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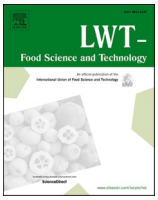

Isolation and characterization of plant-based lactic acid bacteria from spontaneously fermented foods using a new modified medium

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ABSTRACT

The development of starter cultures tailored to different plant-based materials plays a crucial role in achieving better fermented plant-based dairy alternatives (PBDA). Therefore, the isolation and study of new plant-based lactic acid bacteria (LAB) strains is essential. In this study, we developed a new medium for LAB isolation. Results showed this medium supported faster growth for all the 23 tested model strains, compared to the growth on M17 or MRS. Using the newly developed medium, 136 LAB strains belonging to 23 species and 11 genera were isolated from 73 spontaneously fermented food samples after a rigorous de-replication process. In addition, uptake of plant-based carbohydrates were studied. Only four strains were able to ferment all tested sugars efficiently. Furthermore, the production of butter aroma compounds in fermented commercial PBDA, including coconut, oat, rice, hemp, pea, hazelnut, and soy milk was also determined. These results demonstrated significant variations in the performance of fermenting PBDA by using different strains, with *E. casseliflavus* and *L. sakei* identified as efficient butter aroma producers in most of the tested conditions. Notably, certain strains also exhibited different behaviors when fermenting different substrates. The findings may provide valuable insights into developing new non-dairy fermented cultures with desired attributes.

1. Introduction

The food system has a huge impact on climate change, therefore, there is a trend to decrease the intake of animal-derived products and replace them with plant-based dairy alternatives (PBDA) (Christensen et al., 2022; Huang et al., 2022; Tilman & Clark, 2014). Plant-based Foods Association showed PBDA have became an engine for the milk industry (*Plant Based Foods Association*, 2021). PBDA such as soy, almond, oat, and rice milks are some of the most popular ones, yet differing in their appearance, mouthfeel, flavor, texture, and nutritional profile (Reyes-Jurado et al., 2021; Tangyu et al., 2019). However, PBDA often provide less nutritional value than cow milk, and have undesirable flavors, color, texture as well as anti-nutrients derived from the corresponding raw materials (Sethi et al., 2016; Tangyu et al., 2019). To solve this problem, commercial PBDA products are often supplemented with artificial flavors, sweeteners, vitamins, and minerals (Sethi et al., 2016; Tangyu et al., 2019). However, since the growing trend towards clean-label products started, it is necessary to deliver PBDA that provide advanced nutritional values and taste without adding food additives (Asioli et al., 2017). To accomplish this target, fermentation has demonstrated to be a promising solution. Fermented PBDA provide several advantages; for instance, increased bioavailability of certain micronutrients, allowing them to easily be absorbed by humans (Samtiya et al., 2021). It also increases the number of beneficial bacteria, which can help improve gut health and boost the immune system (Kechagia et al., 2013). The fermentation process also breaks down the sugars and fibers, enabling higher digestibility scores (Sharma et al., 2020). Additionally, fermentation can improve the flavor and texture of PBDA, making them tangy and more like milk fermented products such as yogurt and cheese (Coda et al., 2012; Madsen, Priess, et al., 2021; Madsen, Thulesen, et al., 2021).

Lactic Acid Bacteria (LAB) are among the best-studied

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microorganisms due to the number of potential applications in the food and biotech industry (Konings et al., 2000; Liu et al., 2019). The selection of LAB strains for mono- or co-cultured plant-based matrices is crucial to achieve the desired properties in the development of novel and sustainable products(Sedó Molina et al., 2022). However, there are still bottlenecks when mimicking texture and nutritional profiles similar to dairy fermented products (Harper et al., 2022; Sethi et al., 2016; Tangyu et al., 2019). One of the most critical issues is the limited knowledge of plant-based LAB strains in contrast to the ones used for dairy fermented products. Considering that most knowledge and industrial cultures have been developed for the dairy industry, more plant-based LAB need to be isolated and studied to develop better cultures for the next generation of PBDA (Mcclements & Grossmann, 2022; Molina et al., 2023).

LAB represents a diverse group of bacteria, and their nutritional requirements can vary significantly on strain level. Therefore, specific selective media have been developed to isolate different genera of LAB. Among them, the most commonly used media are MRS and M17. MRS is ideal for cultivating *Lactobacillus*, while M17 is more suitable for *Lactococcus* and *Streptococcus* (De MAN et al., 1960; Terzaghi & Sandine, 1975). Süle et al. have demonstrated that there can be significant variation in the growth of different strains on MRS and M17 media(Süle et al., 2014). Thus, using only one medium for isolation will potentially result in loss of isolates. Using both media for isolation is an alternative solution, but it can be a laborious task, especially when dealing with a large quantity of samples. Prolonging the incubation time is another option, but this may increase the risk of overgrowth of other undesirable spore-forming or ropy slimy microorganisms, particularly when processing fermented food samples. In this study, a new medium named MLS (Modified LAB Selective) was developed, which is a modification from MRS and M17 formulas, aiming to support the fast growth of general LAB. By using MLS medium, in this study, a total of 136 strains belonging to 23 species, 11 genera were isolated. Additionally, the capability of the isolates for fermenting plant-based sugars and their effectiveness in fermenting different types of PBDA were investigated. Our study also presents a method for detecting butter aroma compounds in non-transparent PBDA using a VP assay combined with Biolog color analysis.

2. Materials and methods

2.1. Preparation of fermented foods

We employed two methods to isolate LAB from spontaneously fermented foods. The first approach involved fermenting vegetables and fruits in a brine solution, which we referred to as kimchi. The second approach involved the use of sourdough. Kimchi samples were prepared at 14 different households using a standardized protocol (Table S1). These ingredients were cut into 1 cm^2 pieces and filled up in 50 ml tubes with half volume of prefilled 4% sterile NaCl solution and incubated for 2 days at room temperature (22–25 °C). The samples were then shifted to a temperature of 10 °C for an additional 3 days. Sourdough was prepared by 3 households using wheat, rice, or maize flour, either alone or mixed, with a water and flour ratio of 1:1. Fermentation took place at room temperature (22–25 °C) for 3 days with daily refreshments. The refreshment was carried out by transferring one-fifth of the existing sourdough into a new mixture of fresh dough. After fermentation, a final concentration of 20% glycerol was added to the water-diluted sourdough or directly to the supernatant of the kimchi samples and saved at −80 °C for future use.

