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Exploring the Diversity and Potential Use of Flower-Derived Lactic Acid Bacteria in Plant-Based Fermentation: Insights into Exo-Cellular Polysaccharide Production

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Abstract: Isolation of new plant-derived lactic acid bacteria (LAB) is highly prioritized in developing novel starter cultures for plant-based fermentation. This study explores the diversity of LAB in Danish flowers and their potential use for plant-based food fermentation. 46 flower samples under 34 genera were collected for LAB isolation. By introducing an enrichment step, in total of 61 LAB strains were isolated and identified by using MALDI-TOF and 16S rRNA sequencing. These strains represent 24 species across 9 genera, predominantly *Leuconostoc mesenteroides*, *Fructobacillus fructosus*, *Apilactobacillus ozensis*, and *Apilactobacillus kunkeei*. Phenotypic screening for exo-cellular polysaccharide production revealed that 40 strains exhibited sliminess or ropiness on sucrose containing agar plate. HPLC analysis confirmed that all isolates produced exo-cellular polysaccharides containing glucose, fructose or galactose as sugar monomer. Therefore, the strains were glucan, fructan and galactan producers. The suitability of these strains for plant-based fermentations was characterized by using almond, oat and soy milk. The results showed successful acidification in all three types of plant-based matrices but only observed texture development in soy by *Leuconostoc*, *Weissella*, *Lactococcus*, *Apilactobacillus*, and *Fructobacillus*. The findings highlight the potential of flower-derived LAB strains for texture development in soy-based dairy alternatives.

Keywords: exo-cellular polysaccharide; plant-based fermentation; isolation; flower; lactic acid bacteria; texture

1. Introduction

Lactic acid bacteria (LAB) are among the most important and beneficial groups of microorganisms found in various environments, including plants, terrestrial and marine animals, fermented foods, and the mucosal surfaces of humans. Traditionally, they have been associated with fermented foods for their health benefits and close ties to human culture. Therefore, LAB is the primary group of microbes responsible for many fermentation processes [1–3]. In recent years, there has been a growing interest in studying plants as a source for LAB screening. However, LAB derived from flowers have been less isolated and studied, yet they hold great potential for creating new starter cultures for plant-based fermentation [4]. LAB is utilized in fermentation to enhance the stability of raw materials, sensory qualities, and nutritional value by producing lactic acid and other metabolites. Due to limitations in developing fermented plant-based products using traditional starter cultures, isolating and culturing specific LAB strains from natural sources could be solution. Therefore, it is

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intriguing to isolate and characterize strains from flowers to develop innovative plant-based starter cultures [5–7].

It is well-known that LAB can synthesize a variety of polysaccharides, attracting attention due to their safe use and potential probiotic properties [8]. Polysaccharides are high molecular weight polymeric carbohydrate structures formed of monosaccharide units linked together by glycosidic bonds. They exhibit a variety of structures, functional properties, and biological activities. Microbial polysaccharides can be soluble or insoluble, found in the form of capsules tightly attached to the cell wall, mucus loosely attached to the cell, or completely released into the environment [9,10].

Polysaccharides produced by bacteria are classified based on their biological functions. These include intracellular cytoplasmic storage polysaccharides like glycogen, and cell surface associated polysaccharides. In lactic acid bacteria (LAB), the exocellular polysaccharides can be classified into two categories: exopolysaccharides (EPS) and capsular polysaccharides (CPS), both are important for texture development in the food industry [11]. CPS are closely linked to the cell surface, form the outermost layer of the bacterial cell and provide a mechanism to protect the cell, mediating direct interactions with the environment, whereas EPS are polysaccharides that are loosely associated with the cell surface or released into the extracellular medium, They can either be produced extracellularly by enzymes secreted by the bacterium, or synthesized intracellularly and secreted outside the cells and often forming a slime layer [1,9,10].

Polysaccharides are one of the main components involved in the formation of the extracellular biofilm matrix. Exo-cellular polysaccharides are among the main techno-functional metabolites of LAB species. They play an important role in protecting bacteria from adverse environmental factors, such as protection against abiotic or biotic stress, competition, pH, and temperature. Exo-cellular polysaccharides have physicochemical properties that have potential for the food and pharmaceutical industries. like xanthan, alginate, and cellulose, that are important for biofilm formation and pathogenicity [10,12].

Exo-cellular polysaccharides can be classified as homo-polysaccharides when composed of a single type of monosaccharide, or hetero-polysaccharides if composed of two or more different sugars in their repeating unit. Moreover, the sugars may be modified by non-carbohydrate moieties such as acetate, pyruvate, sulfate, and succinate. Bacterial exo-cellular polysaccharides vary according to their source. LAB can produce exo-cellular polysaccharides in both hetero-polysaccharides and homo-polysaccharides structure. Exo-cellular polysaccharides in hetero-polysaccharides structure are produced at a much lower rate because they have a more complex biosynthesis mechanism and require more enzymes for production. The homo-polysaccharides produced by LAB classified as glucans, fructans, and galactans, which consist of D-glucose, D-fructose, or D-galactose, respectively. For the production of exo-cellular polysaccharides in homo-polysaccharides structure, a simpler metabolic pathway is followed and the presence of sucrose as a carbon source and glucan sucrase or fructan sucrase as an enzyme is sufficient. In some studies, galactan is also detected as a major residue of EPS produced by LAB, however, the genes and enzymes behind this are not very clear yet [13]. In contrast, hetero-polysaccharides are composed of several repeating units of sugars, such as pentose (D-ribose, D-arabinose, D-xylose), hexose (D-glucose, D-galactose, D-mannose), N-acetylated monosaccharides (N-acetylglucosamine and N-acetyl-galactosamine), or uronic acids (D-glucuronic acid, D-galacturonic acid) [8–10].

