ARTICLE

Exploratory authentication of lactic acid bacteria in commercial probiotic drinks sold in the Philippines

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ABSTRACT

Food fraud in various products, including dairy products, is prevalent worldwide, raising concerns about consumer health. With their respective microorganism declaration, fermented food products, like yogurt and milk, are probable targets for product mislabeling, adulteration, and substitution. To address this concern, food authentication through analytical methods ensures improved food safety and information for consumers. Hence, an initial assessment was conducted using morphological and molecular tests to authenticate the reported probiotic species in randomly selected milk and yogurt drinks sold in the Philippine market. Lactic acid bacteria (LAB) were isolated from six different brands and were grown and identified through Gram-staining and biochemical testing. For further testing, the 16S rRNA gene region was sequenced and subjected to BLAST and phylogenetic analyses. Morphological and biochemical tests revealed that all samples were characterized as Gram-positive, catalase-negative, nonmotile, and lactic acid-fermenting LAB isolates. The molecular analyses identified two LAB species, namely *Lacticaseibacillus paracasei* and *Leuconostoc mesenteroides*, confirming the species isolated from all the brands are characterized as LAB. However, only one brand matched its exact label, two with uncertain matches, two with nonspecific matches (unspecified labels), and one mismatch. These could indicate possible food adulteration by substitution and enhancement, counterfeiting, and mislabeling from these various probiotic brands. However, inconsistencies may also stem from variations in isolation techniques, environmental conditions, and incubation parameters, which can influence microbial growth dynamics and species viability. Despite these limitations, this study provides valuable insights into the reporting and labeling accuracy of probiotic products sold in the Philippine market.

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INTRODUCTION

Food fraud and safety have been persistent global issues dating back to ancient times (Everstine et al. 2024). These practices involve misrepresentation and mislabeling food products for economic gain, thus posing significant health risks to consumers (Visciano and Schirone 2021). Factors like climate change, population growth, food competition, and political instability exacerbate the challenge of ensuring food safety (Montgomery et al. 2020). Developing countries are particularly vulnerable to these malpractices due to limited awareness and regulation, as seen in the Association of Southeast Asian Nations (ASEAN) countries (Owolabi and Olayinka 2021). Recently, an ASEAN-

KEYWORDS

Lactic acid bacteria (LAB), 16S rRNA gene sequencing, morphological identification, biochemical tests, PCR, probiotic authentication, milk, yogurt

wide survey by Soon-Sinclair et al. (2023) regarding consumer perception of food fraud revealed that Vietnam and Malaysia are significantly concerned about food fraud. There was less concern for food fraud in Indonesia, Thailand, and the Philippines, thus being more susceptible to health risks, potential economic losses, and regulatory challenges.

Several studies have demonstrated adulterations, contaminations, and substitutions in food and medicinal products sold locally in the Philippines. Various herbal medicinal products, such as *lagundi* (*Vitex negundo*) and *bignay* (*Antidesma bunius*), were tested for their authenticity and found cases of food fraud (Olivar et al. 2016; Alfeche et al. 2019). Mislabeling was also detected in seafood products, specifically in sushi restaurants. Results showed consistent mislabeling with a rate of 47% (151 out of the 323 samples) from 2012 to 2015. Species such as halibut, red snapper, and yellowfin tuna had higher mislabeling rates, reaching up to 77%. The study highlighted that all restaurants in the population had at least one instance of mislabeling (Willette et al. 2017). Sarmiento et al. (2018) assessed the popular street foods fish, shrimp, and squid balls, and detected pig and chicken DNA instead. Given the prevalence of food and medicinal product fraud in the country, it is vital to implement robust authentication measures for a broader range of products to help safeguard consumer health and ensure the integrity of the food, nutraceutical, and pharmaceutical industries.

Probiotic food and drinks are widely gaining attention due to their marketability and digestive health benefits. Numerous companies constantly innovate and improve their methods, especially in providing other health-beneficial components in their products by adding prebiotic substances and/or probiotic bacteria (García-Burgos et al. 2020). Commonly produced fermented foods are dairy products such as cultured milk and yogurt, also known as probiotic drinks that can be processed through lactic acid bacteria (LAB) such as *Lactococcus*, *Pediococcus*, *Lactobacillus*, and *Leuconostoc* species. Studies show that these species aid in improving the quality and nutrient bioavailability of milk (Shah et al. 2023). Aside from fermented milk, yogurt and yogurt-related products are considered the most popular fermented dairy foods, generally using *Streptococcus thermophilus* and *Lactobacillus* spp. commercially available and claimed by food companies. A study by Widyastuti et al. (2021) focused on the health-promoting properties of Lactobacilli, specifically in fermented dairy products, which were assessed and classified according to the product's geographical continent. In Asia, Yakult, a popular fermented milk developed in Japan, is reported to have improved the symptoms of lactose-intolerant patients; Koumiss, a product from China and Central Asia, also has claims of having an immunomodulating effect and cholesterol-lowering ability in treating hypercholesterolemia. Alan et al. (2022) and Kowalczyk et al. (2024) presented the commonly used microorganisms with recently claimed probiotic properties, which include *Leuconostoc, Lactobacillus, Bifidobacterium, Enterococcus*, and *Streptococcus.* Notably, quality assessment of probiotic products was also highlighted in the study, stating that most of these products do not go through pre-market approvals and that there are issues with the correct strain identification of each probiotic. This study elaborated that there is a need for the expansion of internationally recognized culture collections of taxonomically classified and deposited probiotics.