2.2. LAB screening and isolation

LAB were screened using MLS-agar supplemented with 1% glucose. The composition of MLS medium is 5 g/L meat extract, 2.5 g/L meat peptone, 5 g/L KH_2PO_4, 8 g/L soy peptone, 10 g/L tryptone, 4 g/L yeast extract, 5 g/L sodium acetate, 2 g/L ammonium citrate, 0.1 g/L ascorbic acid, 0.3 g/L $MgSO_4$, 0.1 g/L $MnSO_4$, 0.034 g/L $FeSO_4$, 1 ml Tween 80 and 15 g/L agar. After adjusting the pH to 6.2, 0.4 g/L cycloheximide was added to agar-plates for inhibiting yeast and fungal growth. After inoculation, the plates were incubated at 30 °C for up to 2 days, plates were transferred to fridge (4 °C) for 2–3 days. Then, 10–15 colonies were picked mainly based on their morphological features, such as size, color, and shape (Fig. S1). Subsequently, the picked colonies were re-streaked on a new MLS-agar plate at least one time for colony purification.

2.3. Strains identification by using Maldi-Tof and de-replication

Purified colonies grown on MLS-agar plate within 24 h were picked loaded onto ground steel MALDI target plate for identification by using Maldi-Tof Biotyper according to the manufacturer's manual (Bruker Daltonics, Bremen, Germany). In order to remove potential duplicate strains from the isolates, a strict de-replication procedure was then employed. If two strains originating from the same sample received the same Maldi-Tof ID with similar scores, only one of the strains would be saved and characterized for further studies.

2.4. Evaluation of plant-based sugar utilization

The utilization of plant-based sugar was evaluated by measuring the biomass accumulation in 24 h. Each strain was inoculated at an optical density (OD_{600}) of 0.02 in MLS medium, which was individually supplemented with 0.5% distinct sugars such as sucrose, raffinose, galactose, xylose, maltose, and arabinose or without any sugar as a control, all in duplicates. After 24 h of incubation at 30 °C, cell density was determined using a microplate reader (Tecan, Infinite 200 Pro) at 600 nm wavelength. Strains grown in sugars were compared with their corresponding controls that did not contain sugar. After subtracting the values in the corresponding control, values greater than 0.3 (account for $5*10^8$ cells) were considered as positive, values greater than 0.05 (account for 10^8 cells) but less than 0.3 considered as weak positive, values less than 0.05 considered as negative. To evaluate sugar consumption at the species level, a scoring method was created as follows:

$$Y = \frac{0.5W + P}{N}$$

Where Y stands for the assigned score to a particular species, W represents the number of weak positive strains within that species, P represents the number of positive strains in one species, and N represents the total number of strains in that species. Species within more than three characterized strains are evaluated by using this method. If a species receives a score greater than 0.75, it means that over 50% of the strains have been identified as positive for sugar consumption. Conversely, if the score is less than 0.25, it indicates that over 75% of the strains are unable to utilize the sugar.

2.5. Evaluation of butter aroma compounds production

Each strain was precultured overnight and inoculated into a deep well plate containing a chemical-defined medium(Ruhdal & Hammer, 1993) with 1% glucose, at an optical density of 0.05. The cultures were grown for 24 h at 30 °C. A Voges-Proskauer (VP) test was then performed as follows: 83 μl of the culture was transferred to a microplate, followed by the addition of 50 μl of α-naphthol and 16 μl of 40% potassium hydroxide. After incubation in a fume hood for 30 min, the absorbance was measured at OD_{510}. For conducting VP test in plant-based milk, we followed the same VP procedure as described above. Additionally, we introduced an extra step when evaluating rice and oat milk, by adding 1.5% arginine prior to the addition of α-naphthol to eliminate false negative results. Then a color development assessment was conducted using the Other Plate program in OmniLog (Biolog Inc. Hayward, California), aiming to eliminate false positive

results caused by the turbidity of PBDA. The performance of color development was evaluated by kinetic data in 40 min. After subtracting the background value in reference cells, a judicious balance between sensitivity and specificity was sought. To achieve this balance and minimize the risk of false positives, a VP positive threshold was set at 35% of the maximum value obtained from each type of PBDA, and a negative threshold was set at 15 for all cultures. The commercial PBDA used in this study as well as their labeled composition are listed in Table 1.

2.6. GC-MS analysis of diacetyl and acetoin

To assess diacetyl and acetoin concentrations in fermented plant-based samples, strains are inoculated into soy, coconut, and almond milk respectively within 15 ml centrifuge tubes. After incubating at 30 °C, the comprehensive profile of volatile organic compounds is analyzed using a well-defined dynamic headspace analysis method(Sedó Molina et al., 2023).

3. Results

3.1. Evaluation of LAB growth on different agar media

To evaluate LAB growth on the newly modified LAB screening (MLS) medium, various strains were picked from DTU NFICC (National Food Institute Culture Collection)(Genet et al., 2023). Precisely, 23 LAB strains belonging to 17 species were investigated and compared with that on M17 (glucose supplemented) and MRS. As shown in Table 2, different LAB species showed different preferences to grow on specific agar media. In general, *L. curvatus, L. brevis (NFICC975), L. paracasei, L. parabuchneri, F. sanfranciscensis, L. sakei and P. pentosaceus* showed higher preference on MRS, while *L. garlicum, W. confusa, L. plantarum, C. maltaromaticum, L. mesenteroides, L. hilgardii, C. alimentarius*, and *L. lactis* preferred M17.

Remarkably, all the tested LAB displayed similar or larger colony sizes on MLS medium in comparison to their growth on either M17 or MRS. This indicates MLS may support the growth of most LAB while maintaining the selectivity of MRS medium since it contains the same concentration of selective compounds from MRS. Therefore, MLS medium was applied for the following LAB screening and isolation from fermented food samples.

3.2. Strains isolated from spontaneously fermented foods

For plant-based LAB isolation, a total of 73 spontaneous fermented food samples were prepared. As shown in Table 3, after de-replication, a total of 136 LAB strains belonging to 23 species, from 11 genera were obtained. To investigate the correlation between the isolated LAB species and the materials used for sample preparation, the 73 samples were divided into 7 categories: Bulb, Fruit, Leaves, Root, Tuber, Sourdough and Other (described in Table S1).

Fig. 1 shows the incidence of different LAB species isolated from each category. The most prevalent LAB species among all the plant materials is *Leuconostoc mesenteroides*, with 36 strains isolated and had the highest incidence in leaves (36%), roots (37%), and tubers (33%). *Lactobacillus plantarum* was another dominant species, with 18 strains isolated and a high incidence in bulbs (33%). Other notable LAB species included

Table 2
Evaluation of LAB growth on different selective agar plates.