Recently research is focused on the application of exo-cellular polysaccharides in the food industry, due to their structural properties, such as texturization, emulsification, gelling, sweetening, water-binding capacity, and bioactive properties. Also studies have demonstrated the health-promoting potential of exo-cellular polysaccharides, including immunomodulatory, prebiotic, anti-inflammatory, anti-biofilm, and antioxidant activities [2,6]. In recent years, interest in vegetarian and vegan diets has increased for many reasons. The challenge for manufacturers of plant-based milk alternatives is to produce products with acceptable taste and texture for customers. The application of exo-cellular polysaccharide producing strains to improve the sensory and organoleptic analysis of these products, can positively influence texture, mouthfeel, and decrease syneresis in plant-based milk alternatives [14]. In the production of functional and fermented products, LAB that produce EPS

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can lead to a reduction in the use of food additives by enhancing texture, as a natural thickener [8,15,16].

The aim of our study was to identify novel LAB strains, from various Danish flowers that produce exo-cellular polysaccharides. We focused on strains that produce more exo-cellular polysaccharides on MLS agar medium, as these have the best performance in fermentation. To achieve this, we applied a high-throughput texture screening method based on plant drink fermentations in microtiter plates (MTP) combined with Total Aspirate Dispense Monitoring (TADM) pressure measurements. Additionally, we investigated the structural characteristics of the exo-cellular polysaccharides produced by all strains.

2. Materials and Methods

2.1. Preparation of Flowers

As the source of LAB species, 47 flower samples were collected from different locations in Denmark on summer season and have remained in 3.5% NaCl solution (saline solution) in 15 ml. After 5 days incubation at 22 °C, glycerol was added to each flower sample and store at -80 °C until use.

2.2. Screening and Isolation of LAB Strain from Flowers

The frozen flower samples were inoculated into MLS-agar supplemented with 1% glucose and 0.5% fructose for LAB screening. 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 pH was adjusted to 6.2, 0.4 g/L Cycloheximide was also added to agar-plates for inhibiting yeast and fungi growth [5]. For isolation, the plates were made by directly streaked of the flower samples supernatant after brief vortex. After incubating the plates at 30 °C for 48 h, plates were transferred to fridge for 24 h for showing more distinctive morphology. Then 10 to 15 single colonies were picked mainly based on their colony morphology, followed by re-streak, and sub cultured on the MLS-agar at least one time for colony purification [5,17].

2.3. Identification of Isolated LAB Strains from Flowers Using MALDI-TOF, PCR, and De-Replication

The species identification of the isolated LABs was performed by protein extraction from 24 h grown cultures on MLS-agar plates from purified single colony using MALDI-TOF Biotype (Bruker Daltonics, Bremen, Germany), which can identify species based on the protein mass to charge (m/z) spectra. The sample preparation process for MALDI-TOF identification is described as follows: Fresh pure cultures from an agar plate are picked using inoculation loops and spread out on spots on a target MALDI-TOF plate. The plate is then treated with 1 µl 75% ethanol, mixed well and left to dry. Then 1µl of 70% formic acid is added to each spot and the plate is left to dry again. Finally, 1µl of a saturated matrix is then added to each spot. Matrix is prepared by mixing α-cyano-4-hydroxycinnamic acid with 475µl of water, 500µl of 100% Acetonitrile, and 25µl of Trifluoroacetic acid. The plate is left to dry completely before being scanned using a MALDI-TOF Biotype for protein mass spectra detection and identification at the species level by matching with its integrated spectral database library. The identification process will provide a species ID along with a score indicating the similarity between the protein mass spectrum and the database. The instrument gives a score of 1-3–score values from 1 to 1.69 are considered not reliable, and thus the genus/species cannot be determined; scores between 1.70 and 1.99 refer to identifications that are only reliable on a genus level; and scores above 2 correspond to identifications that are reliable on a species and genus level [5]. The not reliable strains (14 strains) with low score were identified by 16S rRNA sequencing. The fragment (300 bp) was amplified by using a universal primer: following forward (5′ TGGCTCAGGACGAACGCTGGCGGC 3′) and reverse (5′ CCTACTGCTGCCTCCCGTAGGAGT 3′). The PCR procedure is as follows: using PCR master mix (2X) 25 µl, forward primer 1 µM, reverse primer 1 µM, template DNA 10 pg-1µg, nuclease-free water to 50 µl. Gently vortex the samples. and

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perform PCR using thermal cycling conditions. The PCR program was carried out in a thermal cycler as 5 min of initial denaturation at 95°C, followed by 35 amplification cycles of denaturation at 95°C for 30 s, annealing at 58°C for 30s and extension at 72°C for 40 s. The final elongation was set as 72°C for 7 min. The PCR products were analyzed via 1% agarose gel electrophoresis, stained with DNA Gold Viewer Dye, and visualized under UV light using mini gel documentation device (VMR,). Then the samples (11 strains) were sent for sequencing. To remove 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 will be saved and characterized for further studies [5]. After dereplication, strains were saved to DTU National Food Institute Culture Collection (NFICC) with a designated NFICC number.

2.4. Screening for Exo-Cellular Polysaccharides Producers

A modified MLS-agar medium supplemented with 1% glucose, 0.5% fructose as control, and 2% or 6% of sucrose was prepared to observe the exo-cellular polysaccharide production abilities of 61 selected strains and compare the exo-cellular polysaccharide production in 2% and 6% sucrose by control. Overnight cultures were inoculated to modified MLS agar plates and the slime formation was monitored following the incubation period of 24, 48, and 72 h at 30 °C, described previously by [3], with slight modifications. Morphologically slimy colonies were further selected for exo-cellular polysaccharides production on modified MLS-agar medium supplemented with 2% sucrose to observe the exocellular polysaccharides production abilities of 40 selected strains and compare the Exo-cellular polysaccharide production. Exo-cellular polysaccharide production was assessed through visual inspection of colonies.

2.5. Isolation and Purification of Exo-Cellular Polysaccharides

The 40 selected LAB strains were grown in modified MLS-broth medium supplemented with 2% sucrose, at 30 °C for 48 h. Isolation of exo-cellular polysaccharide was conducted by following the method as depicted previously [8]. Briefly, following the incubation period, 2 vol of chilled ethanol was added to the culture supernatants obtained following the centrifugation of the bacterial cultures, and supernatants were left at 4 °C overnight to precipitate the exocellular polysaccharides. The exocellular polysaccharides pellet was then recovered by centrifugation at 10,000×*g* for 20 min at 4 °C and subjected to resuspension process with distilled water. This process was repeated twice with the use of less distilled water at each time for the resuspension process.

