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ORIGINAL ARTICLE

A novel selective medium for isolation of *Limosilactobacillus reuteri* from dietary supplements

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Abstract

***Limosilactobacillus reuteri* is a probiotic bacterium known for its numerous beneficial effects on human health and is commonly utilized in various dietary supplements. Previously, we encountered difficulties in isolating *L. reuteri* from retail dietary supplements containing complex probiotic compositions by using non-selective media such as de Man, Rogosa, and Sharpe (MRS) agar. Our findings reveal that MRS agar with D-gluconic acid as the carbon source and peptone from soymeal as the nitrogen source provides a growth advantage for *L. reuteri*. Furthermore, all the tested *L. reuteri* strains exhibit higher resistance to oxacillin compared with non-*L. reuteri* strains, and the recovery of *L. reuteri* is significantly higher than that of non-*L. reuteri* strains on modified MRS agar (MRS-GSOT agar) supplemented with either 4 or 10 μg/mL oxacillin. Results of spiking tests indicate that MRS-GSOT agar with 10 μg/mL oxacillin can selectively inhibit the growth of species other than *L. reuteri* in single culture or mixed bacterial broth within food matrices. However, the recovery of *L. reuteri* is relatively low when subjected to the spiking tests with various ratios of non- *L. reuteri*. Testing results of 15 retail dietary supplements also show that MRS-GSOT agar could efficiently isolate *L. reuteri* from retail dietary supplements with complex compositions of probiotic bacteria. In addition, we observe that *L. reuteri* exhibits two different colony morphologies on MRS-GSOT agar with 10 μg/mL oxacillin, yet they shared a common feature: a noticeable metallic (golden) sheen on the colony surface when the plate is slightly tilted, which can be used to distinguish them from non-*L. reuteri* species, such as *Lactiplantibacillus plantarum* subsp. *plantarum*, *Levilactobacillus brevis*, and *Bifidobacterium longum* subsp. *longum*. In conclusion, we have developed MRS-GSOT agar containing D-gluconic acid, peptone from soymeal, oxacillin, and 2,3,5-triphenyltetrazolium chloride for efficient isolation of *L. reuteri* from dietary supplements.**

Keywords: D-Gluconic acid, Oxacillin, Peptone from soymeal, 2,3,5-Triphenyltetrazolium chloride

1. Introduction

Limosilactobacillus reuteri was first isolated in 1962, named and proposed as *Lactobacillus reuteri* in 1980 [1], and reclassified to the genus *Limosilactobacillus* in 2020 [2]. *L. reuteri* is a lactic acid bacterium (LAB) that naturally colonizes the gastrointestinal tract of humans and animals, fluctuating in abundance among individuals and diversifying into host-adapted lineages over time [3–5]. *L. reuteri* has multiple probiotic properties that promote human health and attenuate various diseases. *L. reuteri* prevents gut colonization and microbial translocation of pathogens [6]. It produces broad-spectrum antimicrobial molecules such as reuterin [7], reutericin [8], and reutericyclin [9]. *L. reuteri* also produces health-promoting metabolites that remodel the composition of the commensal microbiota [10,11] and modulate the immune system [12] and enteric nervous system [13] in the gut. Other studies have shown that *L. reuteri* can promote dental health [14], protect against bone loss in postmenopausal women [15], and lower cholesterol levels in patients [16]. Until now, *L. reuteri* has been employed in diverse dietary supplements, food preservatives, and antibacterial additives for clinical purposes.

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In recent years, several reports have indicated *L. reuteri* can harbor plasmids with genetic determinants of antibiotic resistance [17−19] which is transferred to other species in the gastrointestinal tract [20,21]. These reports show that *L. reuteri* has the potential to transmit antibiotic resistance, and that the safety of *L. reuteri* in the food industry should be prioritized. For example, a commonly used strain DSM 17938 was obtained through the removal of plasmids carrying *tet(W)* tetracycline and *lnu(A)* lincosamide resistance determinants from the previous commercially available strain ATCC 55730 [22]. By monitoring the labeling of dietary supplements in Taiwan, we have noticed that, while only a few imported probiotic products claimed *L. reuteri* did not contain transferable resistance genes or that resistance genes affecting human health had been removed, most retail dietary supplements containing *L. reuteri* did not provide sufficient information about the safety of the *L. reuteri* strains used. Therefore, our laboratory has been committed to isolating *L. reuteri* strains from retail dietary supplements to evaluate the potential of these *L. reuteri* strains in transmitting antibiotic resistance.