NFICC number	MALDI-TOF ID	M17		MRS		MLS	
		1d	2d	1d	2d	1d	2d
NFICC31	*Leuconostoc mesenteroides*	+-	+	–	+-	+-	+
NFICC 40	*Weissella confusa*	+	++	–	+-	++	++
NFICC 61	*Pediococcus pentosaceus*	+-	+-	+	++	+	++
NFICC 80	*Leuconostoc garlicum*	–	+	–	+-	+	+
NFICC 132	*Lactococcus lactis*	+	+	–	+-	+	+
NFICC 328	*Latilactobacillus curvatus*	–	–	–	+-	+-	++
NFICC 733	*Lactiplantibacillus plantarum*	+	+	+-	+	+	+
NFICC 857	*Lentilactobacillus hilgardii*	+-	+	–	+-	+	+
NFICC 858	*Companilactobacillus alimentarius*	+-	+	–	+-	+	+
NFICC 864	*Limosilactobacillus fermentum*	–	+-	–	+-	+-	+
NFICC 883	*Lactococcus lactis* ssp *cremoris*	+	+	–	+-	+	++
NFICC 928	*Lactococcus lactis* ssp *tructae*	+-	+	–	+-	+	+
NFICC 964	*Lactococcus lactis* ssp *lactis*	+	++	+-	+-	+	++
NFICC 975	*Levilactobacillus brevis*	–	+-	+-	+	+-	++
NFICC 997	*Lactococcus lactis* ssp *hordniae*	+	+	–	+-	+	++
NFICC 1433	*Lacticaseibacillus paracasei*	–	+	+-	+	+-	++
NFICC 1509	*Lacticaseibacillus pantheris*	+-	+	+-	+	+	++
NFICC 1523	*Carnobacterium maltaromaticum*	+	+	–	–	+	+
NFICC 1553	*Lentilactobacillus parabuchneri*	–	–	–	+-	–	+
NFICC 1555	*Levilactobacillus brevis*	+-	+	+-	+	+-	++
NFICC 1536	*Fructilactobacillus sanfranciscensis*	–	–	–	+	+-	++
NFICC 1541	*Leuconostoc citerum*	+-	+	+-	+	+	++
NFICC 1773	*Latilactobacillus sakei*	–	+-	+	+	+	+

Growth of different LAB strains were evaluated by measuring the size of single colony after incubation at 30 °C for 1 or 2 days, where "-" stands for not visible; "+-" stands for less than 1 mm diameter; "+" stands for more than 1 mm diameter; "++" stands for more than 2 mm diameter. NA, data not investigated.

Table 1
Plant-based milk compositions labeled by manufacturers.

Every 100 ml	Pea	Soya	Coconut	Rice	Oat	Hemp	Hazelnut
Manufacturer	DRYK	Naturli	Ecomil	Naturli	DRYK	Ecomil	Ecomil
Energy (kJ)	176	153	139	220	256	165	133
Fat (g)	3	2.1	2.7	1.3	2.8	2.9	2.6
-here saturated fatty acid (g)	0.3	0.3	2.5	0.2	0.2	0.3	0.4
Carbohydrate (g)	1.8	0.6	2	10	7.8	2.2	1.3
-here Sugars (g)	1.8	0.6	0.15	7	3.5	0.3	0.3
Fiber (g)	–	0.6	0	–	–	–	–
Protein (g)	2	3.7	0.2	0.1	1	1	0.6
Salt (g)	0.2	0.09	–	0.11	0.1	0.1	0.1
Sodium (mg)	–	–	40	–	–	40	40
Vitamin D (μg)	1	–	–	–	1	–	–
Riboflavin (mg)	0.21	–	–	–	0.21	–	–
Vitamin B12 (μg)	0.38	–	–	–	0.38	–	–
Calcium (mg)	120	–	–	–	120	–	–

Table 3
Overview of the isolates from different types of spontaneously fermented food samples.

	Tm	Pp	P	Sp	Squ	Syb	Spn	Rd	Rd, Mu, L	Mu	Ro, Mu, L	Ro	Ro, Gn	Gn	Wo	Rcb	Pep	Pr	Lev	Ltt	Ja	Gar	Cuc, Ro	Cuc	Cel	Cr	Cb	Ap	Sod	Total
E. casseliflavus						1				1				1											1					4
E. durans																													1	1
E. faecium			1																1								1			3
L. brevis			1					1				1																	2	5
L. curvatus			2									1															1	1	3	8
L. fermentum								1										1												2
L. paracasei	1		2													1	1	1								1				7
L. paraplantarum			2				1					1																		4
L. plantarum	1		1					1	1	1	1	2		1		1	1			1		1	1				1		3	18
L. rhamnosus					1																									1
L. sakei			1		1		1			1									1								1			6
P. vaccinostercus					1																									1
L. garvieae										1			1	1																3
L. lactis	1		2		1									1				1			1									7
Leu. citreum																													4	4
Leu. lactis																						1					1			2
Leu. mesenteroides			6	2	2			1						2	1	1		1	1	1	1		1	1	2	4	4		5	36
Leu. pseudomesenteroides					1																					4				5
P. acidilactici																													1	1
P. pentosaceus								1										1											7	9
W. cibaria								1														1				1			2	5
W. hellenica		1					1																		1					3
W. viridescens																											1			1
No. Isolated strains	3	1	18	2	7	1	3	6	1	4	1	5	1	6	1	3	2	5	3	2	2	3	2	1	4	10	10	1	28	136
No. Samples	1	1	7	2	4	1	2	2	1	2	1	2	1	3	1	2	2	2	1	1	1	3	1	1	3	7	6	1	11	73
No. Isolated species	3	1	9	1	6	1	3	6	1	4	1	4	1	5	2	3	2	5	3	2	2	3	2	1	3	4	7	1	9	

Tm- Tomato; Pp- Purple potato; P- Potato; Sp- Sweet potato; Squ- Squash; Syb- Soybean; Spn- Spinach; Rd- Radish; Mu- Mushroom; L- Leek; Ro- Red onion; Gn- Ginger; Wo- White onion; Rcb- Red cabbage; Pep- Pepper; Pr- Pear; Lev- Levet; Ltt- Lettuce; Ja- Jerusalem Artichoke; Gar- Garlic; Cuc- Cucumber; Cel- Celery; Cr- Carrot; Cb- Cabbage; Ap- Apple; Sod-sourdough.

