	Fruity [1]	1	13 [14]	MS
12	2-Nonenal	15.88	Green [1, 16], musty[16], fatty [1],	6	0.02 [8]	MS
13	Nonanal	14.48	Citrus [7-10, 17], flora [4, 7, 8, 17], fatty [8], green [17], smoky [6]	1	0.34 [14]	MS, STD
14	2,4-Decadienal[4]	19.60	Grass [10],fatty [1], melon [10], aldehyde [10]	6	0.07 [14]	MS
15	2-Decenal	13.83	Fatty [4], orange [4], earthy [4]	3	0.43 [5]	MS
16	Decanal	17.01	Fatty [3], floral [4]	3	0.10 [14]	MS
Alcohols						
1	[S,S]-2,3-Butanediol[1,2]	5.65	Creamy [1, 18], buttery[1] [18]	3	49 [18]	MS
2	3-Methyl-2-buten-1-ol [2]	5.48	Fruity [1]	1	173 [4]	MS
3	3-Methyl-1-butanol [2,4]	4.56	Fruity [10, 19], banana [10], whiskey [4, 10], floral [3, 19], Fermented [1]	1	68 [20]	MS
4	2-Methyl-1-butanol [1,4]	4.60	Ethereal [1], floral [19]	1	68 [20]	MS
5	1-Pentanol	5.31	Fermented [1], sweet [4], fruity [17], balsamic [17], alcoholic [7]	1	43 [8]	MS, STD
6	1-Hexanol	8.03	Fruity [1, 17, 19] lemon [3, 10], nice green [8-10, 17]	1	6 [2]	MS, STD
7	2-Heptanol[1,3,4]	8.89	Citrus [1], fruity [18, 21], herbal [21]	1	41 [14]	MS
8	1-Heptanol	10.87	Light green [10], mushroom [7], rancid [10]	6	3 [14]	MS, STD
9	Benzyl Alcohol[2,3]	12.61	Floral [19]	2	1×10^4 [14]	MS
10	2-Methyl-3-hexanol	7.74	Unknown	9		MS
11	Phenylethyl alcohol[2,4]	14.71	Flora [1, 10], rose [10], honey [10]	2	0.02 [14]	MS, STD
12	3,5-Xylenol[1]	19.57	Balsamic [1]	5	1×10^3 [14]	MS
13	1-Octen-3-ol	11.12	Earthy [1], mushroom [3, 10]	7	0.52 [8]	MS
14	2-Octen-1-ol[1,2,3,4]	13.53	Cucumber [10], nice green [1, 10]	2	40 [14]	MS
15	3-Octanol[4]	11.59	Earthy [1]	7	27 [2]	MS
16	2-Ethyl-1-hexanol	12.47	Citrus [1], floral [21]	1	130	MS