Authentication of probiotic dairy products in the UK and European markets was performed by Farahmand et al. (2021), testing 36 products. Results showed inconsistencies between the declared probiotic strains and those that were actually present in the product, whereby specific bacterial strains were not fully disclosed on the label. Furthermore, only 22 out of the 36 tested products contained more than 10^6 colony-forming units (CFU) per gram at the end of their shelf life, aligning with the recommended minimum therapeutic level for probiotics. This finding underscores concerns regarding labeling accuracy and consumer transparency. Although the study did not identify the minimum recommended probiotic counts for each specific brand of milk and yogurt drink, the primary aim was to assess the health benefits of these products, evaluating the viability and culturability of probiotic counts is crucial. Such assessments can determine whether the products contain sufficient viable and culturable probiotic counts, thereby ensuring their efficacy and reliability for consumers. Hence, there is continuous growth in probiotic drink development, evaluating their microbiological quality, dependability, and efficacy based on the set international standards. Lestari et al. (2022) assessed the sensory profile of drinkable yogurts made with prebiotics and probiotics to further aid yogurt manufacturers in providing information to incorporate specific novel value-added ingredients, as this approach enhances the health benefits and boosts market appeal for consumers.

As the food market spurs with the development of new probiotic drink products, it is crucial to establish a standard and/or protocol for the authenticity of their microbial content, especially in the Philippines, where it is widely popular and available. Although international standards exist, developing a specific local and standardized protocol, per Sustainable Developmental Goal (SDG) 2 (Zero Hunger) while also addressing the rising interest in probiotics for their health benefits following SDG 3 (Good Health and Well-being), is essential as an added precautionary measure for enhancing food testing, safety, and assessment in the country.

To set a baseline for its development, this study authenticated probiotic species in commercially available milk and yogurt drinks sold in the Philippines through basic morphological, biochemical, and molecular methods. Specifically, the study aims to 1) isolate and culture probiotic species from locally sold milk and yogurt drinks, 2) identify and characterize isolates through morphological and biochemical methods, and lastly, 3) perform molecular authentication using the 16S rRNA gene barcode through BLAST and tree-building methods.

MATERIALS AND METHODS

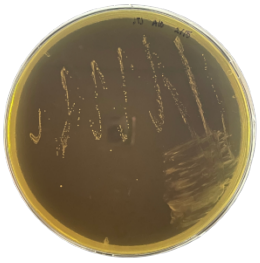
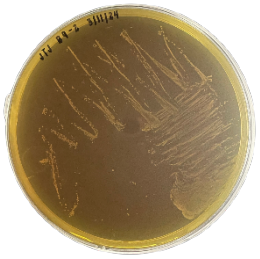
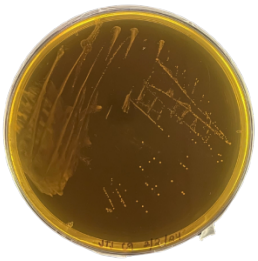
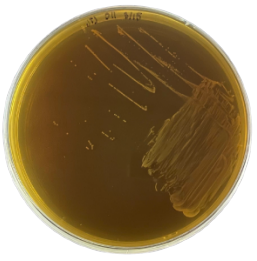
Product sampling and isolation

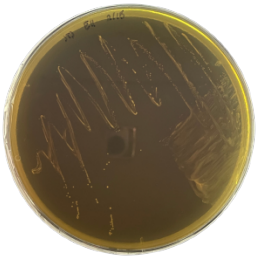
Six (6) milk and yogurt drink brands commercially available in the Philippines were randomly selected and procured from a reputable supermarket in Manila City, which was chosen due to its adherence to standardized storage and handling protocols that minimize variability due to environmental factors. Sample collection was conducted in February 2024, and for each brand, two (2) product samples from different production batches (as indicated by distinct batch numbers) were acquired. This was done to ensure adequate biological replicates and to capture inter-batch variation, thus enhancing the study's reproducibility and reliability. The expiration dates of the samples ranged from one (1) to two (2) months post-purchase, consistent with the standard shelf life of such products, thereby confirming product freshness and viability for analysis.

Upon procurement, all samples were labeled, recorded, and transported under cooled conditions to the laboratory. Immediately upon arrival, samples were stored in a refrigerator maintained at 4°C, in accordance with the storage recommendations for dairy-based beverages and as described by Wassie and Wassie (2016).

LAB were isolated following the methods of Wassie and Wassie (2016) and Taye et al. (2021). While the pH of the samples was not specifically recorded prior to processing, adjustments were made to control oxygen availability and incubation temperature to support LAB growth. The cultures were incubated under both aerobic and anaerobic conditions, and at temperatures suitable for mesophilic LAB (typically around 30–37 °C), to increase the likelihood of recovering diverse LAB taxa. These modifications were based on known growth requirements of commonly declared probiotic strains. To optimize the isolation protocol, which was necessary because multiple attempts to culture LAB species under anaerobic incubation failed to support LAB growth, 5 ml of each milk and yogurt sample was transferred into a sterilized falcon tube containing 5 ml of Tryptic Soy Broth (TSB). This adjustment was made to improve bacterial recovery during the pre-enrichment step prior to selective culturing. Although TSB is a non-selective enrichment medium, it was intentionally used in this study as an initial step to promote the general growth of bacteria, including stressed or low-abundance LAB, before subjecting the samples to more selective conditions. As mentioned, this pre-enrichment allowed viable LAB to recover and multiply. This alternative approach proved effective in our culturing attempts, as it increased the likelihood of successful LAB isolation upon subsequent transfer to selective media - De Man–Rogosa–Sharpe (MRS) agar. The tubes were then incubated at 37°C for 48 hours. Afterward, the tubes were vortexed, and 50–100 µl of each sample suspension was spread-plated on a 2-channel MRS agar media plate before incubation at 37°C for another 48 hours. Finally, once bacterial growth was achieved, the observed bacterial strain per sample was restreaked (via multiple interrupted streak method) in a 1-channel MRS agar plate for bacterial isolation. These restreaked plates were incubated at 37°C for 48 hours before morphological identification.