2.6. Determination of Monosaccharides by High Performance Liquid Chromatography (HPLC) Analysis

The monosaccharide composition is determined by HPLC after treatment for the purified polysaccharides as previously described [8]. HPLC (ThermoFisher, Boston, US) analysis is set up in a system equipped with an Aminex HPX-87H (Bio-Rad, Hercules, CA) and a Shodex RI-101 refractive index detector (Showa Denko K.K., Tokyo, Japan). The mobile phase was 5 mM sulfuric acid with flow rate 0.5 mL/min. The column oven temperature was maintained at 60°C. Glucose, galactose, and fructose were used as standard sugars for determining the composition of purified polysaccharides. Chromatograms for samples and standards were analyzed using Chromeleon 2.0 software (ThermoFisher, Boston, US).

2.7. High-Throughput Screening for Texturing Strains in Plant-Base Drink

The ability of strains to acidify milk was investigated using color of pH method and their texturing abilities were investigated using TADM as described in [18]. Here, three types of commercial plant-based milk were used, and their nutritional content is listed in Table 1. TADM results (pressure versus time curves) were converted into single descriptors (TADM area) by accumulating all the measured pressure points below zero. The pressure was measured every 0.01 s for 3 s. Strains resulting in fermented samples with high texture were represented by large TADM

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areas, whereas non texturing strains, by small TADM areas. Strains were considered texturing when giving rise to elevated texture in fermented milk samples (TADM area ≥ 800,000 Pa × ms).

Table 1. Nutrient content of used plant-based drink.

	Oat, Isola Bio	Almond, Ecomil	Soy, Naturli
Energy	215 kJ / 51 kcal	134.00 kJ / 32.00 kcal	153kJ/37 kcal
Fat	1.0 g	2.10 g	2.1 g
Carbohydrate	9.0 g	3.30 g	0.6 g
-Here sugar	4.2 g	<0.30 g	0.6 g
Protein	1.0 g	1.00 g	3.7 g
Salt	0.08 g	0.14 g	0.09 g

3. Results and Discussion

3.1. Diversity of plant-based LAB strains according to MALDI-TOF, PCR, and de-replication

To isolate flower-derived LAB, we collected 46 flower samples representing 34 genera from various locations in Denmark during the summer season. By employing MALDI-TOF and 16SrRNA identification, a total of 61 LAB strains belonging to 24 species were isolated after a strict de-replication process (Table 2, Figure 1). The strains belong to 9 genera under *Apilactobacillus*, *Fructobacillus*, *Levilactobacillus*, *Lactiplantibacillus*, *Latilactobacillus*, *Lactococcus*, *Leuconostoc*, *Pediococcus*, and *Weissella*. At the species level, the most prevalent LAB species found among all flowers were *Leuconostoc mesenteroides*, with 12 isolated strains. Followed by other frequently occurring species such as *Fructobacillus fructosus* with 8 isolates, *Apilactobacillus ozensis* with 5 isolates and *Apilactobacillus Kunkeei* with 4 isolates. Our results indicate a wide spread of LAB species on flowers.

In other studies focusing on LAB isolation from flowers, similar occurrences at the species level have been described, which aligns well with the findings of our study [19,20]. In contrast, LAB isolated from fermented vegetables or dairy sources is typically abundant in other species such as *Lactiplantibacillus plantarum*, *Lacticaseibacillus casei*, *Lacticaseibacillus paracase*, *Latilactobacillus curvatus*, *Latilactobacillus sakei*, and *Lactobacillus delbrueckii* [5,21,22]. This indicates flowers harbor a niche for distinct LAB species, with high abundance of fructophilic LAB. Interestingly, such species are also frequently found inside of honeybee gut [23], suggesting a potential microbiota exchange between flowers and pollinators. Unlike LAB derived from dairy and fermented vegetables, flower-derived LAB is less studied. Considering their distinctiveness, it could be a promising resource for investigating novel LAB in food applications.

Although LAB is ubiquitous in nature, isolating and identifying plant-derived LAB strains can be challenging due to their low abundance on plant surfaces and meticulous cultivation conditions [24]. We attempted to directly use flower-washed water for plating but only yielded poor results. To get better isolation from flower samples, an enrichment procedure for LAB in each sample needs to be employed before isolation. In this study, inspired by the preparation of fermented vegetables, we introduced a simple method that preferment the flower samples in 3.5% NaCl solution at room temperature for 5 days. The pre-fermentation resulted in a low pH around 4 for most of the samples, indicating an enrichment of anaerobes. Additionally, the successful isolation of LAB from different flower samples confirmed the robustness and efficiency of this method.

Regarding the isolation and dereplication process, approximately 10 strains per sample were selected based on the appearance of the colonies on agar plates. Therefore, the isolates from each sample only represented the prevalent strains, not the entire LAB community. Dereplication is crucial during isolation as it enables high-quality outputs during strain isolation, furthermore, it allows people to identify new strains without wasting time and resources on strains that have already been discovered.

Overall, the study highlights the potential of flowers as a promising resource for LAB isolation. In comparison to other sources, flowers have received less attention, however, our findings

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emphasize the distinctness of LAB strains found in flower samples. To develop new starter cultures for plant-based fermentation, it's important to study plant-derived LAB, as they are likely equipped with the capability to metabolize plant-derived sugars and protein, and detoxify phenolic compounds present in plant-based materials [25].

Table 2. Overview of the diversity of plant-based LAB strains isolated from flowers according to de-replication process.