17	1-Octanol	13.60	Waxy [1], aldehyde [1, 10], fruity [9], flora [9]	3	2.7 [14]	MS
18	2-Nonen-1-ol	12.49	Mint [10], melon [10] nice green [10], fatty [1]	2	130 [14]	MS
19	1-Nonanol[2,4]	16.21	Citrus, rose [10]	1	0.9 [2]	MS
20	Eugenol[4]	20.83	Pleasant spicy [1, 10], clove-like [1, 10]	2	6 [14]	MS
21	Cherry propanol	16.53	Fruity [1]	1		MS
22	Isopinocarveol	12.75	Woody [1], balsamic [1]	5		MS
23	laevo-Pinocarveol	15.38	Woody [1], balsamic [1]	5		MS
24	*cis*-Verbenol	16.08	Balsamic [1], pine [1]	5		MS
25	(-)-Myrtenol	16.83	Woody [1], minty [22], Camphoraceous [22],	5	0.32 [21]	MS
26	Myrtenol	17.02	Woody [1], herbal [1], floral [21]	5	0.32 [21]	MS
27	Linalool	14.42	Floral [1, 19, 21, 23], fruity [1, 19, 21]	2	4 [14]	MS
28	Terpinen-4-ol	16.35	Balsamic [19], woody [19, 23], green [19], fatty [19], fruity [23], floral [23], spicy [1]	5	150 [24]	MS
29	3-Ethyl-4-nonanol[2,3]	14.24	Unknown	9		MS
30	Phytol	20.24	Flora [1]	2		MS
Ketones						
1	Methyl vinyl ketone	4.59	Pungent [1], sweet [1]	8		MS
2	2,3-Butanedione[2,3,4]	2.43	Buttery [1, 12], sweet [1], creamy [1]	3	0.3 [14]	MS
3	3-Hydroxy-2-butanone[2,4]	3.96	Buttery [1, 10], creamy [1, 4, 10], sweet [11], toasted [11]	3	0.3 [14]	MS
4	2,3-Pentanedione[3]	3.74	Buttery [1, 12], sweet [12]	3	12 [4]	MS
5	2-Hexanone[1]	6.10	Fruity [1]	1	24 [14]	MS
6	2-Methyl-3-pentanone[2]	6.13	Unknown	9		MS
7	1-(2-Furanyl)-ethanone[2]	5.49	Balsamic [1], almond [1], sweet [1]	5	10 [14]	MS
8	2,3-Heptanedione[1]	7.10	Buttery [1]	3		MS
9	3,6-Heptanedione[2]	13.39	Buttery [1]	3		MS
10	2-Heptanone	8.63	Fruity [3, 8], flora [12], sweet [8], cheesy [1]	1	0.76 [25]	MS
11	1-(2-Furanyl)-1-propanone[2]	12.54	Fruity [1]	1		MS
12	3,5-Octadien-2-one	14.18	Fruity [1], fatty [1], green [1, 10], earthy [10]	1	5 [14]	MS
13	6-Methyl-5-hepten-2-one[3]	11.34	Citrus [1, 15], musty [4, 15], green [15]	1	1 [5]	MS
14	5-Methyl-3-hepten-2-one	12.75	Unknown	9		MS
15	3-Octanone	11.34	Earthy [3], mushroom [1], fresh [1], herbal [1]. ripe banana [1]	7	21 [14]	MS
16	Acetovanillone	15.88	Vanilla [1]	1		MS
17	2-Nonanone[4]	14.17	Sweet [4, 15], fruity [8, 15], floral [8], green [11, 15], hot milk [11], soap[11]	1	5 [14]	MS
18	1-(4-Ethylphenyl)-ethanone	20.05	Flora [1]	2		MS
19	5,6-Dehydrocamphor	14.27	Unknown	9		MS
20	Pinocarvone	16.00	Camphoraceous [26], fresh [26]	5		MS
21	D-Verbenone	17.15	Camphoraceous [1], minty [1, 26], spicy [1, 26]	5		MS
Organic acids						
1	Acetic acid[1,2,3,4]	2.40	Acidic[1, 3], sour [1], vinegar [1, 4, 27]	4	6 [2]	MS
2	3-Methyl-butanoic acid[1,3]	7.30	Cheesy [3, 27]	4	0.08 [2]	MS