In 2020, we collected 19 retail dietary supplements that listed *L. reuteri* as an ingredient. *L. reuteri* was recovered from only two of these products, one of which contained *L. reuteri* as the sole probiotic bacteria and the other had a simple composition, with *L. reuteri* as the predominant probiotic species. Most retail dietary supplements containing *L. reuteri* have complicated probiotic bacterial compositions, with *L. reuteri* not being the major species. It is extremely difficult to isolate *L. reuteri* from such products by de Man, Rogosa, and Sharpe agar (MRS agar), which is commonly used to enumerate lactobacilli. Previous studies provided the strategies for isolating certain probiotic strains of *L. reuteri* from fecal samples [23,24], including the pH modified MRS agar or a modified MRS agar containing vancomycin and sodium acetate [25,26]. We attempted to isolate *L. reuteri* from retail dietary supplements by such media, but *L. reuteri* was isolated from only few retail products owing to the media's poor selectivity for *L. reuteri*. Nonetheless, no studies have developed an approach for efficiently isolation of *L. reuteri* in the presence of multiple probiotic species.

The development of a new culture medium requires a better understanding of metabolism of the targeted bacteria. Carbon sources can serve as the main energy source for bacterial growth, and different carbon sources can have diverse effects on growth and probiotic functions in LAB [27,28]. A redox indicator, 2,3,5-triphenyl tetrazolium chloride (TTC), was applied to evaluate the ability of lactobacilli to ferment the tested sugars [29]. In addition, LAB mainly use amino acids and peptides to fulfill their need for complex nitrogen sources [30]. Each LAB strain responds differently to each protein source because of the uniqueness of the enzyme systems involved [31]. Moreover, previous studies on the antimicrobial susceptibility of *Lactobacillus* strains commercially available revealed that some species had resistance for oxacillin evaluated by disc diffusion [32,33]. The data of minimal inhibitory concentration (MIC) of oxacillin indicated that *L. reuteri* strains display significantly high level of resistance to oxacillin (MIC 256 μg/mL). However, low resistance towards this antibiotic has also been observed among other probiotic strains tested [34−36]. Owing to *L. reuteri* commonly demonstrating higher resistance to oxacillin compared with other probiotic species, the use of the appropriate concentration of oxacillin may provide selective pressure to inhibit the growth of species other than *L. reuteri*. In this study, we investigated the optimal components of carbon and nitrogen sources, as well as the appropriate dosage of oxacillin, to develop a selective culture medium (MRS-GSOT agar) that would specifically provide growth advantages for *L. reuteri*, and further apply it to isolate *L. reuteri* from retail dietary supplements with complex compositions of probiotic bacteria.

2. Materials and methods

2.1. Bacterial strains and growth conditions

Supplementary Table 1 [https://doi.org/10.38212/2224-6614.3507] lists the strains of *L. reuteri* and other probiotic species obtained from the Bioresource Collection and Research Center (BCRC, the Food Industry Research and Development Institute, Hsinchu, Taiwan). Ten strains of *L. reuteri* (TFDA-Lreu-01 to TFDA-Lreu-10) were isolated from retail dietary supplements before 2021. The strains of the genus *Lactobacillus*, *Levilactobacillus*, *Lacticaseibacillus*, *Limosilactobacillus*, *Lactiplantibacillus,* and *Ligilactobacillus* were grown on MRS agar (Difco Laboratories Inc., Detroit, MI, USA) and cultivated anaerobically at 37 °C for 24−48 h.

2.2. API 50 CHL system

API 50 CHL system (bioMérieux, Marcy l'Etoile, France) was used to determine carbohydrate fermentation according to the manufacturer's instructions. Based on the color change in the API strip reaction, which represents the fermentation of

the examined carbohydrates, the results were classified as positive, negative, or borderline.