No.	Species	NFICC ID	City	Flower
1	Apilactobacillus kunkeei	2373	Odense	Verbascum
2	Apilactobacillus kunkeei	2324	Copenhagen	Rosa
3	Apilactobacillus kunkeei	2359	Aarhus	Fallopia
4	Apilactobacillus kunkeei	2362	Silkeborg	Rubus
5	Apilactobacillus ozensis	2370	Odense	Potentilla
6	Apilactobacillus ozensis	2374	Odense	Jacobaea
7	Apilactobacillus ozensis	2363	Silkeborg	Rubus
8	Apilactobacillus ozensis	2366	Silkeborg	Lavandula
9	Apilactobacillus ozensis	2368	Odense	Jacobaea
10	Apilactobacillus sp.	2412	Silkeborg	Lavandula
11	Fructobacillus fructosus	2323	Copenhagen	Rosa
12	Fructobacillus fructosus	2321	Copenhagen	Geranium
13	Fructobacillus fructosus	2361	Silkeborg	Rubus
14	Fructobacillus fructosus	2347	Copenhagen	Rosa
15	Fructobacillus fructosus	2325	Copenhagen	Rubus
16	Fructobacillus fructosus	2319	Copenhagen	Syringa
17	Fructobacillus fructosus	2376	Odense	Convolvulus
18	Fructobacillus fructosus	2393	Silkeborg	Lavandula
19	Fructobacillus pseudoficulneus	2222	Aarhus	Betonica
20	Fructobacillus tropaeoli	2365	Silkeborg	Lavandula
21	Levilactobacillus brevis	2055	Aarhus	Eschscholzia
22	Lactiplantibacillus paraplantarum	2184	Odense	Cirsium
23	Lactiplantibacillus pentosus	2185	Odense	Cirsium
24	Lactiplantibacillus plantarum	2183	Odense	Cirsium
25	Latilactobacillus sakei	2317	Copenhagen	Stellaria
26	Lactococcus garvieae	2331	Copenhagen	Hyacinthus
27	Lactococcus lactis	2358	Aarhus	Erythranthe
28	Lactococcus lactis	2332	Copenhagen	Hyacinthus
29	Lactococcus lactis	2336	Copenhagen	Trifolium
30	Leuconostoc mesenteroides	2012	Copenhagen	Forsythia
31	Leuconostoc mesenteroides	2011	Copenhagen	Prunus
32	Leuconostoc mesenteroides	2008	Copenhagen	Aubrieta
33	Leuconostoc mesenteroides	2181	Aarhus	Telekia
34	Leuconostoc mesenteroides	2333	Copenhagen	Prunus
35	Leuconostoc mesenteroides	2343	Copenhagen	Trifolium
36	Leuconostoc mesenteroides	2329	Copenhagen	Ranunculus
37	Leuconostoc mesenteroides	2327	Copenhagen	Hottonia
38	Leuconostoc mesenteroides	2334	Copenhagen	Trifolium
39	Leuconostoc mesenteroides	2350	Copenhagen	Bellis
40	Leuconostoc mesenteroides	2182	Aarhus	Telekia
41	Leuconostoc mesenteroides	2377	Aarhus	Campanula

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42	Leuconostoc miyukkimchii	2224	Copenhagen	Rosa
43	Leuconostoc sp.	2228	Aarhus	Dasiphora
44	Leuconostoc sp.	2378	Aarhus	Black mullein
45	Leuconostoc sp. THK-X10	2246	Copenhagen	Rosa
46	Pediococcus acidilactici	2053	Aarhus	Betonica
47	Pediococcus acidilactici	2057	Aarhus	Fuchsia
48	Pediococcus acidilactici	2357	Aarhus	Erythranthe
49	Pediococcus pentosaceus	2051	Aarhus	Hydrangea
50	Pediococcus pentosaceus	2048	Aarhus	Dasiphora
51	Pediococcus pentosaceus	2379	Aarhus	Teucrium
52	Pediococcus pentosaceus	2369	Odense	Artemisia
53	Weissella bombi	2356	Aarhus	Erythranthe
54	Weissella bombi	2346	Aarhus	Teucrium
55	Weissella bombi	2371	Odense	Agastache
56	Weissella minor	2054	Aarhus	Betonica
57	Weissella paramesenteroides	2341	Copenhagen	Trifolium
58	Weissella thailandensis	2056	Odense	Convolvulus
59	Weissella thailandensis	2364	Silkeborg	Lavandula
60	Weissella viridescens	2320	Copenhagen	Cardamine
61	Weissella viridescens	2318	Copenhagen	Cotoneaster

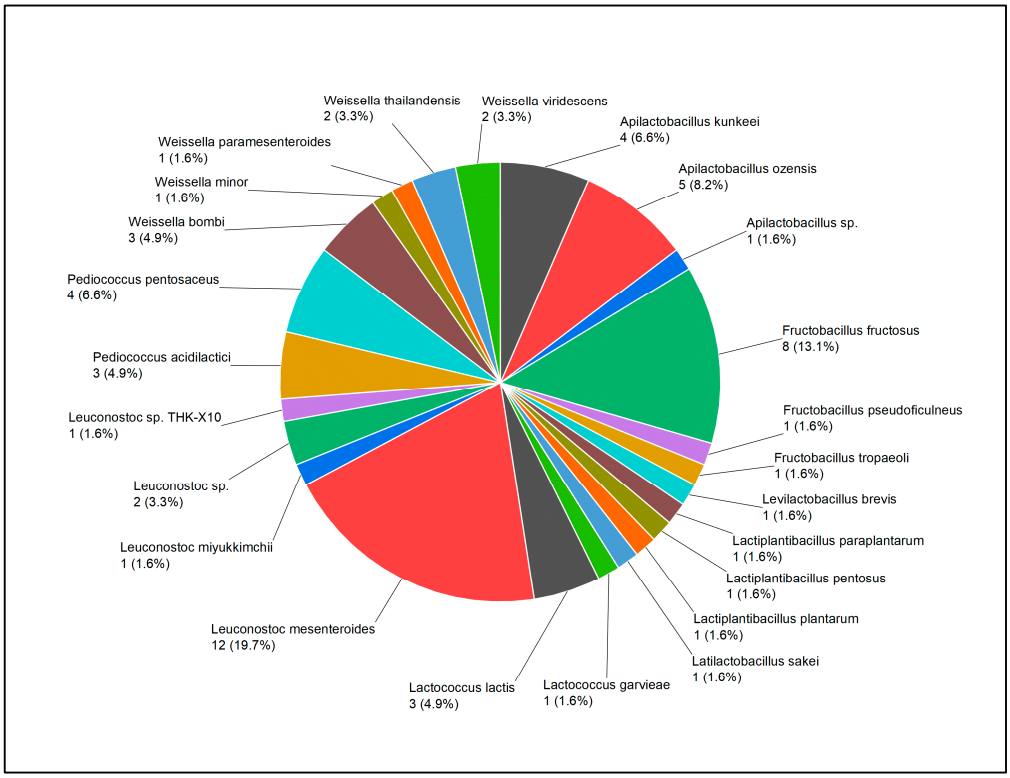

Figure 1. The abundance percentage, and diversity of plant-based LAB within the overall LAB community strains isolated from flowers.