3	2-Methyl-butanoic acid[2,3]	7.58	Sour [1], cheesy [1], fermented [1]	4	0.04 [2]	MS
4	Pentanoic acid[1]	8.37	Cheesy [1], acidic [1], unpleasure [4]	4	8.90 [20]	MS
5	Hexanoic acid[2,3]	11.21	Cheesy [1, 18, 21], fatty [1, 18, 21], sour [10], sharp [10], rancid [10, 21]	4	0.60 [2]	MS
6	Octanoic acid[2,3]	16.11	Cheesy [4, 18], fatty [1, 18], sweaty [10]	4	27 [18]	MS
7	Nonanoic acid	18.61	Waxy [1], earthy [4]	3	1.9 [25]	MS
Esters						
1	Ethyl Acetate	9.33	Sweet [1, 15], fruity [1, 15], Mild [4]	1	870 [2]	MS
2	Ethyl lactate[1]	5.90	Fruity [1], buttery [1]	1	5×10^4 [14]	MS
3	Ethyl 2-methylbutyrate	7.50	Fruity [1]	1	0.01 [14]	MS
4	Hexyl acetate	12.08	Fruity [1, 15], sweet [15]	1	1.8 [2]	MS
5	Myrtenyl acetate	10.39	Woody [1], herbal [1]	5		MS
6	Verbenyl acetate	15.53	Unknown	9		MS
7	Bornyl acetate[2]	16.97	Balsamic [1], camphoraceous [1]	5	75 [14]	MS
8	Epoxy-.alpha.-terpenyl acetate	15.18	Unknown	9		MS
9	Linalyl propionate[4]	12.47	Flora [1]	2		MS
Furans						
1	2-Ethyl-furan[3]	3.81	Chemical [1], sweet [28], coffeelike [28]	8	1.3×10^6 [28]	MS
2	2-Acetyl-5-methylfuran	7.64	Nutty [1]	5		MS
3	2-n-Butyl furan[1]	8.64	Spicy [1], fruit [1], wine [1]	8	1×10^5 [28]	MS
4	2-(1-Pentenyl)-furan[3]	11.73	Roasted [1]	5		MS
5	2-Pentyl-furan	11.46	Beany[9, 10], green[11] [10], grassy[13], nutty[9] fatty [3]	6	3.4 [8]	MS
6	2-Methyl-benzofuran	16.46	Phenolic [1]	8		MS
7	2-n-Heptylfuran	16.71	Green [1], fatty [1]	6		MS
Alkanes						
1	Undecane	14.36	Unknown	9	620 [14]	MS
2	Dodecane	16.86	Unknown	9	110 [2]	MS
3	Tridecane	19.21	Unknown	9		MS
Alkenes						
1	a-Pinene	9.80	Herbal [1], fresh [1], fruity [29, 30], woody [1], pine [6, 11, 22, 29]	5	18 [2]	MS
2	Camphene	10.30	Woody [1], herbal [1], camphoreous [1, 26, 30, 31]	5	130 [5]	MS
3	β-Terpinene	11.01	Terpenic [31], fatty [31]	5	130 [5]	MS
4	3-Carene	11.96	Citrus [1], sweet [26]	1	140 [5]	MS
5	p-Cymene	12.35	Fruity [29], fresh [1], citrus[1, 23], terpenic [1], flora [31], fragrant [31]	1	57 [14]	MS
6	D-Limonene	12.46	Citrus [1, 3, 4, 13, 23], fresh [1], sweet [1]	1	38 [2]	MS
7	γ-Terpinene	13.28	Terpenic [1, 23], citrus [23], herbal [23]	5	5×10^4 [24]	MS
8	α-Thujene[2]	14.94	Woody [1], herbal [23, 26], green [23, 26]	5		MS
9	Alloocimene[1]	15.12	Flora [1], sweet [1], nut [1]	2	1.8×10^4 [32]	MS
10	2,4-Dimethyl-1-decene	13.94	Unknown	9		MS
1	β-Gurjunene	22.25	Unknown	9		MS
Others						
1	Methyl isocyanate	11.40	Sharp, strong	8		MS

2	Dimethyl ether[2,3,4]	1.87	Ethereal [1]	1	5×10^5 [14]	MS
3	4-Ethenyl-1,2-dimethyl-benzene	14.10	Unknown	9		MS
4	4-Methyl-2-propylphenol[1]	19.66	Unknown	9		MS
5	α-Limonene diepoxide	12.54	Citrus [1]	1		MS
6	5-Methoxy-1,3-dimethyl-1H-pyrazole	14.02	Unknown	9		MS

* The number represents new forming volatiles during fermentation of oat milk (1), sunflower seed milk (2), pea milk (3), and faba milk (4).