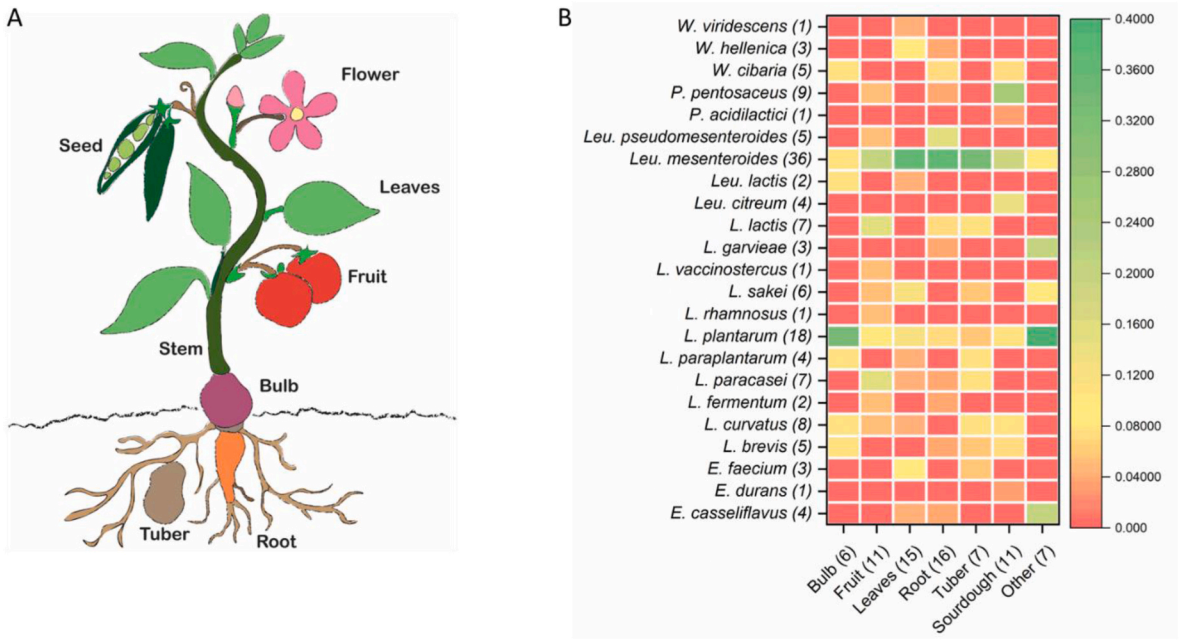

Fig. 1. Correlation between LAB species and isolation sources. A. Sketch for illustrating the different parts of plants, including root, stem, leaves, bulb, tuber, and fruits. B. A heatmap displaying the incidence of each LAB species isolated from particular plant material. Each color represents the percentage of strains of a particular species that were isolated from a specific part of the plant, with the sum of each vertical column equaling 1. The number in horizontal axis indicates the quantity of investigated samples from different plant parts, the number in vertical axis indicates the quantity of isolated strains of that species. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

No. of strains	Species	Arabinose	Galactose	Maltose	Raffinose	Sucrose	Xylose
4	*E. casseliflavus*	0.00	0.25	0.13	0.00	0.38	0.75
1	*E. durans*	0.00	1.00	1.00	0.00	1.00	0.00
3	*E. faecium*	1.00	1.00	0.50	0.50	0.67	0.00
5	*L. brevis*	0.70	0.70	1.00	0.10	0.80	0.80
8	*L. curvatus*	0.00	0.81	0.69	0.00	1.00	0.00
2	*L. fermentum*	1.00	0.50	1.00	1.00	1.00	0.25
7	*L.paracasei*	0.36	0.86	0.64	0.14	1.00	0.21
4	*L. paraplantarum*	0.25	1.00	0.88	0.38	1.00	0.00
18	*L. plantarum*	0.36	1.00	1.00	0.44	0.94	0.08
1	*L. rhamnosus*	0.00	1.00	0.00	0.00	1.00	1.00
6	*L. sakei*	1.00	1.00	0.33	0.00	1.00	0.17
1	*L. vaccinostercus*	1.00	0.50	0.50	0.00	1.00	1.00
3	*L. garvieae*	0.17	0.50	0.67	0.33	0.50	0.00
7	*L. lactis*	0.29	0.86	0.57	0.14	1.00	0.86
4	*Leu. citreum*	0.63	0.63	0.75	0.13	1.00	0.13
2	*Leu. lactis*	0.25	0.25	1.00	0.75	1.00	0.00
36	*Leu. mesenteroides*	0.90	0.63	0.97	0.85	0.99	0.57
5	*Leu. pseudomesenteroides*	0.80	0.30	1.00	0.70	1.00	0.70
1	*P. acidilactici*	0.50	1.00	1.00	0.00	1.00	0.50
9	*P. pentosaceus*	0.61	1.00	1.00	0.67	1.00	0.50
5	*W. cibaria*	0.90	0.90	1.00	0.00	1.00	0.80
3	*W. hellenica*	0.83	0.50	0.83	0.00	1.00	0.00
1	*W. viridescens*	0.50	0.00	0.00	0.00	1.00	0.50

Fig. 2. Evaluation of sugar consumption in species level. Scores indicate the capability of consuming each of the sugar, the higher the better. Green: more than 50% of strains can efficiently utilize the indicated sugar. Yellow: 25–50% of strains can utilize indicated sugar. Red: less than 25% of strains can utilize the indicated sugar. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

Pediococcus pentosaceus, *L. lactis*, *L. sakei*, *L. paracasei*, *L. curvatus*, etc. were found only prevalent in specific materials. For example, 9 *P. pentosaceus* strains were isolated in total, and they only showed a high incidence in sourdough (25%) but none were isolated from bulbs, leaves, or tubers. In contrast, 7 *L. paracasei* strains obtained only showed relatively high incidence in fruits (16%) and tubers (11%), but none isolated from bulbs or sourdough. Some LAB species, such as *P. acidilactici, W. viridescens, Leu. lactis* and *L. rhamnosus*, were only isolated from specific plant materials with low incidence, indicating a more restricted distribution. Overall, the results suggest that different plant materials host diverse LAB communities, with some species being more prevalent in certain parts of the plant than others.

3.3. Characterization of growth in plant-derived carbon sources

An overview of the fermentability of different sugars for each strain is shown in Table S2. Among the 136 strains, 122 efficiently grew in sucrose, 86 in maltose, 80 in galactose, 65 in arabinose, 48 in raffinose, and 31 in xylose. Only 4 strains, one *L. plantarum* (NFICC1649), one *Leu. mesenteroides* (NFICC1736), and 2 *P. pentosaceus* (NFICC1651 and NFICC1648) were shown able to ferment all 6 sugars efficiently. Our results suggested isolates belonging to the same species generally exhibited similar behaviors on sugars consumption, though a few strains exhibit unique characteristics. Furthermore, the similarities on sugar consumption for different strains within same species was also performed.