Table 1: Results of LAB grown in MRS agar

Product	Sample	SP ⟶ MI	Documentation (Representative plates only)
Brand A	**A1**	+ +	
	A2	+ +	
	A3	+ +	
	A4	+ +	
Brand B	**B1**	+ +	
	B2	+ +	
	B3	+ +	
	B4	+ +	
Brand C	**C1**	+ +	
	C2	+ +	
	C3	+ +	
	C4	+ +	
Brand D	**D1**	+ +	
	D2	+ +	
	D3	+ +	
	D4	+ +	

Brand E	**E1**	+	+	
	E2	+	+	
	E3	+	+	
	E4	+	+	
Brand F	**F1**	+	+	
	F2	+	+	
	F3	+	+	
	F4	+	+	

Legend:
+ (with growth); **-** (without growth)
SP - spread plate; **MI** - multiple-interrupted streak method

Morphological identification and biochemical characterization
Isolates were physically and biochemically characterized for initial identification (Ibraheim et al. 2023; Ismail et al. 2018). As stated by Mokoena (2017) and Taye et al. (2021), LAB was established to be Gram-positive, catalase-negative, lactic acid-producing, and usually seen as cocci or rods in groups or colonies. Therefore, the following tests were applied to verify these characteristics: Gram-staining, catalase test, methyl reduction, and motility test (Thakur et al. 2017; Ismael et al. 2018). These were performed in duplicates.

A modified protocol of Tripathi and Sapra (2022) was applied in the Gram-staining method. Prior to colony transfer, these colonies were assessed and described, noting their distinctions. After checking the consistency of the bacterial colonies, a single colony was obtained from the culture and inoculated on the sterile water drop on the slide. Once completely dried, it was heat-fixed, and staining was performed. Initially, the Gram-staining results were inconsistent, with poor retention of stain or unclear differentiation between Gram-positive and Gram-negative bacteria. To address this, modifications were made, including adjusting the smear thickness, extending the crystal violet and iodine application times, and ensuring more effective decolorization control. The specimens were observed under oil immersion objective (OIO) under a compound light microscope. Gram-positive bacteria should appear purple or bluish-purple.

The chosen bacterial colonies were subjected to various biochemical tests to validate the genus identity of the bacteria following similar protocols to the studies of Ismail et al. (2018) and Taye et al. (2021). For the catalase test, hydrogen peroxide (H_2O_2) was added to each of the isolated 24-hour cultures on glass slides, where the formation of bubbles was evaluated. Next, the methyl red test was performed. The isolated strains were inoculated to Tryptic Soy Broth (TSB) and incubated for 48 hours at 37°C. Afterward, these were subcultured in Methyl Red-Voges Proskauer (MR-VP) broth, followed by adding methyl red to observe a color change. Lastly, a motility test was conducted by inserting a colony of bacteria into a motility indole ornithine (MIO) media inside a test tube, followed by incubation for 48 hours at 37°C.

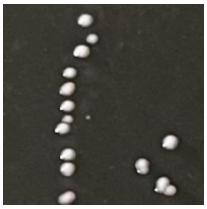
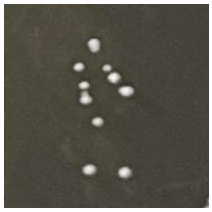
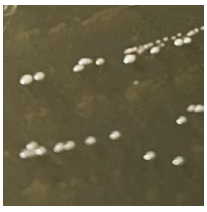
DNA isolation and tree-building analyses
Using unique morphotypes as a basis for colony differentiation, replicates that vary in colony morphology within a single brand were selected. Pure cultures of these samples from each brand were sent to Macrogen, Korea, for DNA isolation, amplification by polymerase chain reaction (PCR), and bi-directional sequencing, targeting the 16S rRNA gene region. The seven newly generated sequences were subjected to BLASTn search (Altschul et al. 1990) and were included in the alignment generated by the software MEGA ver. 11 (Tamura et al. 2021), containing 31 accessions from the genera *Lacticaseibacilius*, *Leuconostoc*, and *Streptococcus*, with *Bacillus subtilis* as the outgroup (Moiseenko et al. 2023). Phylogenetic trees by neighbor-joining (NJ) and maximum likelihood (ML) methods were generated using the same software with 1,000 bootstrap replicates. Node support inferences are as follows: < 50% are unsupported, 50–74% weak support, 75–84% moderate support, and 85–100% strong support (Fu et al. 2022).

RESULTS AND DISCUSSION

Sample isolation and colony morphology
All replicates per sample had bacterial growth in both isolation methods, specifically spread plate and multiple-interrupted streak (Table 1). For the spread plate method, nearly all plates yielded colonies that were too numerous to count (TNTC), indicating a high bacterial load. While this limited the ability to determine exact CFU values, the observation still confirmed the presence of viable LAB in the products. Moreover, since CFU counts are typically not declared on the labels of commercially available probiotic drinks, direct authentication of CFUs based on label claims was not feasible. MRS agar is a selective culture medium for the cultivation of *Lactobacillus* spp., and initial results showed that LAB were identified to their generic level through the observation of "white, small to large size, and circular margin on MRS media" (Taye et al. 2021). LAB species are facultative anaerobic organisms that may thrive with or without oxygen. Thus, the method of aerobic incubation in this study still yielded bacterial growth since LAB can grow in MRS agar in either aerobic or anaerobic conditions, as also observed in the study by Matejčeková et al. (2016). However, this could have hindered the microbial growth of some colonies, promoting the fastest-growing species to proliferate given its environmental conditions, as not all lactic acid bacteria are aerotolerant, and some species prefer anaerobic conditions, thereby limiting colony selection in the succeeding protocols. This was mentioned in the study by Sionek et al. (2024), highlighting the essential physicochemical conditions (such as the food matrices and technological processes involved in its production) on LAB and its effect on their survival in food