** Odor groups: 1) fruity, citrus, sweet, melty, ethereal; 2) floral, nice green; 3) buttery, fatty, waxy, creamy; 4) cheesy, sour; 5) nutty, woody, minty, toasted, turpentine, balsamic, camphoraceous; 6) green, grassy, beany, herbal; 7) earthy, mushroom; 8) pungent, spicy, sharp, phenolic, and 9) unknown odor.

Aroma descriptions and odor thresholds were collected from previous studies: [1] (The Good Scents Company Information System 2021), [2] (Nagata and Takeuchi 2003), [3] (Akkad et al. 2019), [4] (Liu et al. 2022), [5] (Yan et al. 2020), [6] (Fu et al. 2020), [7] (Lv et al. 2011), [8] (Yang et al. 2008), [9] (Zeng et al. 2008), [10] (Xu et al. 2019), [11] (Verginer et al. 2010), [12] (Boatright and Lei 1999), [13] (Xiao et al. 2017), [14] (Peyer et al. 2016), [15] (El Youssef et al. 2020), [16] (Bott and Chambers IV 2006), [17] (Sabatini et al. 2008), [18] (Cai et al. 2014), [19] (Osorio et al. 2006), [20] (van Gemert 2011), [21] (Diarra et al. 2005; Wu et al. 2016), [22] (Costa et al. 2008), [23] (Schmidt et al. 2008), [24] (Tamura et al. 2001), [25] (Xu et al. 2014), [26] (Jirovetz et al. 2005), [27] (Aznar et al. 2001), [28] (Maga and Katz 1979), [29] (Högnadóttir and Rouseff 2003), [30] (Van Opstaele et al. 2012), [31] (Ounamornas et al. 2017), [32] (Yang et al. 2019).

6.3.7 Flavor study: PCA loading plot

Table S7. Loading plot of unfermented and fermented oat milk.

	PC1 (24.10%)	PC2 (17.51%)	PC3 (14.06%)
Acetic acid	-1.09	-0.11	0.46
Butanoic acid, 3-methyl-	-1.56	0.58	0.49
Pentanoic acid	-0.10	0.89	0.65
Hexanoic acid	-0.90	1.26	1.08
Octanoic Acid	1.01	-0.43	0.91
Nonanoic acid	1.39	-0.72	-0.52
[S,S]-2,3-butanediol	-0.94	0.96	-1.23
1-Butanol, 2-methyl-	-1.15	0.29	0.98
1-Pentanol	1.22	-0.74	0.34
1-Hexanol	1.71	0.69	0.18
2-Heptanol	-0.34	2.66	-0.74
1-Heptanol	0.69	2.71	-0.06
3-Hexanol, 2-methyl-	-1.15	-0.87	0.47
3,5-Xylenol	-0.67	-0.88	0.29
1-Octen-3-ol	1.12	0.36	0.64
2-Octen-1-ol, (Z)-	0.11	2.71	0.90
1-Octanol	0.85	2.43	0.99
1-Nonanol	0.87	1.45	0.29
3-Ethyl-4-nonanol	0.19	-1.34	0.55
Butanal, 3-methyl-	-0.42	-1.19	0.80
Butanal, 2-methyl-	-0.34	-1.21	0.88
Hexanal	-0.54	-1.38	1.99
2-Heptenal, (Z)-	0.34	-1.38	-0.04
Heptanal	0.31	-0.76	0.99
Benzaldehyde	-0.74	-0.16	2.01
2-Octenal, (E)-	1.01	-2.06	-0.13
Benzaldehyde, 4-ethyl-	0.47	-0.38	0.81
2-Nonenal, (E)-	0.47	-1.32	0.41
Nonanal	-0.90	-1.19	0.79
2,4-Decadienal	1.48	-1.58	-0.63
Dodecane	0.96	-0.49	1.02
Tridecane	1.48	1.09	0.12
α-Pinene	0.91	2.34	0.39
3-Carene	1.55	1.03	1.05
p-Cymenene	1.06	2.15	0.11
D-Limonene	1.33	0.25	0.71
Alloocimene	1.31	-0.34	-1.24
Ethyl Acetate	-0.18	-1.08	0.72
Ethyl lactate	0.20	2.44	-0.08
Butanoic acid, 2-methyl-, ethyl ester	1.13	-1.49	0.48
Acetic acid, hexyl ester	0.78	1.37	1.91
2-n-Butyl furan	-0.42	2.62	-0.83