Fig. 2 showed that sucrose can be well metabolized by most of the characterized species, except some strains of *E. casseliflavus*, *E. faecium* and *L. garvieae*. Similarly, galactose and maltose were also able to be well metabolized by most of the species. Nevertheless, it was shown that more than half of *Leuconostoc* strains cannot efficiently utilize galactose and more than half of *Lactococcus* strains cannot efficiently utilize maltose.

For arabinose, most *E. faecium*, *L. fermentum*, *L. sakei*, *P. vaccinostercus*, *Leu. pseudomesenteroides*, *Leu. mesenteroides*, *W. cibaria* and *W. hellenica* were shown to be able to ferment it efficiently. For raffinose, only *L. fermentum, Leu. lactis* and *Leu. mesenteroides* were shown able to ferment efficiently. On the other hand, a few strains of *E. faecium*, *L. paraplantarum*, *L. plantarum*, *L. garvieae*, *Leu. pseudomesenteroides* and *P. pentosaceus* were also shown able to efficiently consume raffinose.

For xylose, most *L. brevis*, *L. rhamnosus*, *P. vaccinostercus*, *L. lactis*, and *W. cibaria*, as well as some *Leu. mesenteroides*, *Leu. pseudomesenteroides* and *P. pentosaceus* were shown able to metabolize it efficiently. The findings indicate some species are better at fermenting certain sugars than others, which highlights the feasibility of considering the species-level characteristics when selecting LAB strains for specific applications.

3.4. Screening for aroma compounds producers

Aroma compounds are highly interesting given that they contribute to the taste and flavor of foods. Acetoin and diacetyl are known to be produced by LAB and contribute to the butter flavor and aroma. In this study, the production of acetoin and diacetyl are characterized for different strains by using a chemical defined medium (SA) supplemented with 1% glucose and incubation at 30 °C for 24 h. VP test is a colorimetric approach for detecting both acetoin and diacetyl. As shown in Table 4, 59 strains belonging to 15 species were characterized as VP positive, including 3 *E. casseliflavus*, 1 *L. brevis*, 3 *L. paracasei*, 1 *L. paraplantarum*, 15 *L. plantarum*, 1 *L. rhamnosus*, 3 *L. sakei*, 1 *P. pentosaceus* that had superior color development. All the 59 VP-positive strains were picked for further characterization in fermenting different commercial PBDA.

Table 4
VP test in defined medium.

Species	Strain ID	VP test	Species	Strains ID	VP test
E. casseliflavus	NFICC1611	+		NFICC 1603	+
	NFICC 1622	+		NFICC 1672	+-
	NFICC 1781	+		NFICC 1619	+
E. faecium	NFICC 1772	+-		NFICC 1649	+
L. brevis	NFICC 1723	+-		NFICC 1665	+
	NFICC 1667	+-		NFICC 1660	+
L. curvatus	NFICC 1637	+-		NFICC 2031	+
	NFICC 1675	+		NFICC 2020	+
	NFICC 1684	+-	*L. rhamnosus*	NFICC 2041	+
	NFICC 1732	+-	*L. sakei*	NFICC 1747	+
	NFICC 1671	+-		NFICC 1683	+-
	NFICC 1656	+-		NFICC 2018	+
L. paracasei	NFICC 1745	+-		NFICC 2042	+
	NFICC 1741	+-		NFICC 1773	+-
	NFICC 1743	+-		NFICC 1786	+
	NFICC 1746	+-	*L. garvieae*	NFICC 1625	+
	NFICC 2032	+	*L. lactis*	NFICC 1721	+
L. paraplantarum	NFICC 1722	+-		NFICC 2043	+
	NFICC 1602	+-	*L. citreum*	NFICC 1658	+-
	NFICC 1803	+	*Leu. mesenteroides*	NFICC 1666	+-
L. plantarum	NFICC 1628	+		NFICC 2029	+-
	NFICC 1636	+-	*P. pentosaceus*	NFICC 1645	+-
	NFICC 1740	+-		NFICC 1651	+-
	NFICC 1718	+		NFICC 1644	+-
	NFICC 1616	+		NFICC 1663	+-
	NFICC 1681	+		NFICC 1655	+-
	NFICC 1715	+		NFICC 1650	+
	NFICC 1694	+		NFICC 1734	+-
	NFICC 1608	+-	*W. cibaria*	NFICC 1646	+-
	NFICC 1607	+-			

3.5. Acidification of plant-based milk and production of butter aroma compounds

Regarding the VP assay, none of the unfermented commercial milk resulted in color development. However, it is worth mentioning that distinct color development was observed in oat and rice milk samples only after supplementing with arginine (Fig. S2). This indicates a low composition of guanidine compounds in oat and rice milk, since guanidine compounds are required for the VP assay. Fig. 3 provides information about the acidification and VP test results of 59 pre-screened