products. It was still noted that the targeted species in the study, as indicated in product labels, were characterized as facultative anaerobic, with only the *Bifidobacterium* group as an obligate anaerobe, which was not specified in the label of the brands in the study (Jiang et al. 2022). With this, molecular analysis identified *L. mesenteroides* in most products, which have been demonstrated comparatively to have faster growing rates than target lactic acid bacteria species, especially in lower temperatures (Seo et al. 2021). The products' claimed species also exhibit specific conditions for optimal growth, such as pH ranges (pH 6.5 for *S. thermophilus* and pH 5.8 to 6 for *L. bulgaricus*), and oxygen requirements (microaerophilic for *L. bulgaricus*) (Mustafa et al. 2019). Furthermore, one product's claimed species is a heat-killed strain, *Lactobacillus paracasei* MCC1849, identified as a paraprobiotic, indicating its non-viability and non-culturability (Arai et al. 2018; Murata et al. 2018).

Table 2: Colony morphology of the different brands in MRS agar

Sample	Colony Morphology	Description	Sample	Colony Morphology	Description
(+) *L. rham* CLR1506		**Form:** Circle **Size:** Small (≤ 2 mm) **Color:** Milky white **Texture:** Smooth **Elevation:** Convex **Margin:** Entire	C2		**Form:** Circle **Size:** Small (≤ 2 mm) **Color:** Milky white **Texture:** Smooth **Elevation:** Convex **Margin:** Entire
A		**Form:** Circle **Size:** Small (≤ 2 mm) **Color:** Milky white **Texture:** Smooth **Elevation:** Convex **Margin:** Entire	D		**Form:** Circle **Size:** Small (≤ 2 mm) **Color:** Milky white **Texture:** Smooth **Elevation:** Convex **Margin:** Entire
B		**Form:** Circle **Size:** Small (≤ 2 mm) **Color:** Milky white **Texture:** Smooth **Elevation:** Convex **Margin:** Entire	E		**Form:** Circle **Size:** Small (≤ 2 mm) **Color:** Milky white **Texture:** Smooth **Elevation:** Convex **Margin:** Entire
C1		**Form:** Circle **Size:** Small (≤ 2 mm) **Color:** Beige/cream **Texture:** Smooth **Elevation:** Convex **Margin:** Entire	F		**Form:** Circle **Size:** Small (≤ 2 mm) **Color:** Milky white **Texture:** Smooth **Elevation:** Convex **Margin:** Entire
	5 mm			5 mm	

Representative bacterial colonies were also initially identified as *Lactobacillus* spp. by observing their colony morphology (Table 2). All isolates were observed to be circular in form, smooth in texture, convex in elevation, and had an entire margin based on the appearance of colonies. Additionally, the colors of the colonies varied from milky white to beige or cream. LAB colonies commonly observed are small, specifically 2 mm in approximate diameter (Escobar-Ramírez et al. 2020).

Gram staining and biochemical tests

The bacterial colonies from the different samples were evaluated into Gram-positive and Gram-negative species and differentiated into their respective shapes through microscopic examination. As shown in Figure 1, all isolates were Gram-positive. Gram-positive bacteria have thick peptidoglycan walls that retain the added crystal violet color (Sizar and Unakal 2022). LAB were characterized as Gram-positive bacteria with distinct cell wall components that exhibit fundamental properties essential for their diverse functions. Probiotic strains have unique surface proteins in their cell walls observed to facilitate mucosal colonization and persistence in the gastrointestinal tract (Lopez de Felipe et al. 2021). Moreover, by facilitating direct contact with the gut mucosa, they could promote cross-communication with immune cells (Du et al. 2019). These contributed to LAB's probiotic nature of functioning to improve intestinal immune responses and promote better digestion.