Furan, 2-pentyl-	0.81	1.55	1.83
Benzofuran, 2-methyl-	1.54	-1.28	-0.36
2-n-Heptylfuran	-0.18	0.45	1.51
2,3-Butanedione	1.02	-1.04	-1.08
2-Butanone, 3-hydroxy-	0.46	-1.02	0.13
2-Hexanone	1.01	1.53	-0.96
2,3-Heptanedione	0.86	-0.33	-0.93
2-Heptanone	0.29	-2.03	0.50
1-Propanone, 1-(2-furanyl)-	-0.52	-0.53	1.80
3,5-Octadien-2-one, (E,E)-	1.20	0.19	1.35
5-Hepten-2-one, 6-methyl-	-0.09	-0.75	0.94
Ethanone, 1-(4-ethylphenyl)-	1.76	-0.79	-0.31
Dimethyl ether	-0.93	1.27	-1.14
4-Methyl-2-propylphenol	-0.05	1.16	1.69
Growth	-0.95	1.27	-0.13

Table S8. Loading plot of unfermented and fermented sunflower seed milk.

	PC 1 (40.60%)	PC 2 (15.04%)	PC 3 (9.68%)
Acetic acid	-0.30	1.53	-0.63
Butanoic acid, 2-methyl-	2.04	0.04	-0.49
Hexanoic acid	1.62	1.09	-0.79
Octanoic Acid	-0.05	0.50	-2.03
Nonanoic acid	1.81	0.29	-1.34
[S,S]-2,3-butanediol	-0.03	-0.18	-1.72
2-Buten-1-ol, 3-methyl-	2.43	-0.20	0.55
1-Butanol, 3-methyl-	2.43	-0.20	0.54
1-Butanol, 2-methyl-	0.15	1.42	0.07
1-Pentanol	-0.04	1.74	0.72
1-Hexanol	0.75	1.41	-0.95
1-Heptanol	1.01	0.66	-1.56
Benzyl Alcohol	2.43	-0.22	0.56
Phenylethyl Alcohol	2.42	-0.24	0.57
1-Octen-3-ol	1.15	0.36	1.25
2-Octen-1-ol, (Z)-	1.69	-0.42	0.56
1-Octanol	1.01	0.88	-0.54
1-Nonanol	-0.80	-0.99	0.00
Cherry propanol	1.81	0.78	0.01
Isopinocarveol	2.41	0.16	0.66
L-Pinocarveol	2.42	-0.24	0.57
cis-Verbenol	2.32	0.00	0.62
(-)-Myrtenol	2.43	-0.21	0.56
Myrtenol	2.42	-0.07	0.62
Terpinen-4-ol	2.28	0.59	0.08
3-Ethyl-4-nonanol	0.96	-0.89	0.24
Butanal, 3-methyl-	-0.73	-0.26	1.76
Butanal, 2-methyl-	-0.82	-0.29	1.75
Hexanal	-0.55	-0.23	1.80
Heptanal	-0.52	-0.38	1.65
Benzaldehyde	0.37	-0.98	1.04
Benzeneacetaldehyde	-0.50	-0.54	1.17
Nonanal	-0.78	0.09	1.61
Undecane	1.27	-0.64	-0.30
Dodecane	-2.19	-0.17	-0.44
Tridecane	1.37	-0.43	0.01
α-Pinene	-1.64	0.95	1.04
Camphene	-1.47	1.04	1.14
β-Terpinene	-1.56	1.06	1.15
3-Carene	-1.62	0.68	0.82
p-Cymenene	2.42	0.31	0.34
D-Limonene	-1.36	-0.32	1.55
γ-Terpinene	2.24	0.02	0.68
α-Thujene	2.42	-0.17	0.52