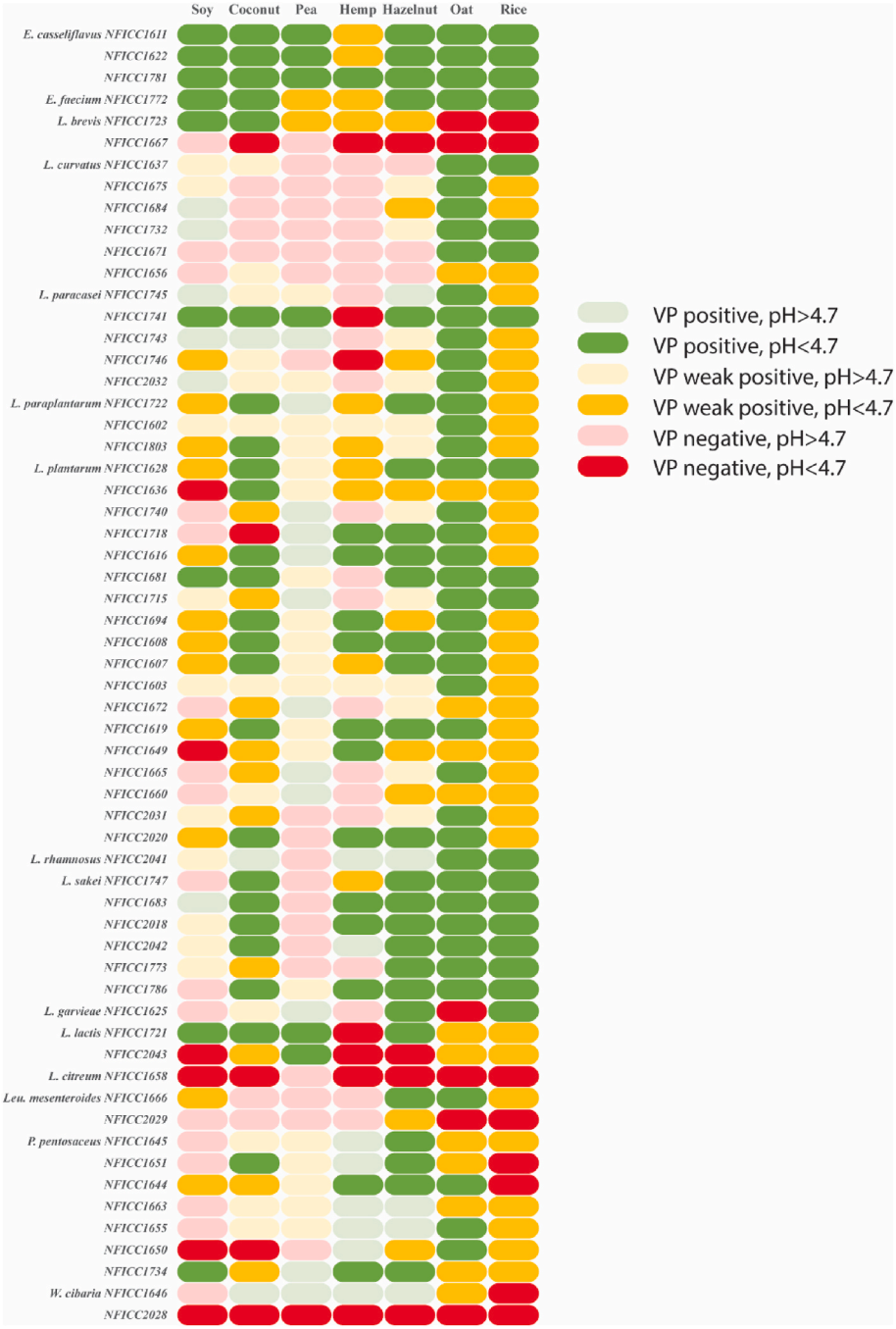

Fig. 3. Characterization of 59 strains in acidification and butter aroma production after fermenting different plant-based substrates. The green color in the figure indicates a positive result in the VP test with superior color development, the yellow color indicates a positive result in the VP test with weak color development, and the red color indicates a negative result in the VP test. The saturated color indicates a final pH below 4.7, indicating good acidification, while the fading color indicates a final pH over 4.7. All data were investigated after fermenting different plant-based milks at 30 °C for 24h. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

strains after fermenting different plant-based products. Although two strains NFICC1677 and NFICC1658 exhibited VP negative in all tested plant-based milks, most of the strains showed VP positive after acidifying the substrates.

We observed fewer strains characterized as VP positive in soy and pea milk. In species level, *E. casseliflavus* and *L. sakei* were found to have superior color development in most of the substrates, suggesting they are efficient in producing butter aromas in various conditions. Interestingly, some strains also exhibited VP-positive results without acidifying their substrate. For example, *L. rhamnosus* NFICC2041 was characterized as VP positive without acidification after fermenting coconut, hemp and hazelnut milks.

It is worth noting that VP results in some strains that were substrate dependent when acidification occurred. For instance, *L. lactis* NFICC2043 was able to acidify all tested PBDA, but color development in the VP assay was only detected after fermenting coconut, pea, oat, and rice milks. A similar pattern could also be observed in *L. brevis* NFICC1723, *L. plantarum* NFICC1636, NFICC1718, NFICC1649, *L. paracasei* NFICC1745, etc.

To further validate these findings, three strains, that make different

Table 5
Validation of acetoin and diacetyl production determined by GC-MS.

Treatment	Acetoin (mM)	Diacetyl (mM)	VP
Soy control	0 ± 0	0 ± 0.01	–
Soy NFICC1684	7.81 ± 0.63	0.50 ± 0.15	+
Soy NFICC1718	0.30 ± 0.03	0 ± 0	–
Soy NFICC2043	0.06 ± 0.05	0 ± 0	–
Hazelnut control	0.01 ± 0.01	0 ± 0	–
Hazelnut NFICC1684	0.62 ± 0.04	0.02 ± 0.03	+-
Hazelnut NFICC1718	2.78 ± 0.67	0.27 ± 0.01	+
Hazelnut NFICC2043	0.25 ± 0.19	0.01 ± 0.01	–
Coconut control	0 ± 0	0 ± 0	–
Coconut NFICC1684	0.10 ± 0.08	0 ± 0	–
Coconut NFICC1718	0.32 ± 0.01	0.07 ± 0.03	–
Coconut NFICC2043	0.79 ± 0.22	0.12 ± 0.09	+-

VP results after fermenting soy, hazelnut, and coconut milk, were randomly selected (NFICC1684, NFICC1718, and NFICC2043) and validated by using GC-MS. The results (Table 5) showed that higher levels of acetoin and diacetyl were detected in NFICC1684-fermented soy milk, NFICC1684- and NFICC1718-fermented hazelnut milk, and NFICC2043-fermented coconut milk, which were all characterized as VP positive. These findings suggest that the VP assay combined with the color analysis by the Biolog system is a useful tool for detecting the presence of acetoin and/or diacetyl in LAB fermented PBDA.

Furthermore, we also characterized the buffering capacity in different plant-based products, Fig. 4 shows that pea and soy milks have a significantly higher buffering capacity compared with other type of milks. Specifically, to achieve a pH reduction from 7 to 4 in different substrates, the volume of added HCl was determined by the following ratios: 1 for rice, 1.5 for hemp and coconut, 2 for oat, 3 for hazelnut, 8 for soy, and 10 for pea. This reveals a strong buffering capacity in both soy and pea milk and probably explains why fewer strains were detected able to acidify soy and pea milk to a pH below 4.7 (Fig. 3).

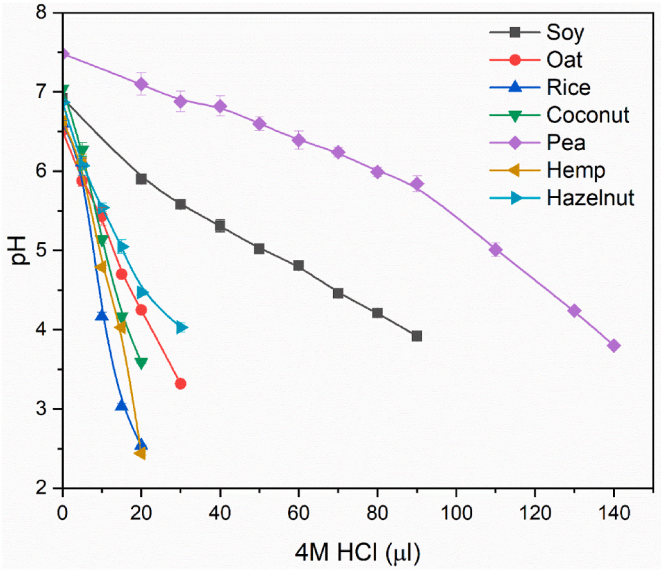

Fig. 4. Buffering capacity of different commercial plant-based milks. pH titration using 4M HCl in soy, oat, rice, coconut, pea, hemp and hazelnut milks, which are indicated by different colors. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

4. Discussion

4.1. Satisfactory performance using newly modified medium for LAB isolation

LAB is a large group of micro-organisms that are generally known as fastidious in nutritional requirements and cultivation conditions (Hayek et al., 2019). Isolating and culturing specific LAB strains from natural sources can be challenging, and different media have been developed over the years to facilitate their growth and isolation.