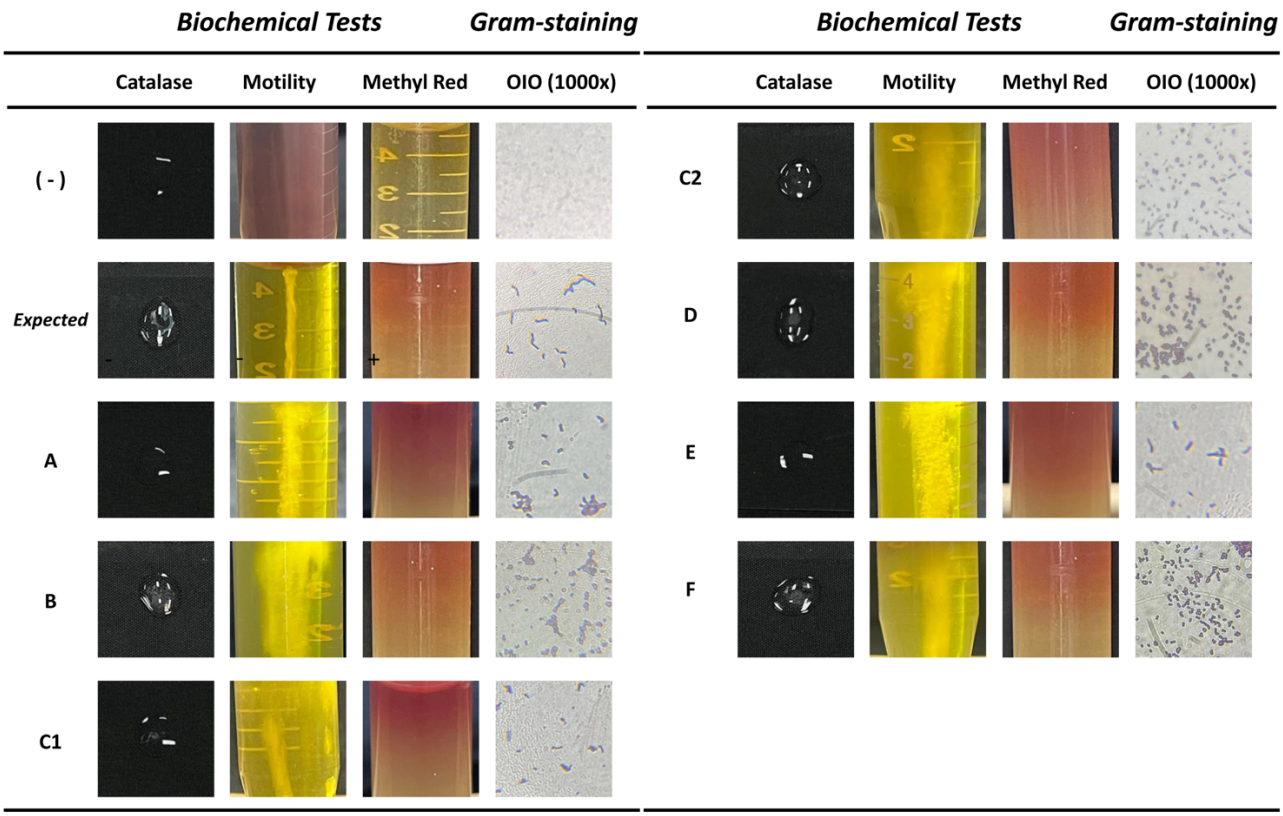


Figure 1: Gram-staining and biochemical results of the different brands through representative bacterial colonies

Similar bacterial shapes with their respective arrangements were observed among the bacterial samples. All the samples were primarily observed as smaller cocci or rod-shaped bacteria, occurring in pairs and chains. These findings agreed with the characteristics of LAB. Khushboo et al. (2023) reported that probiotic LAB species from dietary sources were generally characterized as having rod or cocci shapes. In the study of Wassie and Wassie (2016), six genera of LAB were isolated from raw cow milk. They reported *Lactobacillus* were rod-shaped, while *Streptococcus*, *Lactococcus*, *Enterococcus*, *Leuconostoc*, and *Pediococcus* were cocci, successfully isolating them from dairy. Upon careful examination, samples B, C2, D, and F resembled each other, exhibiting some cocci irregularities. These observations corroborated with species belonging to the genus *Leuconostoc*, which have shown similar characteristics of irregular coccoid morphology (Vyas et al. 2017).

Following microscopic evaluation, the samples were characterized through biochemical characterization. The biochemical test results in Figure 1 identified distinctive properties that represented the isolated samples from each brand as LAB. Catalase, motility, and methyl red tests were the biochemical tests performed in the study.

In the catalase test, the presence of catalase enzyme that chemically converts hydrogen peroxide (H_2O_2) to water (H_2O) and oxygen (O_2) was tested. The formation of bubbles, indicating the occurrence of the reaction, was uncharacteristic of a LAB species, while the absence of bubbles showed the absence of catalase. All samples were catalase-negative, matching an attribute of LAB. Results showing bubble-like appearances were light reflections during documentation, while white-colored smudges were the sample colonies tested. A well-known characteristic of LAB species was the lack of the catalase enzyme, inhibiting the decomposition of hydrogen peroxide into its products. While some bacteria needed oxygen to produce hydrogen peroxide—a byproduct of aerobic metabolism, several species of LAB, especially those used in the food industry, were anaerobic or facultatively anaerobic, allowing them to thrive in the absence of oxygen (Rombouts et al. 2020). Most aerotolerant LAB species commonly used in probiotics as involved in this study test negative for the enzyme catalase. The absence of bubble formation in all samples showed a negative result, which was the expected result of this test.

The motility test determined the species' motility, indicating distinguishing characteristics functioning for movement, such as flagella. As shown in Figure 2, isolates from all brand samples were nonmotile, indicated by the distinct line of growth along the line of inoculation in the test tubes. The results implied that the isolated species lack flagella, a characteristic of most lactic acid bacteria. LAB isolates from food and drink sources (milk, curd, fermented vegetables) were identified as nonmotile (Islam et al. 2020; Khushboo et al. 2023).