β-Gurjunene	0.42	-1.02	-1.00
Myrtenyl acetate	2.21	0.76	0.27
Verbenyl acetate	2.38	0.42	0.44
Bornyl acetate	2.43	-0.21	0.55
Epoxy-.alpha.-terpenyl acetate	2.45	-0.06	0.57
2-Acetyl-5-methylfuran	0.44	1.52	0.18
Furan, 2-pentyl-	-1.09	1.24	0.95
2,3-Butanedione	2.10	-0.27	0.35
2-Butanone, 3-hydroxy-	0.64	1.20	-1.39
3-Pentanone, 2-methyl-	0.00	0.41	-1.15
Ethanone, 1-(2-furanyl)-	-0.69	0.50	0.23
3,6-Heptanedione	1.69	-0.42	0.56
2-Heptanone	0.14	1.36	1.10
1-Propanone, 1-(2-furanyl)-	-0.48	0.23	1.16
2-Nonanone	0.21	1.63	-0.41
Ethanone, 1-(4-ethylphenyl)-	-0.66	1.43	1.41
5,6-Dehydrocamphor	1.86	0.21	-0.58
Pinocarvone	2.12	0.74	0.70
D-Verbenone	1.39	0.41	0.12
Methane, isocyanato-	-2.32	0.40	0.13
Dimethyl ether	-0.26	-0.37	-1.98
1H-Pyrazole, 5-methoxy-1,3-dimethyl-	0.74	0.35	-0.01
Benzene, 4-ethenyl-1,2-dimethyl-	1.57	-0.08	0.37
α-Limonene diepoxide	-2.01	-0.07	0.05
Growth	0.86	-0.11	-1.49

Table S9. Loading plot of unfermented and fermented pea milk.

	PC 1 (29.80%)	PC 2 (2323)	PC 3 (10.12%)
Acetic acid	-1.95	0.61	0.10
Butanoic acid, 3-methyl-	0.06	1.52	1.09
Butanoic acid, 2-methyl-	0.28	1.13	1.65
Hexanoic acid	-0.21	1.34	1.57
Octanoic Acid	-0.28	1.83	-0.02
Nonanoic acid	-1.04	1.28	0.51
1-Pentanol	1.07	0.73	-0.13
1-Hexanol	0.97	1.61	0.23
2-Heptanol	-1.09	1.18	-1.33
1-Heptanol	0.61	1.79	-0.49
Benzyl Alcohol	0.35	1.55	0.19
1-Octen-3-ol	1.46	0.62	0.09
2-Octen-1-ol, (Z)-	0.18	1.87	-0.81
1-Hexanol, 2-ethyl-	1.05	0.84	-1.87
1-Octanol	1.17	1.32	0.63
1-Nonanol	-0.63	-0.57	2.12
3-Ethyl-4-nonanol	1.28	-0.12	1.77
Phytol	1.00	0.90	1.25
2-Propenal	-1.15	-0.75	1.51
Hexanal	1.03	-0.58	-1.78
2,4-Heptadienal, (E,E)-	1.76	0.27	1.14
2-Heptenal, (Z)-	1.42	-0.45	0.61
Heptanal	0.99	-0.15	-2.02
Benzaldehyde	0.48	-0.92	1.33
2-Octenal, (E)-	1.87	0.13	0.02
Benzeneacetaldehyde	1.22	-0.54	-1.74
Benzaldehyde, 4-ethyl-	1.48	-0.61	-0.93
Nonanal	1.82	0.11	-0.97
2,4-Decadienal	1.89	0.10	0.83
Decanal	1.47	-0.19	1.58
Furan, 2-ethyl-	-0.48	-0.66	0.18
Furan, 2-(1-pentenyl)-, (E)-	-1.18	1.03	-0.36
Furan, 2-pentyl-	1.25	-0.67	-0.49
2,3-Butanedione	1.39	0.71	-0.10
2,3-Pentanedione	1.40	0.73	-0.28
2-Heptanone	0.85	-0.71	1.68
1-Propanone, 1-(2-furanyl)-	0.07	-1.49	0.16
3,5-Octadien-2-one, (E,E)-	1.70	-0.40	0.08
5-Hepten-2-one, 6-methyl-	-0.61	0.12	2.04
3-Hepten-2-one, 5-methyl-	1.62	0.11	-0.72
Acetovanillone	1.77	0.49	1.23
Dimethyl ether	-1.31	1.16	-0.87
Growth	-1.19	1.00	0.01