3.2. Screening for Polysaccharides Producing LAB Strains on Different Sucrose-Supplemented Media

Exo-cellular polysaccharides, including exopolysaccharides (EPS) and capsular polysaccharides (CPS) are particularly important in the food industry. An efficient way to assess exo-cellular polysaccharides production is to visually observe the phenotypic characteristics of the colonies such

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as sliminess or ropiness [8]. The slimy phenotype is recognized by mucilaginous colonies, while the ropy phenotype is identified by the formation of long filaments when an inoculation loop is lifted from the colony surface or cell pellet [26]. As sucrose is usually used for stimulating homo-EPS production in LAB, the screening of homo-EPS production for 61 LAB strains was characterized on the MLS plates containing 2% or 6% sucrose. In total 40 of the tested strains showed varying degree of sliminess and ropiness, indicating the production of homo-EPS at various levels. Additionally, more slime was observed by using 2% sucrose compared to that with 6% sucrose (Figure 2). No sliminess or ropiness observed in the control plates with no sucrose added for all tested strains, only the growth of colonies. Hence, we focus on these 40 slime-producing strains for further studies.

Sugar metabolism significantly influences exo-cellular polysaccharides production in LAB. Studies have demonstrated that exo-cellular polysaccharides can be varied in both amount and composition when LAB grows on different sugars [27,28]. Unlike dairy products, which mainly contain lactose as fermentable sugar, the sugar composition in plant-based materials is more complex. Hence, we attempted to investigate exo-cellular polysaccharides production in LAB under mixed sugar conditions. To simplify the model, exo-cellular polysaccharides production was evaluated in 2% sucrose plate supplemented with 1% glucose (exemplified in Figure 3). As summarized in Table 3, The results revealed a highly species-dependent pattern in sliminess production. For example, most *Apilactobacillus ozensis*, *Pediococcus pentosaceus*, *Weissella viridescens*, *Fructobacillus fructosus* hampered in slime formation in the presence of glucose. Interestingly, some species behaved completely in an opposite way, like *Apilactobacillus kunkeei*, *Leuconostoc miyukkimchii*, *Lactococcus lactis*, *Lactococcus garvieae*, *Leuconostoc mesenter*oides, *Weissella bombi*, and *Weissella minor*, an enhanced homo-EPS production was detected in the presence of glucose.

The quantity of exo-cellular polysaccharides synthesized by LAB largely depends on several parameters, including pH, temperature, oxygen tension, incubation period, metabolic activity, and microbial growth conditions. Nevertheless, the most important factor is the composition of the culture medium and its sugar compounds [26,29]. Exo-cellular polysaccharides production usually can be induced by adding sucrose, which plays a key role in the EPS biosynthesis pathway [30]. However, in this study, an inhibited slime formation production is observed in some strains when glucose is present. This inhibition may be attributed to carbon catabolite repression, which may inhibit sucrose uptake when glucose is present [31]. Conversely, some strains showed enhanced homo-EPS production in the presence of glucose, which may benefit from accelerated sugar metabolism. This suggests a diverse regulation mechanism in EPS production among different LAB, which has been reviewed in other studies [11]. Although the mechanism behind the inconsistent slime formation with and without glucose remains unclear, our findings provide valuable insights when selecting LAB strains for texturizing plant-based materials with different sugar compositions.

Table 3. Evaluation of homo-EPS production of the LAB strains on modified MLS-agar medium supplemented with 2% sucrose and also MLS-agar medium supplemented with 2% sucrose and 1% glucose.

NFICC codes	Species	2% Sucrose		2% Sucrose and 1% Glucose	
		Slimy	Ropy	Slimy	Ropy
2324	Apilactobacillus kunkeei	-	+	+	+
2362	Apilactobacillus kunkeei	+	+	++	-
2370	Apilactobacillus ozensis	++	+	-	+
2374	Apilactobacillus ozensis	+++	-	++	-
2363	Apilactobacillus ozensis	-	+	-	+
2366	Apilactobacillus ozensis	-	+++	-	++
2347	Fructobacillus fructosus	+	-	-	+
2185	Lactobacillus pentosus	+-	-	-	-
2183	Lactiplantibacillus plantarum	+-	-	-	-
2331	Lactococcus garvieae	+-	+	+-	+

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2336	Lactococcus lactis	+	++	++	+
2012	Leuconostoc mesenteroides	-	+	++	+-
2011	Leuconostoc mesenteroides	+-	+-	+-	+
2008	Leuconostoc mesenteroides	-	+	+	+
2181	Leuconostoc mesenteroides	+-	-	++	-
2333	Leuconostoc mesenteroides	+	+	++	+
2329	Leuconostoc mesenteroides	+	-	+	-
2327	Leuconostoc mesenteroides	+	-	+	-
2334	Leuconostoc mesenteroides	+-	-	+	-
2350	Leuconostoc mesenteroides	-	+-	-	-
2182	Leuconostoc mesenteroides	+	-	++	-
2224	Leuconostoc miyukkimchii	-	+	+	++
2228	Leuconostoc sp.	+	+	+	+
2246	Leuconostoc sp. THK-X10	+	-	+	+
2057	Pediococcus acidilactici	-	+++	-	+++
2051	Pediococcus pentosaceus	+	-	-	-
2048	Pediococcus pentosaceus	+	+	-	-
2369	Pediococcus pentosaceus	-	+++	-	+++
2379	Pediococcus pentosaceus	-	+	-	+
2371	Weissella bombi	++	-	++	-
2054	Weissella minor	+	+	++	+
2341	Weissella paramesenteroides	+	+	+	+
2056	Weissella thailandensis	+	-	++	-
2318	Weissella viridescens	+	-	-	+-
2393	Fructobacillus fructosus	+	-	-	-
2378	Leuconostoc sp.	+	-	++	+
2377	Leuconostoc mesenteroides	+	-	+	+
2412	Apilactobacillus sp.	+	-	-	-
2365	Fructobacillus tropaeoli	+	-	-	-
2364	Weissella thailandensis	+	-	-	++