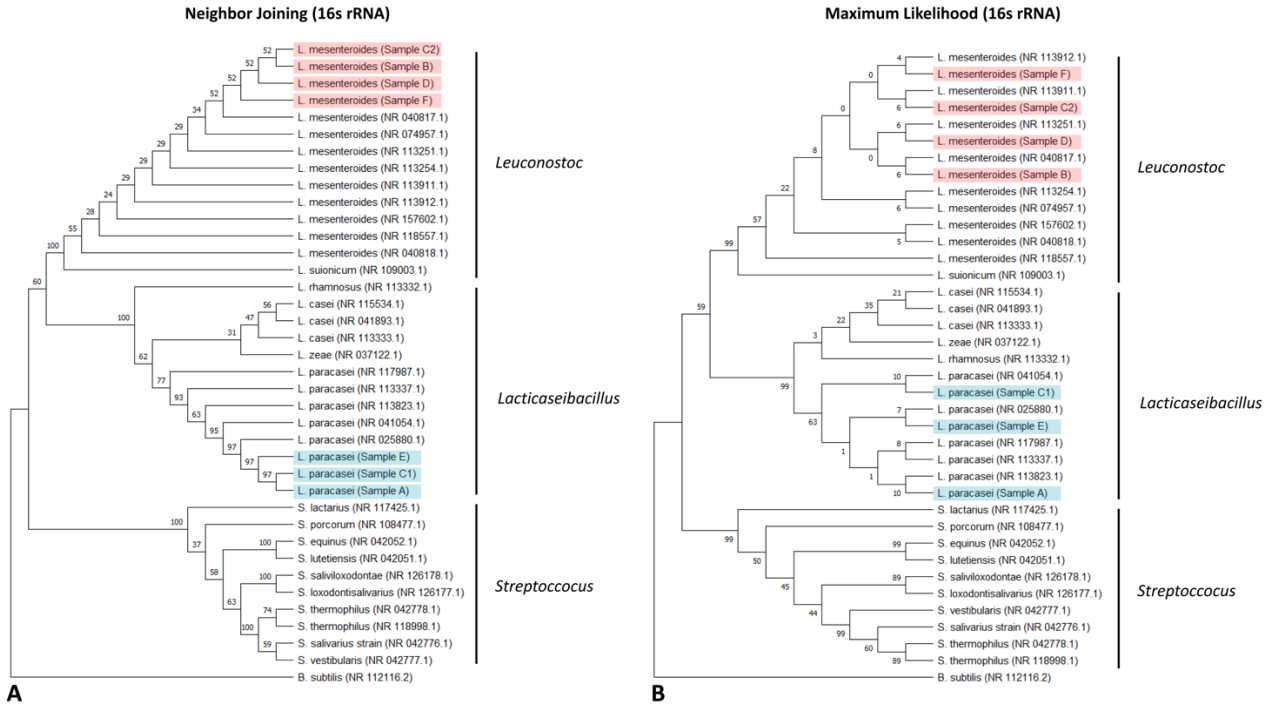


Figure 2: Phylogenetic trees generated through NJ method (A) and ML method (B). Generated sequences from this study are highlighted in color (*L. mesenteroides* – pink; *L. paracasei* - blue)

The methyl red test determined the LAB species' ability to ferment glucose and convert it to lactic acid. LAB are expected to yield a positive result by observing the red color, indicating successful fermentation (Islam et al. 2020). Otherwise, a yellow color (negative) will be observed. Generally, LAB metabolizes glucose to pyruvic acid first, and through the mixed acid pathway, the pyruvic acid is then metabolized into a stable acid, lactic acid. Through the production of the lactic acid, the pH of the Methyl Red–Voges Proskauer (MRVP) broth decreases to 4.5 or below, resulting in a change of color from yellow (negative) to red (positive) after the addition of the methyl red. With this, Figure 1 shows that all samples showed a positive result.

Samples from all brands corroborated with the LAB characteristics. LAB are a group of Gram-positive, catalase-negative, nonmotile, nonsporulating bacteria that produce lactic acid as a fermentation product (Mokoena 2017; Ramadhanti et al. 2021). Some LAB species closely associated with dairy products included, genera such as *Lactobacillus*, *Streptococcus*, *Enterococcus*, *Bifidobacterium*, and *Leuconostoc* (Ağagündüz et al. 2021). These findings presented the possible identification of the bacterial isolates up to their genera.

Molecular analyses of LAB in probiotic products

BLASTn analyses reveal *Leuconostoc mesenteroides* was identified in brands B, D, and F rather than the species indicated in the product labels. Brands C (represented by two samples) and E have no LAB species indicated on the product labels; however, they were identified as *Lacticaseibacillus paracasei* and *L. mesenteroides*. It is worth noting that both representative samples from Brand C have different LAB species, as supported by the variations in the morphological examination. Only Brand A has a definite match with its product label. Table 3 provides the complete BLASTn results relative to the only LAB species declared by the manufacturer.

Table 3: BLASTn results of the 16S rRNA gene sequences

Product	Claimed Taxon	BLAST Result	Length (bp)	% Identity	Accession Number
A	*Lacticaseibacillus paracasei* Shirota	*Lacticaseibacillus paracasei*	1522	100.00	NR_025880.1
B	*Lactobacillus bulgaricus* *Streptococcus thermophilus*	*Leuconostoc mesenteroides*	1476	100.00	NR_113912.1
C	no specific strains specified in the product label	C1: *Lacticaseibacillus paracasei*	1522	99.93	NR_025880.1
		C2: *Leuconostoc mesenteroides*	1476	100.00	NR_113912.1
D	*Lactobacillus bulgaricus* *Streptococcus thermophilus*	*Leuconostoc mesenteroides*	1476	100.00	NR_113912.1

E	no specific strains specified in the product label	*Lacticaseibacillus paracasei*	1522	100.00	NR_025880.1
F	*Lactobacillus paracasei* MCC1849	*Leuconostoc mesenteroides*	1476	100.00	NR_113912.1

Aligned sequences of the 16S rRNA gene yielded 1,587 basepairs (bp), with 1127 conserved, 456 variable, and 346 parsimony informative sites. The trees generated from both NJ and ML yielded highly similar topologies (Figure 2), with all three genera being highly supported (BS ≥ 99%). The LAB samples B, C2, D, and F, identified as *L. mesenteroides* in BLASTn results, are all nested within the *Leuconostoc* group in both NJ (BS = 100%) and ML (BS = 99%) analyses. The LAB samples A, C1, and E are all nested within the *Lacticaseibacillus* group in both NJ (BS = 100%) and ML (BS = 99%) analyses, consistent with the BLASTn identifications. While deeper node supports and interspecies relationships range from moderately low to unsupported due to low sequence variations among the taxa, the newly generated sequences still exhibit a high affinity in their expected placements within both trees.