In 1950s, a medium known as tomato juice was favorable for culturing lactobacilli (COX & BRIGGS, 1954). However, tomato juice medium had limitations due to its low selectivity towards LAB and failed to give consistent results. To address this, Rogosa et al. developed a highly selective medium for Lactobacilli called Rogosa medium, which contained over 25 g/L acetate (ROGOSA et al., 1951). However, this medium was shown to be unsatisfactory as some lactobacilli were inhibited due to its high selectivity, therefore it was optimized by diminishing acetate concentration, resulting in MRS medium (De MAN et al., 1960). Although most of lactobacilli grow well in MRS medium, studies showed it poorly supports the growth of some other LAB like lactococci and streptococci. Therefore in 1970s, M17 was developed to isolate and cultivate these microorganisms (Süle et al., 2014; Terzaghi & Sandine, 1975). Since then, both media are mostly used for isolating and culturing LAB because they combined support the growth of most LAB. Nevertheless, for the purpose of isolating general LAB from environmental samples, especially when processing a large number of samples, it is laborious to use both media, as strains may appear in both, resulting in more than doubled workloads. Developing a highly selective medium for general LAB can be challenging as it requires a compromise between inhibiting unwanted organisms and supporting the growth of desired organisms. One strategy is to develop a general medium that supports most LAB growth and then add selective compounds to make it selective to LAB.

In this study, we developed the MLS medium based on the formulas of MRS and M17, which have been successfully used for LAB isolation for many decades. The MLS medium contains selective compounds like sodium acetate, ammonium citrate, and ascorbic acid, which are known for either stimulating LAB growth or antimicrobial activity(Journal et al., 1980; Lino T et al., 2002; Mousavi et al., 2019). Other selective compounds that can potentially be used for increasing the selectivity of the medium like sodium azide and antibiotics such as vancomycin have also been considered (Efthymiou & Joseph, 1974; Lichstein & Soule, 1944; Süle et al., 2014). However, several studies reported that their use may also inhibit the growth of some LAB strains, particularly lactococci. Therefore, their inclusion in the medium must be carefully considered and evaluated. Alternatively, using a fungicide like cycloheximide or natamycin could be a suitable approach to minimize the risk of causing any interference with the growth of LAB (Pedersen, 1992; Petkova et al., 2021; Shah et al., 2020). Moreover, the medium was formulated with an acidic pH, which plays a crucial role in ensuring that the LAB strains can outcompete other unwanted microorganisms. Nevertheless, it is important to maintain an optimal pH level, as fast acidification of the culture can result from growth of the LAB strains. Hence, a robust buffering capacity is able to delay growth arrest due to the medium acidification and result in higher biomass. In this study, we doubled the amount of buffering agent in MLS compared to that in MRS. From our observation, MLS medium supported a similar or better growth for all the 17 tested LAB species compared with those growing on MRS or M17. By using MLS medium, a total of 136 LAB strains which belonged to 23 species and 11 genera, were isolated from spontaneously fermented food samples, demonstrating its satisfactory performance on LAB isolation.

4.2. Rigorous de-replication procedures are applicable for high-quality LAB isolation

In this study, most of the LAB strains were isolated from spontaneously fermented kimchi, a type of food that has a long history and is widely consumed in many countries. Conventional kimchi is typically dominated by LAB such as *Leuconostoc*, *Lactobacillus*, and *Weissella*, with *Leu. mesenteroides* and *L. plantarum* being the most frequently observed species (Cho et al., 2006). This is also confirmed in our study that *Leu. mesenteroides* or *L. plantarum* were found in 42/62 kimchi samples and contribute to 46/108 of the strains. Interestingly, only in 4 of the 42 samples both *Leu. mesenteroides* and *L. plantarum* were found together. Furthermore, both species were less abundant in sourdough samples.

Some community studies indicated that the LAB found from fermented food samples are mostly culturable (Calabrese et al., 2022; Hong SW et al., 2016). However, it is quite challenging to isolate the strains that do not belong to one of the dominants in a sample. Studies showed such minority strains can only be isolated by picking up hundreds of colonies from agar-plates (Calabrese et al., 2022; Maoloni et al., 2020; Se-Hui Lee et al., 2015). This is likely because LAB always form white, smooth, and round colonies that are difficult to distinguish from each other on agar-plates, especially when identifying minority strains in the presence of the dominants. In this study, instead of isolating thousands of colonies from few samples, we only picked 10–15 strains per sample based on the appearance of the colonies on the agar-plates. Consequently, the isolates from each sample only represented the prevalent strains, but not the whole LAB community.

De-replication is an important procedure during isolation because it allows people to effectively identify new strains that avoid wasting time and resources on strains that have already been discovered. To avoid processing a large number of duplicated strains, a harsh de-replication strategy was employed. Since it was only saved one strain that belong to the same species isolated from one sample, it is likely some strains were lost due to this rigorous de-replication procedure. However, this guaranteed the uniqueness for each of the isolated strains. Whole genome sequencing data analysis (data not shown) confirmed that none of the two isolated strains are the same after de-replication.

Regarding the sample preparation, most of the published data use conventional kimchi preparation recipe that usually consists of mixing different raw materials like cabbage, radish, red pepper, garlic and ginger together with seasonings (Hong SW et al., 2016; Maoloni et al., 2020; Se-Hui Lee et al., 2015). Differently, in this study, a simple workflow was applied that most of the samples were prepared using a single raw material in brine condition. These results showed that all the 25 used vegetables, fruits, and even mushrooms can be fermented alone without adding any starter culture. The results showed that such spontaneously fermented samples are suitable for LAB isolation. Some studies showed there is a strong correlation between the type of LAB strains isolated and the raw materials used (Leech et al., 2020; Seon et al., 2020). Similar correlations were also detected in this study, however, to make a solid conclusion more samples from the same type of plants would need to be analyzed and community data should be considered as well.