Table S10. Loading plot of unfermented and fermented faba milk.

	PC 1 (20.94%)	PC 2 (19.91%)	PC 3 (15.05%)
Acetic acid	0.99	1.24	-0.81
Butanoic acid, 3-methyl-	-0.59	1.92	0.33
Nonanoic acid	0.21	1.11	-1.22
1-Butanol, 3-methyl-	1.18	1.55	-1.29
1-Butanol, 2-methyl-	-1.69	-0.31	0.60
1-Pentanol	0.21	2.23	1.06
1-Hexanol	-1.37	1.92	1.02
2-Heptanol	-0.43	1.58	0.15
1-Heptanol	-0.17	2.26	0.81
Benzyl Alcohol	0.95	1.37	-1.79
Phenylethyl Alcohol	0.04	-0.01	-1.94
1-Octen-3-ol	0.23	2.28	0.05
2-Octen-1-ol, (Z)-	-0.79	1.81	0.61
3-Octanol	0.03	-0.51	-1.02
1-Octanol	0.75	1.32	0.55
2-Nonen-1-ol, (E)-	2.04	0.83	-0.32
1-Nonanol	-2.00	0.77	0.73
Eugenol	1.16	1.01	-1.66
Linalool	0.14	-0.21	-1.40
Butanal, 3-methyl-	1.47	-1.18	1.46
Butanal, 2-methyl-	2.21	-0.04	0.87
Hexanal	1.27	-1.24	1.72
2-Heptenal, (Z)-	-1.57	0.14	1.32
Heptanal	1.47	-1.18	1.46
Benzaldehyde	1.65	-0.42	-1.04
2-Octenal, (E)-	0.40	-0.97	-0.03
Benzeneacetaldehyde	2.07	0.35	1.24
Nonanal	1.60	-1.33	1.44
2,4-Decadienal	-1.39	-0.19	0.80
2-Decenal, (E)-	-0.43	-2.01	-1.03
Decanal	1.89	0.45	0.92
p-Cymenene	1.74	1.36	0.68
1-Decene, 2,4-dimethyl-	0.13	2.14	0.86
Linalyl propionate	-1.02	0.62	1.11
Furan, 2-pentyl-	1.88	0.98	-0.62
Methyl vinyl ketone	1.47	-1.18	1.46
2,3-Butanedione	-0.95	-0.69	-0.31
2-Butanone, 3-hydroxy-	-1.47	-0.50	0.46
2-Heptanone	0.80	0.00	0.57
3,5-Octadien-2-one, (E,E)-	0.95	0.59	-0.11
3-Hepten-2-one, 5-methyl-	-0.08	1.02	1.88
3-Octanone	0.05	-0.50	-1.59
2-Nonanone	-0.82	-0.47	0.28
Dimethyl ether	-0.26	1.83	-0.04

Growth	-0.10	1.75	-0.49

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