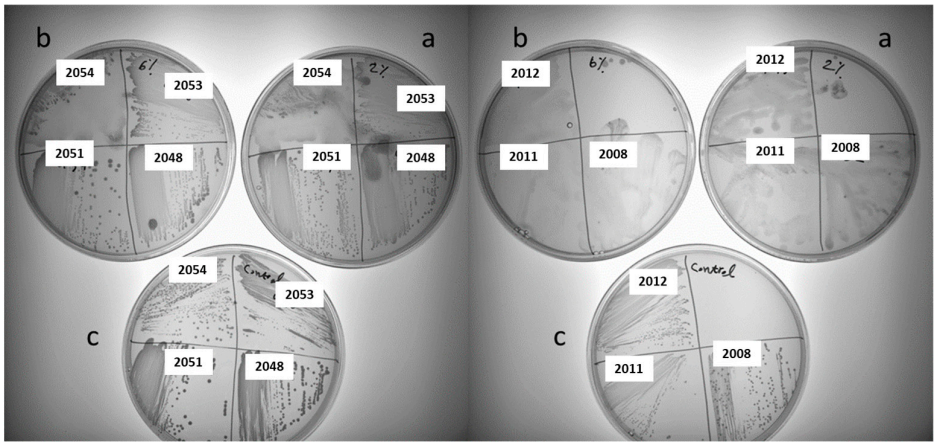

Figure 2. Examples for comparing homo-EPS production of 7 random LAB strains on modified MLS-agar medium supplemented with (a): 2%, (b): 6% sucrose, and (c): control MLS medium.

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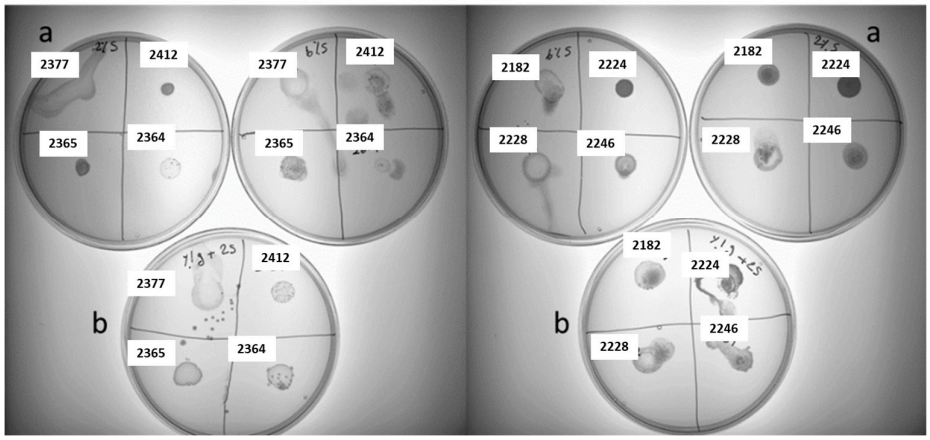

Figure 3. Examples for comparing homo-EPS production of 8 random LAB strains on (a): modified MLS-agar medium supplemented with 2% sucrose and (b): MLS-agar medium supplemented with 2% sucrose and 1% glucose.

3.3. Determination of Monosaccharide Composition by HPLC Analysis

To investigate the monosaccharide composition of exo-cellular polysaccharides, all 40 strains were cultivated in MSL medium supplemented with 2% sucrose. The extraction method used in this study specifically targets EPS, so we focused on analyzing EPS composition. After extraction, 25 strains yielded a satisfactory amount of EPS for compositional analysis. As shown in Figure 4, the sugar composition of EPS produced by each strain is highly dependent on the species level. Specifically, most *Leuconostoc mesenteroides*, *Lactococcus lactis*, and *Weissella minor* produced EPS dominated by glucose, with a small amount of fructose also detected. In contrast, EPS produced from *Fructoacillus tropaeoli*, *Weissella viridescens* and *Weissella thailandensis* contain both large amounts of glucose and fructose. Furthermore, the EPS produced from *Apilactobacillus kunkeei*, *Apilactobacillus ozensis*, *Apilactobacillus sp.*, *Fructobacillus fructosus,* and *Weissella paramesenteroides* consisted of a large amount of glucose and galactose. Overall, all strains had glucose monomer in their composition, some of them had fructose or galactose beside the glucose. *Leuconostoc mesenteroides* (NFICC 2011) and *Weissella bombi* (NFICC 2371) had glucose, fructose and galactose in their composition.

LAB strains are well-known for EPS production [8]. Results of HPLC analysis show that all isolates produced EPS containing glucose, fructose or galactose as sugar monomer in the EPS structure. However, it is unclear if they are producing HePS or mixture of HoPS. Glucans are the main backbone structure with different degrees of branching and binding sites that vary from bacterial strain to bacterial strain. Glucans can be classified as either α-glucans (divided into four groups: dextran, mutan, reuteran, and alternan) or β-glucans, and are produced by a variety of LAB species in the genera *Leuconostoc, Lactobacillus, Streptococcus*, and *Weissella* [10,32]. Fructans (divided into 2 groups: levan and inulin) are produced by strains of *Streptococcus salivarius*, *Leuconostoc mesenteroides*, *Lactobacillus reuteri*, *Lactobacillus johnsonii* and *Fructilactobacillus sanfranciscensis* [33]. Galactans are less abundant, and are produced by a few LAB strains belonging to *Weissella confusa*, *Lactococcus lactis* subsp. *lactis* and *Lactobacillus delbrueckii* subsp. *Bulgaricus* [8,10,16,33–35]. In general, LAB that produce HoPS release high amounts of polymers into the environment. However, HePS from LAB are produced in considerably smaller amounts [3,10]. We focused on HoPS-producing LAB as we monitored the quantity of EPS and visual characteristics, including slimy and ropy phenotypes. When comparing the 9 genera identified in this study in terms of EPS production efficiency, it was found that *Leuconostoc* had the highest amount of homo-EPS production, followed by *Apilactobacillus, Fructobacillus, Weissella, Lactococcus, Lactiplantibacillus,* and *Pediococcus*, respectively. According to some studies, a high amount of sugar may contribute to an increase in the production of HoPS. Possible explanations for the increased HoEPS synthesis under the stress of high sugar concentration

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in some strains include osmosis, the unlimited supply of sugar building blocks, and high energy availability [3,8].