4.3. Sugar composition in the substrate is crucial for plant-based fermentation by using different strains

Fermentation is heavily dependent on sugars, as they serve as energy sources for microorganisms. Unlike dairy products, plant-based materials do not have high levels of lactose. Studies indicate that the type of sugar used in fermentation can greatly impact the behavior of LAB and therefore affect the efficiency and end-products of fermentation (Darwin et al., 2018; Fuso et al., 2023; Neves et al., 2005). The ability to ferment a particular sugar depends on the presence, and activation of specific genes in the genome, but these genes are subject to complex regulation. Furthermore, not all sugar metabolism pathways in LAB are fully understood, such as raffinose metabolism pathway (Wang et al., 2022). In this study, we evaluated the capabilities of the isolates for fermenting simple sugars commonly present in plant-based materials. The concentration of sugars employed in this assay was standardized at 0.5%. The results showed that, in general, sucrose and maltose are among the most easily fermentable sugars by the isolated LAB strains. In contrast, only a few strains were found able to metabolize raffinose and xylose efficiently (Fig. 2).

Raffinose, usually found in legume-based products such as soybeans, is a non-digestible sugar and a known antinutrient that can cause digestion problems (Reddy et al., 1984). Although some LAB strains have been reported to ferment raffinose, the exact mechanism of transport and metabolism of raffinose in cells is still not fully understood (Fritsch et al., 2015; Tortuero et al., 1997; Wang et al., 2022).

Similarly, xylose and arabinose are also considered as poorly digestible plant-based sugars (Huntley & Patience, 2018). Xylose and its derivatives are the second most abundant carbohydrates on the planet, after glucose. However, the presented data showed that arabinose is more easily fermented by LAB than xylose, especially in strains of *E. faecium*, *L. fermentum*, *L. sakei*, and *W. hellenica*. In contrast, only *E. casseliflavus*, *L. brevis*, *L. rhamnosus*, and *L. lactis* showed higher performance in fermenting xylose than arabinose. This is because they require different transporters and enzymes to undergo the pentose phosphate pathway, which results in different fermentation performances by different strains (Passerini et al., 2013; Phane Chaillou et al., 1999; Tanaka et al., 2002).

Galactose is another common plant-based sugar. The metabolism of galactose in LAB is well-studied and can be broken down through either the Leloir or Tagatose-6P pathway, depending on whether it is transported by the permease or PTS system mode of transport (Iskandar et al., 2019). However, not all LAB strains possess a functional Tagatose-6P pathway (Iskandar et al., 2019; Wu et al., 2015). Fig. 2 shows that over half of the LAB strains and species can efficiently ferment galactose, indicating that it is a relatively easy fermentable sugar. The findings indicated that sugar composition in PBDA may greatly affect the behavior of LAB and hence determine the efficiency of plant-based fermentation by using different strains.

4.4. Variety of plant-based milk and buffering capacity contribute to different fermentation performance

As a variety of PBDA are rapidly emerging on the market, it is highly interesting to investigate the behaviors of LAB in fermenting different types of PBDA. Acidification is an important characteristic in the process of plant-based fermentations, which could prevent spoilage and prolong shelf life. Regardless the different strains used, initial pH and buffering capacity also act as key factors in the matrix acidification during the fermentation process, whereas in this study, the differences in initial pH and buffering capacity for different commercial PBDA were investigated.

Pea and soy milk were found to have significantly higher buffering capacity compared to other types of plant-based milks. This likely due to the high protein content in these milks, as proteins may acts as buffering agents (Karow et al., 2013). It is important to investigate the LAB growth in different plant-based materials for developing a desired culture. Fig. 3 reveals that different LAB strains can exhibit entirely different performance, which ultimately affects the taste and texture of the final products. Similar phenomenon has been observed in other studies as well (Labba et al., 2020; Sedó Molina et al., 2022).

Additionally, certain LAB strains may behave differently when fermenting different PBDA. For example, some strains may efficiently acidify the substrate, but not produce butter aroma compounds depending on the plant-based materials used for fermentation. Similarly, Demarinis et al. observed different acidification rate and sensory characteristics when they were using the same strains for bean, lupin and pea fermentation in their study(Demarinis et al., 2022). It was suggested that

those differences could be caused by the variety of buffering capacity and nutritional compositions like sugars, proteins, organic/inorganic compounds in different PBDA, as it is known such environmental factors play a key role on switching the metabolic directions in LAB (Braneni & Keenan, 1971; Cogan et al., 1981; Lopez de Felipe et al., 2006; Müller, 1990).

In this study, we demonstrated an applicable VP assay in non-transparent PBDA, in combination with Biolog color analysis, which provides reliable qualitative evaluation for detecting acetoin and diacetyl. This could be useful for fast screening for butter aroma producers under various conditions. However, the supplement of guanidine compounds to certain PBDA, like oat and rice milks, was also found to be necessary for having color development. This is because guanidine compounds are critical components for VP assay, and some plant-based materials like rice or oat, may contain a poor amount of guanidine compound. All these data provide insight into the strains' abilities to ferment different PBDA and their potential for producing stable products with desirable flavors.

In conclusion, our study provides valuable insights into modifying medium and sample preparation for plant-origin LAB isolation. Additionally, our research contributes to the advancement of methods for detecting butter aroma compounds in non-transparent PBDA and sheds light on complexities of plant-based LAB fermentation. These findings have significant implications for identifying good strains that can be potentially used for the development of desired PBDA in future works.

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CRediT authorship contribution statement

Hang Xiao: Data curation, Formal analysis, Methodology, Project administration, Validation, Visualization, Writing – original draft, Writing – review & editing, Investigation, Conceptualization. **Guillermo Eduardo Sedó Molina:** Data curation, Methodology, Validation, Writing – original draft. **Miguel Tovar:** Funding acquisition, Methodology, Writing – original draft, Supervision. **Ha Minh Quoc:** Data curation, Validation. **Egon Bech Hansen:** Methodology, Writing – original draft. **Claus Heiner Bang-Berthelsen:** Conceptualization, Funding acquisition, Project administration, Resources, Software, Supervision, Writing – original draft, Writing – review & editing.

Declaration of Competing interest

Claus Heiner Bang-Berthelsen (CHBB), Egon Bech Hansen (EBH), Minh Quoc Ha (MQH), Guillermo Eduardo Sedó Molina (GESM) and Hang Xiao (HX) have no conflict of interest. The funders had no role in the writing or reviewing of the manuscript or influence on data or interpretation of the results obtained in this study. Miguel Tovar (MT) is currently an employee of Chr. Hansen.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.lwt.2023.115695.

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