2.3. Biolog-based microbial identification and metabolic profiling

Freshly grown colonies on a Biolog Universal Anaerobe agar plate (Biolog Inc., Hayward, CA, USA) were used for Biolog AN Microplates, read by the Biolog semi-automated system (Biolog Inc.) following the manufacturer's instructions. The Biolog AN Microplates were incubated anaerobically at 37 °C for 24 and 48 h before being read by the MicroLog™ plate reader and analyzed by Biolog Gen5 version 2.0 (Biolog Inc.). A color shift based on the redox dye chemistry indicated the growth and utilization of the tested carbon source.

2.4. Medium preparation

The composition of MRS-GSOT agar is listed in Table 1. All the ingredients, except D-gluconic acid, 2,3,5-triphenyl tetrazolium chloride (TTC), and oxacillin, were dissolved in deionized water to the volume of 800 mL prior to autoclaving at 121 °C for 15 min. Stock solutions of D-gluconic acid (0.1 g/mL) (Sigma–Aldrich, Burlington, MA, USA), TTC (30 mg/mL) (Sigma–Aldrich, St. Louis, MO, USA), and oxacillin (10 mg/mL) (Sigma–Aldrich, Burlington, MA, USA) were sterilized by 0.22 μm filtration. Immediately before dispensing the medium onto the plates, 200 mL of D-gluconic acid solution, 1 mL of TTC solution, and 1 mL of oxacillin solution were added. The MRS agar plates were prepared according to the manufacturer's instructions.

Table 1. Composition of MRS-GSOT agar.

Component	Amount per litre
Peptone from soymeal (papain-digested)	20 g
D-Gluconic acid[a]	20 g
Tween 80	1 mL
Dipotassium hydrogen phosphate	2.6 g
Sodium acetate	5.4 g
Ammonium citrate tribasic	2.3 g
Magnesium sulfate heptahydrate	0.25 g
Manganese (II) sulfate monohydrate	0.1 g
Bacto agar	15 g
2,3,5-Triphenyl tetrazolium chloride[a]	30 mg
Oxacillin sodium salt monohydrate[a]	10 mg

[a] The solutions of D-gluconic acid, 2,3,5-triphenyl tetrazolium chloride and oxacillin sodium salt monohydrate were filter-sterilized and added once the autoclaved agar medium was cooled to 50 °C.

2.5. Bacterial identification by matrix-assisted laser desorption ionization–time of flight mass spectrometry

Bacterial isolates were prepared for analysis using a formic acid-based method directly on the plate. Each single colony was spread onto a steel anchor plate for MALDI-TOF MS analysis (BigAnchor 96-well plate; Bruker Daltonics, Bremen, Germany). Each well received 1 μL of 70% formic acid (Fluka; Sigma–Aldrich) followed by 1 μL of matrix solution (α-cyano-4-hydroxycinnamic acid dissolved in a solution of 50% acetonitrile, 47.5% water, and 2.5% trifluoroacetic acid). After drying, samples were analyzed using MALDI-TOF MS Biotyper with FlexControl software (Bruker Daltonics). Calibration was performed with a bacterial test standard (Bruker Daltonics), and analysis parameters included a laser frequency of 60 Hz, MBT-auto-smart mode, and a voltage of 20 kV. Spectra within the range of 2000–20,000 *m/z* were collected and compared with the database for species identification.

2.6. Evaluation of the impact carbon and peptide sources on L. reuteri growth

The modified MRS agar was utilized for selecting carbon and peptide sources to facilitate the cultivation of *L. reuteri*, formulated without carbohydrates (glucose) and peptide sources (peptone, beef extract, and yeast extract). These were substituted with specified carbohydrate and peptide sources (final concentration: 0.02 mg/mL). Strains were streaked on this modified agar and cultivated anaerobically at 37 °C for 48 h. Species identification by MALDI-TOF MS was performed on colonies grown from a mixture of *L. reuteri* and non-*L. reuteri* strains or diluted retail products, spread on the modified MRS agar.