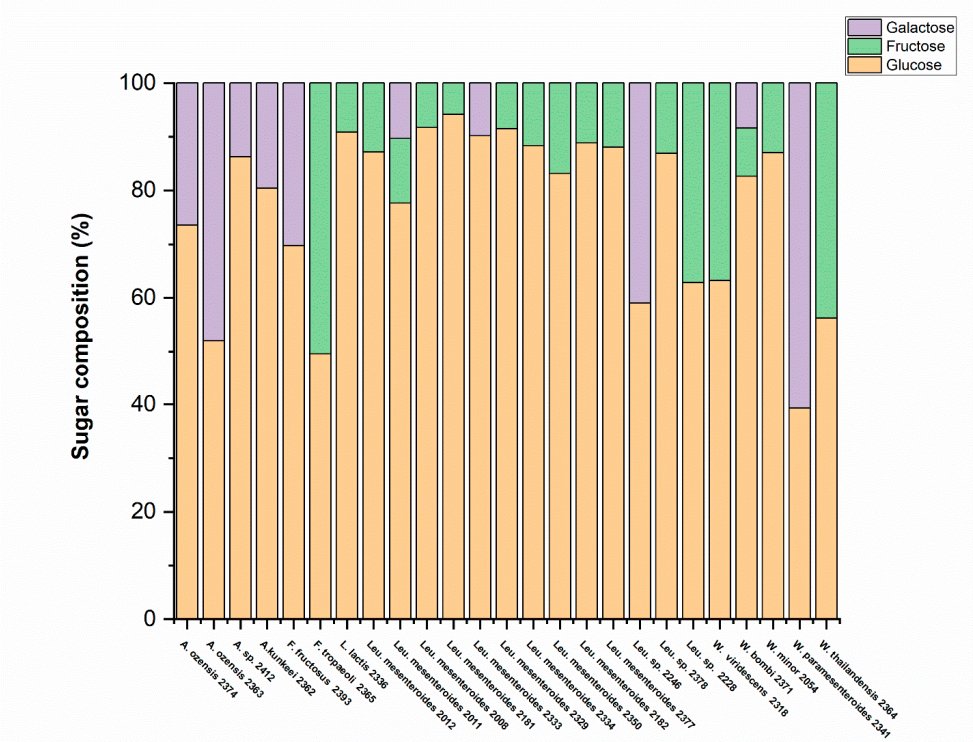

Figure 4. fingerprint of EPS monosaccharide composition of LAB strains by HPLC. Glucose is represented by orange bar color, fructose by a green bar color and last galactose is shown as a purple bar color.

3.4. High-Throughput Screening for Texturing Strains

To characterize whether the strains could texturize plant-based drink, oat, almond, and soy drink were used without additional sugars (Table 1). Here, 25 strains were selected based on their exo-cellular polysaccharide production on the MLS plate. The fermentation was carried out using 96 deep-well plates at 30°C for 1 day. The starting pH was about 7. At the end of fermentation, the endpoint pH and texture in each sample were investigated. The endpoint pH less than 5.5 is regarded as acidification and TADM areas of above 800,000 Pa × ms is regarded as texturing. The results showed that all strains could acidify oat, but none achieved texturing. In contrast, when using soy and almond drink, several strains exhibited a similar acidification tendency in both substrates depending on the strain used (Figure 5). Interestingly, only soy drink observed texturing. This is likely because of the relatively high protein content of soy, opposite to oat and almond. Specifically, 15 strains species texturing, including 7 *Leuconostoc mesenteroides*, 1 *Leuconostoc sp*., 1 *Weissella cibaria*, 1 *Weissella minor*, 1 *Weissella paramesenteroides*, 1 *Apilactobacillus kunkeei*, 1 *Apilactobacillus sp*., 1 *Fructobacillus tropaeoli*, and 1 *Lactococcus lactis*. In addition, texturing was found to be highly dependent on acidification. In acidified samples, TADM areas varied from 800,000 to 1,400,000 Pa × ms, indicating significant differences in texturing ability among strains (Figure 6).

Different types of cow milk have a similar profile of proteins, fats, and sugars. In contrast, plant-based milk differs dramatically in nutrient compositions and physicochemical properties. These differences may significantly impact the microbial fermentation process in different plant-based matrices [5]. Particularly, protein is critical for creating a yogurt-like texture development in fermented drinks. In a food system, proteins are the most significant functional component because of their structuring, texturizing, emulsifying, foaming, hydration, and nutritive properties [36]. In this study, texture development was observed only in fermented soy milk. We speculate this is due

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to the high protein content in soy milk. Nevertheless, in oat and almond milk, elevated TADM aspiration pressures were also detected from all strains after fermentation, compared to the blank samples. This could be a clue for the produced extra-cellular polysaccharides but just far from enough for a yogurt-like texture formation, as the protein content in both samples is around 4 times lower than soymilk.