Authentication of probiotic species in commercially sold products in the Philippines

The sequence of Brand A was identified to be *Lacticaseibacillus paracasei* (formerly known as *Lactobacillus paracasei*), matching the claimed species of *L. paracasei* strain Shirota (LcS) in Brand A (Bengoa et al. 2021). Brands B and D, however, are referred to as mismatches since the claimed species of *Lactobacillus bulgaricus* and *Streptococcus thermophilus* did not match the identified strain of *Leuconostoc mesenteroides*, a claimed probiotic that also belongs to the group of LAB. Multiple studies mentioned that *Leuconostoc* spp. is commonly added to the mixture of dairy products as a non-starter lactic acid bacteria (NSLAB), elucidating flavor development and possessing aromatic traits (Ruppitsch et al. 2021). This is due to their ability to produce diacetyl, acetoin, and carbon dioxide from citrate which all take effect in the aroma and texture formation, hence used as flavor starters or adjunct cultures in various fermented products (de Paula et al. 2015; Ruppitsch et al. 2021; Hamdaoui et al. 2022).

Regarding isolation, LAB species usually occur in mixed microbial populations, causing claimed species to become non-culturable as high quantities of *L. mesenteroides* dominated them. Research has shown that during quality-checking of probiotic products, a portion of its bacterial population may have become viable but non-culturable (VBNC) after storage (Zawistowska-Rojek et al. 2022). Although possible, these products may have previously undergone research to ensure species viability after various storage conditions. Focusing on the study's isolation parameters, the initial isolation in TSB as an enrichment step to aid in culturing the target claimed bacteria could have promoted the growth of other bacterial strains in low numbers in the samples. The isolation of some microorganisms from milk and other complex samples is improved by involving an enrichment procedure with a modified tryptic soy broth. This additional method has successfully recovered and isolated *Y. enterocolitica* and related species from food samples in a study done by Pal et al. 2023. In this study, the addition of the enrichment step also yielded the isolation of microorganisms, which was not possible when samples were directly cultured on MRS agar. Thus, the protocol was optimized to include this step to obtain results of isolating a microbial strain from the probiotic drinks. However, in these experiments, difficulties still arise in recovering targeted strains due to high concentrations of mesophilic and psychrotrophic microbes in milk samples, leading to the isolation of other bacterial species. *Leuconostoc* species are mesophilic and psychrotolerant heterofermentative bacteria, well-adapted to sugary niches (Onyeaka and Nwabor 2022; Ruiz et al. 2024). Contrarily, the claimed probiotic species (*S. thermophilus* and *L. bulgaricus*) are homofermentative facultatively anaerobic, cultivated at 45°C (Bostan et al. 2017). In this case, the isolation conditions performed in the study could have been suboptimal to grow the claimed species, while *L. mesenteroides* could have been provided with the optimal growth conditions to flourish in the population.

Another possible explanation would be the change in environmental conditions during isolation, such as oxygen requirement. In a study, the researchers observed how *L. mesenteroides* as a starter significantly decreased *Lactobacillus* spp. populations during kimchi fermentation (Lee et al. 2020). This is caused by the change of environment over time from weak acidic and less anaerobic conditions, where *L. mesenteroides* thrives, to strong acidic and more anaerobic conditions, an environment where *Lactobacillus* spp. dominates. This transition is reflected in the isolation process where the environment changes from aerobic packaging to less anaerobic conditions; thus, *L. mesenteroides* possibly dominating the population.

In the case of Brand F, it is also considered a mismatch due to the claimed strain, *L. paracasei* MCC1849, not matching its identity with *L. mesenteroides*. However, it must be emphasized that the claimed strain is a paraprobiotic, which are defined as heat-killed microbial cells that still perform its probiotic role in the host's immune system (Murata et al. 2018). In the study of Teame et al. (2020), they stated that the isolation of paraprobiotics involves a different process aside from traditional pre-enrichment and purification methods used for probiotics, particularly cell disruption techniques. This is mainly because of the cells' bacterial components that significantly contribute to the immune regulation function of the host's system. With this, techniques such as thermal treatment, enzymatic treatment, solvent extraction, radiation, high-pressure, and sonication could have been performed to efficiently isolate and purify this non-viable and inactive strain (Teame et al. 2020). This is also reflected in the protocol of Murata et al. (2018) wherein they successfully cultured *L. paracasei* MCC1849 strain in a medium that contained sugar, yeast extract, and salt, followed by pasteurization and freeze-drying of the harvested cells.

Finally, Brands C and E were identified as *L. paracasei* and *L. mesenteroides*, but are referred to as nonspecific matches due to no specific taxa being declared on their product labels. Despite the specification of species not being required in probiotic-based beverages (Koirala and Anal 2021), this fails to provide information for consumers on the microbial populations present in the drink, compromising the safety and trust of consumers. The classification of species offering desirable metabolic capabilities in the dairy industry allows easier identification of these specific organisms and their growth condition requirements for commercial production. The organism's potential to yield products of interest requires precisely specified and reproducible growth parameters (Oberg et al. 2022). Thus, stricter labeling standards in manufacturing are suggested. Proper labeling enhances the communication between and among producers, industries, policy-makers, scientists, and consumers on the prospects of these species, allowing consistency and reproducibility in species' studies and applications in the food industry. This establishes better

awareness and understanding of the probiotic applications of these microorganisms to the products and facilitates approaches to maximize the full potential of using their desired capabilities while controlling unwanted organisms or inverse consequences from product consumption.