2.7. Oxacillin susceptibility test

The MIC for the tested strains was determined using the broth microdilution method with LAB susceptibility test medium (LSM), following ISO 10932/IDF 223 guidelines. The LSM consisted of 90% Iso-Sensitest broth (Oxoid Ltd., Hampshire, UK) and 10% MRS Broth (Difco Laboratories Inc., Detroit, MI, USA) [37]. Oxacillin was serially diluted twofold in distilled water to concentrations ranging from 512 to 0.25 μg/mL. Each well of a 96-well round-bottom culture plate received 50 μL of the antibiotic solution. Strains were inoculated into MRS broth, cultured anaerobically at 37 °C for 16 h, washed twice with 0.85% sterile NaCl solution, and adjusted to a bacterial suspension of approximately

6×10^5 CFU/mL. Fifty microliters of this suspension were added to each well, followed by anaerobic culture at 37 °C for 48 h. The MIC was determined as the lowest concentration of oxacillin preventing turbidity. Each test was performed in triplicate.

2.8. Efficiency of MRS-GSOT agar in selectively supporting L. reuteri growth

2.8.1. Recovery assay

For each strain, a bacterial suspension (approximately 1.5×10^3 CFU/mL) was prepared in PBS. One hundred microliters of the suspension were spread on MRS agar and MRS-GSOT agar plates supplemented with 4 or 10 μg/mL oxacillin, each in triplicate, then anaerobically cultured at 37 °C for 72 h. The recovery on MRS-GSOT agar was calculated as the ratio of colonies on MRS-GSOT agar to those on MRS agar.

2.8.2. Spiking test

A retail dietary supplement containing heat-inactivated *Lactiplantibacillus plantarum* subsp. *plantarum* was used as the matrix sample. Twenty-five grams of the sample were diluted with 0.1% peptone to 250 mL. Bacterial suspensions of *L. reuteri* BCRC14625^T, *L. plantarum* subsp. *plantarum* BCRC10069^T, *Limosilactobacillus fermentum* BCRC12190^T, and *Ligilactobacillus salivarius* BCRC14759^T were prepared in PBS and serially diluted. For single-strain testing, diluted matrix samples were spiked with the indicated strain at approximately 1.5×10^4 CFU/g. For mixed-strain testing, matrices were spiked with *L. reuteri* BCRC14625^T and each non-*L. reuteri* strain at four ratios (1:1, 1:10, 1:100, and 1:1000). Spiked solutions were plated on MRS-GSOT agar and incubated anaerobically at 37 °C for 72 h. Species identification of colonies was done using MALDI-TOF MS.

2.8.3. Selectivity

Overnight broth cultures of each strain were washed twice with sterile PBS and adjusted to 0.5 McFarland standard. A 1 μL loopful (approximately 1.5×10^3 CFU) of bacterial suspension was streaked on MRS-GSOT agar and anaerobically incubated at 37 °C for 72 h. Growth of each strain was then recorded.

2.9. Isolation of L. reuteri from retail products

Fifteen retail dietary supplements containing *L. reuteri* as an ingredient were collected from convenience stores, supermarkets, pharmacies, and electronic commerce (Supplementary Table 2 [https://doi.org/10.38212/2224-6614.3507]). Sample homogenates were prepared following ISO 15214:1998 guidelines [38]. The homogenate was serially diluted tenfold with buffered peptone water, and 100 μL of each diluted sample was spread on MRS agar and MRS-GSOT agar plates supplemented with 10 μg/mL oxacillin, then cultured anaerobically at 37 °C for 72 h. Species identification of colonies on plates with 25–250 colonies was performed using MALDI-TOF MS. The total count of probiotic bacteria in the products was determined based on growth on MRS agar.

2.10. Recoding of colony morphologies

Colonies on MRS-GSOT agar were photographed using a digital camera. Morphological features were recorded with a stereomicroscope (SMZ800, Nikon, Tokyo, Japan) equipped with a Dino-Eye eyepiece camera (Dino-Lite Europe, Almere). Covers were removed to prevent distortion during the recording process.

2.11. Statistical analysis

The experiments for recovery assays and spiking tests were conducted in triplicate and the data were presented as the mean ± standard deviation. One-way ANOVA, followed by Student's *t* test, was used to determine statistically significant differences. Differences were considered statistically significant when p-values were <0.05.