EPS production in LAB is well studied, however, most of the industrial strains are tailored for making yogurt and cheese. Our study showed that several traditional EPS producers such as *Leuconostoc, Weissella, and Lactococcus* enabled texturizing soy milk as well. It is worth noting that *Leuconostoc* normally grows poorly in milk alone and is often cultured with other LAB like *Lactococcus lactis* in dairy fermentation [37]. In this study, 8 *Leuconostoc* strains showed better texture development in soy milk, demonstrating their promising role shift from dairy to the plant-based section. In addition, *Apilactobacillus sp*. and *Fructobacillus tropaeoli* also showed better texture development in soy milk. These LAB species are rarely studied for EPS production and plant-based fermentation. Our results highlight the potential of broadening the LAB diversity used for plant-based fermentation. Furthermore, the plant-based drinks used in this study are free of pre-added sugars from manufacturers. The ability of diverse strains to acidify and texturize plant-based matrices based on their natural nutritional composition highlights their robustness and flexibility in developing plant-based fermented products. To fully understand and harness the fermentation process, comprehensive studies of protein and sugar composition in each plant-based material and genomic analysis for different LAB strains will be essential.

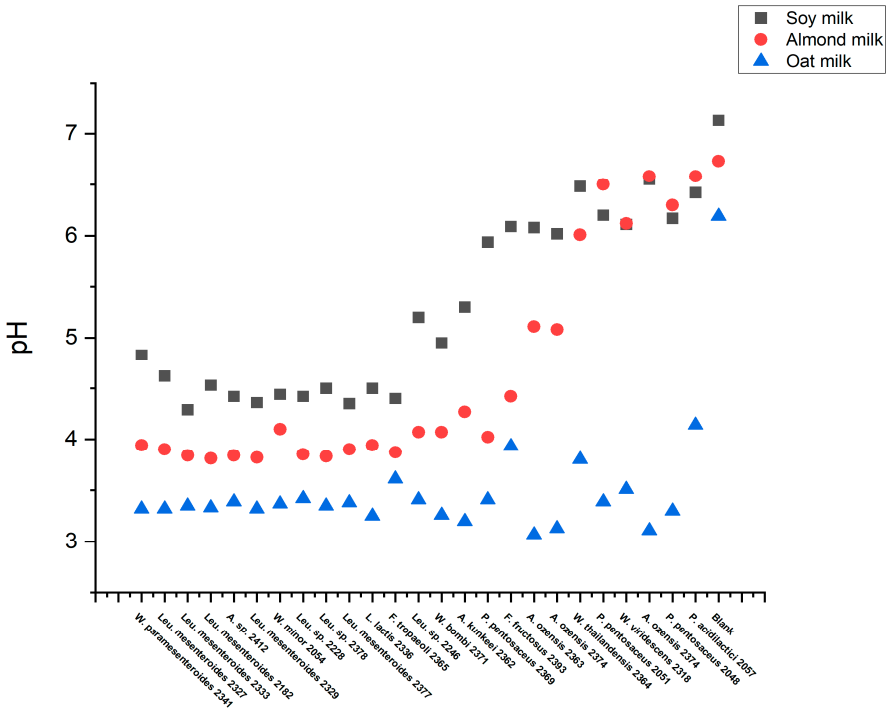

Figure 5. pH values of strains in fermented base-plant milks.

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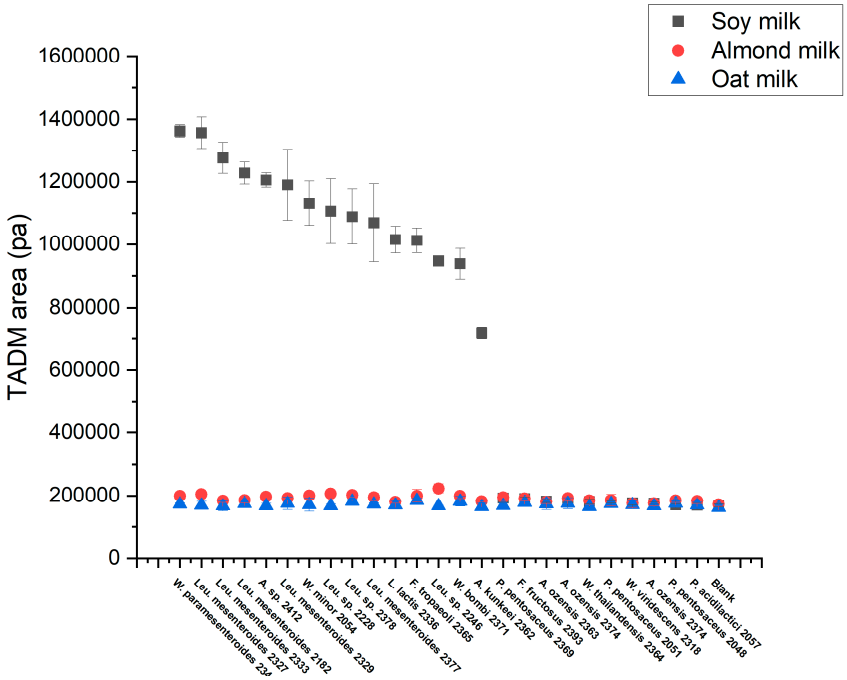

Figure 6. TADM areas of strains in fermented base-plant milks.

4. Conclusions

This study demonstrates a wide distribution of LAB in Danish flowers along with an innovative and efficient method for isolating LAB from flower samples. Additionally, we highlight the potential use of flower-derived LAB for plant-based food fermentation. The robust acidification and texture development, particularly in soy milk, indicate their significant role in enhancing the quality and diversity of plant-based fermented products. Further genomic study of the strains and compositional studies for different plant-based materials are essential to optimize their application in plant-based food fermentation.

Author Contributions: HX and CHBB conceptualized the study. HX, AKH, APW, MT, EBH, and CHBB designed the study. HX, AKH, APW, EBH, CHBB and VKP contributed to the acquisition of data. AKH and HX made the first draft of the manuscript. All authors contributed to the article and approved the submitted version.

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Data Availability Statement: The original contributions presented in the study are included in the article, further inquiries can be directed to the corresponding author.

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Conflicts of Interest: The authors EBH, CHBB, AKH, APW, and HX declare no conflict of interest. Additionally, it can be mentioned that VKP and MT are fulltime employee at Novonesis. The grant providers had no influence on the design and interpretation of the results generated and precented in this study.

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