Product implications in food authentication

Considering the three mismatches (Brands B, D, and F) and two nonspecific matches (Brands C and E), the observed discrepancies may be due to the impact of environmental factors and isolation conditions and effects on microbial growth dynamics within milk and yogurt products. Such variability during isolation and culturing can influence which species are recovered, potentially leading to misinterpretation of product composition. While these inconsistencies raise questions about labeling accuracy, caution must be exercised before concluding food fraud or mislabeling. It is important to verify whether the product labels specify exclusive probiotic strains or manufacturers simply list representative species. According to Koirala and Anal (2021), labels on probiotic foods and beverages should be clear and accurate, and must be validated by the appropriate regulatory agencies. As *L. mesenteroides* can improve the product's flavoring when added to the microbial population, this may have been used as a secondary culture and intentionally added by manufacturers. But these substitutions and additions may also be characterized as counterfeiting, a term used for cases of replacements between ingredients that are functionally identical. This is applicable as most LAB species are physiologically similar, enabling them to still participate during fermentation processes and deliver probiotic purposes. However, these claims are subject to further verification since *Leuconostoc* spp. are naturally occurring, commonly added as starter cultures, and predominant in fermented food, thus being isolated from the product (Rezac et al. 2018). From this, isolation conditions for the claimed strains could have been exclusively optimized for these brands. For instance, *L. paracasei* MCC1849 of Brand F was advertised on their brand website to provide health benefits by strengthening one's immune system against infections. Since a specific strain is highlighted, the isolation conditions could have been modified to the protocol of Murata et al. (2018) as they involved cell disruption techniques such as pasteurization and freeze-drying. As this may be the case for other probiotic drinks claiming specific strains of LAB, it is therefore recommended to include the species that were used as starter cultures in the label to avoid misinterpretations in future species identification studies. Lastly, the concept of mislabeling is also possible, along with Brands C and E, since either not all species were declared or some were misidentified due to their highly identical genetic characteristics in the 16S rRNA gene region, making closely related taxa challenging to distinguish through molecular means (Jones 2017; Huang et al. 2018; Zheng et al. 2020). As regulations only partially require all species declaration in their products, companies like Brand F could choose to present claims of only specific and relevant microorganisms in their finished products to avoid complexities and confusion with consumer understanding and impracticalities. However, appropriately labeling probiotic product packages is still highly encouraged as these probiotic microbes' health effects depend on their strain identification (Zawistowska-Rojek et al. 2022). Proper labeling allows consistency and reproducibility when manufacturing these products and promotes transparency between producers and consumers, encouraging informed choices. Notably, multiple factors must be considered when labeling each product correctly, as probiotic products undergo an intricate process and quality control involving starter cultures, strain production, raw material manufacture, etc. (Fenster et al. 2019). Species strains depend highly on these manufacturing variables, thus implying their relevance for careful consideration, selection, and control.

Although the results suggest that *L. mesenteroides* might have been used as a substitute by manufacturers with probiotic drinks, this may not always be the case since isolation conditions must always be optimized per claimed species to effectively isolate the advertised strain. Despite this, manufacturers must still be aware of the dangers and risks of adulteration by substitution since this could negatively impact the promotion of their product and health of consumers since *Leuconostoc* spp. may cause disadvantages in the long run, such as spoilage and pathogenic potential, as reported in several studies. Additionally, manufacturers must be aware of using the updated nomenclature for probiotic species, despite the convenience of using shortened versions in labels. This is observed in the case of the recently updated genus of *Lacticaseibacillus* from *Lactobacillus* in 2020 (Bengoa et al. 2021) and *L. bulgaricus* that was already reclassified under a subspecies referred to as *L. delbrueckii* subsp. *bulgaricus* (Hawrelak 2020). This is to avoid confusion in future species identification studies that significantly rely on the available and updated species names recorded in the NCBI database.

CONCLUSION

Due to limitations in time, resources, and laboratory facilities available during the conduct of this research, it was not possible to perform all the necessary isolation techniques and meet specialized incubation conditions for a comprehensive profiling of lactic acid bacteria present in the products. As a result, this study provides a baseline report into the types of probiotic species found in commercially available milk and yogurt drinks. However, despite these constraints, the findings still provide valuable insight into the presence of LAB strains in the sampled products. For future studies, we recommend the use of more advanced techniques to overcome the limitations associated with culturing and isolating LAB strains. Future LAB screening should incorporate more advanced, culture-independent methods such as direct molecular authentication or metagenomic analysis to overcome the limitations of culturing and to enable more comprehensive microbial profiling.

Overall, the findings of this study emphasize the importance of enforcing stricter regulations and quality control in the production and labeling of LAB probiotic products. The discrepancies between the authentication results and the product labels indicate that insufficient information (non-specification of all species used in the fermentation process), as well as potential mislabeling and food fraud, remain prevalent within the industry. Though specification of species is not required, these practices not only compromise consumer safety and trust but also extend beyond consumers, impacting supply chains, sellers, and the overall reputation of the food industry. Moreover, it is highly recommended that future studies perform molecular authentication on a broader range of probiotic products with 16S rRNA gene as the standard barcode. Developing a local standardized protocol for screening, validating, and authenticating present species in locally sold products through international standards is strongly recommended. This is to effectively protect consumers and enhance public health safety, not only in the Philippines but around the globe, most especially if products from the Philippines will be exported internationally. Ultimately, it is important to note that the results of this study should be regarded as preliminary and interpreted with caution, as further validation and the use of more advanced techniques are necessary before drawing definitive conclusions.

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CONFLICT OF INTEREST

The authors declare that they have no conflicts of interest.

CONTRIBUTIONS OF INDIVIDUAL AUTHORS

All authors participated in the conceptualization and design of the project. TASE, JHRS, and JJDV performed the experiments, data analyses and interpretation, and writing of the manuscript. Specifically, TASE, JHRS, and JJDV contributed during the optimization of the microbiological processes, while TASE completed the methods under the molecular processes with the assistance of JHRS. MJMC, JADO and ACGC contributed to the data analyses and interpretation, revision of the manuscript, and supervised the entire project.

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