3. Results

3.1. Selection of carbon sources for L. reuteri

In the study, we initially investigated the utilization of various carbon sources. We used the API 50 CHL system and Biolog AN microplate for the strains of *L. reuteri* and 14 other probiotic species (Supplementary Table 1 [https://doi.org/10.38212/2224-6614.3507]). Our results demonstrated that at least four strains of *L. reuteri* exhibited positive reactions to L-arabinose, D-raffinose, potassium gluconate, D-melibiose, and D-gluconic acid, while other non-*L. reuteri* strains, except for *L. plantarum* subsp. *plantarum* BCRC10069^T and *Lacticaseibacillus rhamnosus* BCRC10940^T, predominantly exhibited negative reactions to these five carbon sources (Supplementary Table 3 [https://doi.org/10.38212/2224-6614.3507]). To assess the impact of these carbon sources on *L. reuteri* growth, we incorporated each single carbon source to replace D-glucose in MRS agar. The colonies of *L. reuteri* BCRC14625^T displayed a light red appearance with

a red core on modified MRS agar containing D-raffinose, D-melibiose, potassium gluconate, and D-gluconic acid, but not D-arabinose (Supplementary Fig. 1 [https://doi.org/10.38212/2224-6614.3507]). Among the *L. reuteri* strains tested, colony morphologies were similar on agar plates containing D-raffinose or D-gluconic acid but exhibited diversity on plates containing potassium gluconate or D-melibiose (Supplementary Fig. 2–5 [https://doi.org/10.38212/2224-6614.3507]). In addition, D-raffinose was considerably utilized in nine species, with *L. reuteri* and other species displaying similar colony sizes (Supplementary Table 4 [https://doi.org/10.38212/2224-6614.3507]). In contrast, only six species demonstrated notable metabolism of D-gluconic acid, with distinct colony sizes observed between *L. reuteri* and the other six species (Supplementary Table 5 [https://doi.org/10.38212/2224-6614.3507]). Moreover, a mixture of bacterial culture broth comprising type strains of *L. reuteri* and other species, with approximately equal bacterial counts (approximately 1.0×10^2 CFU/mL), was spread on a plate containing D-gluconic acid. Notably, the colonies of *L. reuteri* were larger than those of the other species (Supplementary Fig. 6 [https://doi.org/10.38212/2224-6614.3507]). In summary, our data indicated that substituting glucose in MRS agar with D-gluconic acid could confer growth advantages for *L. reuteri*.

3.2. Impact of peptide sources in L. reuteri and other species

We investigated the impact of peptide sources on promoting the proliferation of *L. reuteri* using modified MRS agar, supplemented with D-gluconic acid along with the peptide sources commonly used in bacterial culture media, such as peptone, tryptone, peptone from soymeal, yeast extract, proteose peptone no. 3, and beef extract. Both *L. reuteri* strains, BCRC14625^T and BCRC14691, exhibited smaller colony sizes on media containing peptone and tryptone but larger sizes on media supplemented with peptone from soymeal and yeast extract (Supplementary Fig. 7 [https://doi.org/10.38212/2224-6614.3507]). Additionally, notable differences in growth were observed between *L. reuteri* BCRC14625^T and BCRC14691 on media containing proteose peptone no. 3 and beef extract. To compare the effects of peptone from soymeal and yeast extract on the growth of *L. reuteri* and other probiotic species, we prepared a bacterial broth mixture containing equal numbers of *L. reuteri* BCRC14625^T and the type strain of six other probiotic species, which significantly utilized D-gluconic acid (Supplementary Table 5 [https://doi.org/10.38212/2224-6614.3507]). Both *L. reuteri* and *L. rhamnosus* displayed vigorous growth with similar morphologies on plates supplemented with yeast extract. In contrast, *L. reuteri* exhibited larger colony sizes compared with other species on plates containing peptone from soymeal (Supplementary Fig. 8 [https://doi.org/10.38212/2224-6614.3507]). Furthermore, we evaluated the impact of yeast extract and peptone from soymeal on selectively promoting *L. reuteri* proliferation using a retail dietary supplement containing *L. reuteri* and three other species. *L. reuteri* displayed significantly larger colony sizes on plates supplemented with peptone from soymeal, whereas smaller sizes and similar morphologies of other species were observed on plates supplemented with yeast extract (Supplementary Fig. 9 [https://doi.org/10.38212/2224-6614.3507]). These findings suggested that peptone from soymeal, as the sole peptide source in the medium, could offer growth advantages for *L. reuteri*.

3.3. Evaluation of oxacillin resistance in L. reuteri

We determined the MIC of oxacillin in the type strain of *L. reuteri* and in 14 other probiotic species (Fig. 1). Compared with the other probiotic species tested (MIC of oxacillin: 0.25–16 μg/mL), *L. reuteri* exhibited significantly higher resistance to oxacillin (MIC of oxacillin: 64 μg/mL). Additionally, marked resistance to oxacillin was observed in four other *L. reuteri* strains obtained from BCRC (MIC of oxacillin: 32–64 μg/mL), as well as in ten strains isolated from retail products collected before 2021 (MIC of oxacillin: 32–>256 μg/mL) (Supplementary Table 6 [https://doi.org/10.38212/2224-6614.3507]). These findings indicated that the tested *L. reuteri* strains commonly exhibit resistance to oxacillin.

3.4. Assessment of the optimal concentration of oxacillin for selecting the growth of L. reuteri

Our data revealed that *L. reuteri* exhibited higher resistance to oxacillin compared with other probiotic species, implying that an appropriate concentration of oxacillin might selectively inhibit the growth of non-*L. reuteri*. To ascertain the optimal oxacillin concentration for promoting the growth of *L. reuteri*, we inoculated pure cultures of the type strain of *L. reuteri* and 14 other species at approximately 1.5×10^2 CFU separately on MRS agar and MRS-GSOT agar supplemented with 4 or 10 μg/mL oxacillin (Table 2). Among the strains examined, only *L. reuteri*, *L. plantarum* subsp. *plantarum*, *L.*

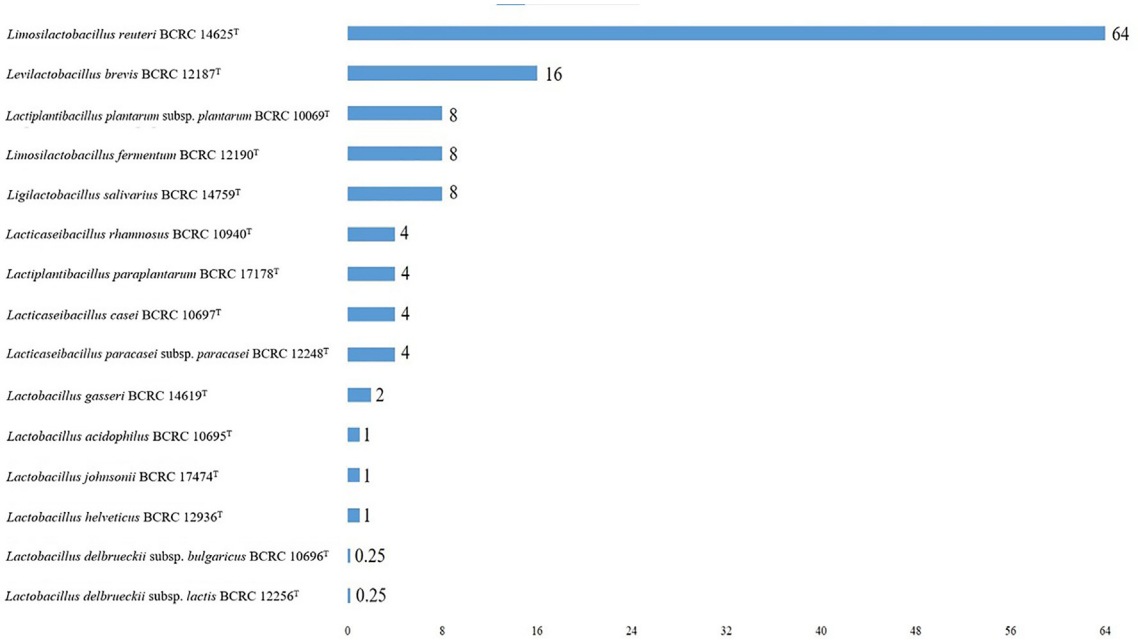

Fig. 1. The minimal inhibitory concentration (MIC) of oxacillin for the type strain of Limosilactobacillus reuteri and 14 other probiotic species commonly in retail dietary supplements.

salivarius, and *L. fermentum* showed growth on MRS-GSOT agar with 4 μg/mL oxacillin, while only *L. reuteri* could colonize on MRS-GSOT agar with 10 μg/mL oxacillin. On agar supplemented with 4 μg/mL oxacillin, the recovery rate of *L. reuteri* (over 85%) was significantly higher than that of *L. plantarum* subsp. *plantarum* (approximately 50%), *L. salivarius* (less than 50%), and *L. fermentum* (about 10%) ($p < 0.01$). On agar containing 10 μg/mL oxacillin, only *L. reuteri* survived, with a recovery rate exceeding 80%. There was no significant difference in the growth of *L. reuteri* across MRS agar, MRS-GSOT agar with 4 or 10 μg/mL oxacillin ($p = 0.71$), indicating that *L. reuteri* maintained

Table 2. The recovery of Limosilactobacillus reuteri and other 14 probiotic species on MRS agar and MRS-GSOT agar.

Species	Strain	MRS agar	MRS-GSOT agar			
		Counts (Log_{10} CFU/mL)	4 μg/mL oxacillin		10 μg/mL oxacillin	
			Counts (Log_{10} CFU/mL)	Recovery (%)	Counts (Log_{10} CFU/mL)	Recovery (%)
Limosilactobacillus reuteri	BCRC 14625^T	2.01 ± 0.05	1.95 ± 0.06	86.9 ± 2.2	1.92 ± 0.04	81.7 ± 1.4
Lactiplantibacillus plantarum subsp. *plantarum*	BCRC 10069^T	2.11 ± 0.04	1.86 ± 0.05	55.9 ± 2.4	NG[a]	0
Limosilactobacillus fermentum	BCRC 12190^T	2.02 ± 0.03	1.10 ± 0.13	11.9 ± 2.8	NG[a]	0
Ligilactobacillus salivarius	BCRC14759^T	2.27 ± 0.01	1.89 ± 0.01	41.2 ± 0.6	NG[a]	0
Lacticaseibacillus rhamnosus	BCRC 10940^T	2.01 ± 0.02	NG[a]	0	NG[a]	0
Lacticaseibacillus casei	BCRC 10697^T	2.09 ± 0.07	NG[a]	0	NG[a]	0
Lacticaseibacillus paracasei subsp. *paracasei*	BCRC12248^T	2.13 ± 0.03	NG[a]	0	NG[a]	0
Levilactobacillus brevis	BCRC12187^T	2.19 ± 0.03	NG[a]	0	NG[a]	0
Lactobacillus acidophilus	BCRC10695^T	2.03 ± 0.01	NG[a]	0	NG[a]	0
Lactobacillus gasseri	BCRC 14619^T	2.16 ± 0.02	NG[a]	0	NG[a]	0
Lactobacillus helveticus	BCRC12936^T	2.02 ± 0.01	NG[a]	0	NG[a]	0
Lactobacillus johnsonii	BCRC17474^T	2.22 ± 0.03	NG[a]	0	NG[a]	0
Lactiplantibacillus paraplantarum	BCRC17178^T	2.24 ± 0.08	NG[a]	0	NG[a]	0
Lactobacillus delbrueckii subsp. *bulgaricus*	BCRC10696^T	2.06 ± 0.00	NG[a]	0	NG[a]	0
Lactobacillus delbrueckii subsp. *lactis*	BCRC12256^T	2.31 ± 0.04	NG[a]	0	NG[a]	0

[a] NG: no growth.

comparable growth rates despite the presence of oxacillin. These findings suggested that 10 μg/mL oxacillin in MRS-GSOT agar could selectively inhibit the growth of non-*L. reuteri* strains.

3.5. Evaluation of MRS-GSOT agar by spiked retail dietary supplement

To evaluate the effectiveness of MRS-GSOT agar with 10 μg/mL oxacillin in selecting for growth between *L. reuteri* and other competing microbes in food matrices, we conducted spiking tests using a retail dietary supplement containing heat-inactivated *L. plantarum* subsp. *plantarum* as the food matrices. Bacterial suspensions of *L. reuteri* and three other species, previously reported to have low-level resistance to oxacillin and partially recovered on MRS-GSOT agar with 4 μg/mL oxacillin (Table 2), were spiked in four different ratios (1:1, 1:10, 1:100, 1:1000), as summarized in Table 3.

Initially, in single-strain tests (spiking at approximately 1.5×10^3 CFU/g) with food matrices, over 80% of *L. reuteri* had recovered, which demonstrated similar results to those of pure *L. reuteri* suspension (Table 2) ($p = 0.285$). However, *L. plantarum* subsp. *plantarum*, *L. salivarius*, and *L. fermentum* did not exhibit growth on MRS-GSOT agar with 10 μg/mL oxacillin. When *L. reuteri* and other three strains were spiked in equal proportions, the recovery of *L. reuteri* was significantly decreased compared with that in the single-strain test ($p = 0.02$) and was similar to that observed when spiking with ten-fold to 1000-fold amounts of each non-*L. reuteri* strains (p value: 0.09–0.25). Spiking at a 1:100 ratio of *L. reuteri* to non-*L. reuteri* strains resulted in numerous tiny colonies on MRS-GSOT agar, including *L. plantarum* subsp. *plantarum* colonies with diameters <2 mm (Supplementary Fig. 10A [https://doi.org/10.38212/2224-6614.3507]). Spiking a 1:1000 mixture of *L. reuteri* and non-*L. reuteri* strains resulted in a small amount (25–40 CFU/plate) of notably larger colonies of *L. plantarum* subsp. *plantarum* (1–3 mm) (Supplementary Fig. 10B [https://doi.org/10.38212/2224-6614.3507]). These results indicated that the presence of matrices did not impact *L. reuteri* recovery on MRS-GSOT agar, while the presence of other species significantly affected *L. reuteri* recovery. Nonetheless, MRS-GSOT agar provided notable growth advantages for *L. reuteri* in the presence of a large number of non-*L. reuteri* strains.

3.6. Selectivity of MRS-GSOT agar

To assess the discriminatory capability of MRS-GSOT agar with 10 μg/mL oxacillin between non-*L. reuteri* (specificity) and *L. reuteri* strains (sensitivity), we evaluated 15 strains of *L. reuteri* (including the type strain BCRC14625^T, four strains obtained from BCRC, and ten strains isolated from retail products before 2021) along with the type strain of 14 other species. Each strain was streaked on MRS-GSOT agar at approximately 1.5×10^3 CFU. We found that all strains of *L. reuteri* exhibited growth on MRS-GSOT agar. However, only few colonies (less than ten colonies) of *L. plantarum* subsp. *plantarum* and *L. salivarius* were visible. In brief, the sensitivity of MRS-GSOT agar in supporting the growth of *L. reuteri* reached 100% (positive in all 15 *L. reuteri* strains), with a specificity of 85.7% (negative in 12 out of 14 non-*L. reuteri* strains tested).

3.7. Isolation of L. reuteri in retail dietary supplements by MRS-GSOT agar

To evaluate the effectiveness of MRS-GSOT agar in isolating *L. reuteri* from dietary supplements, we analyzed 15 retail products, all with expiration dates more than six months prior. Among them, only two products contained no more than five species, while the remaining contained 7–19 probiotic species, with *L. reuteri* listed later in the ingredient list (third to sixteenth) (Supplementary Table 2 [https://doi.org/10.38212/2224-6614.3507]). Table 4 presented the isolation of *L. reuteri* and other species from MRS agar and MRS-GSOT agar with 10 μg/mL oxacillin, with colony morphologies of *L. reuteri* on MRS-GSOT agar depicted in Fig. 2 and Supplementary Fig. 11 [https://doi.org/10.38212/2224-6614.3507]. *L. reuteri* was detected on MRS-GSOT agar but not on MRS agar in all tested products, with *L. reuteri* counts notably lower than total bacterial counts in each product, by up to approximately 10^7 CFU/g. MRS-GSOT agar with 10 μg/mL oxacillin significantly inhibited the growth of non-*L. reuteri* compared with 4 μg/mL oxacillin (Fig. 2A and B). In five products, both *L. reuteri* and non-*L. reuteri* were detected on MRS-GSOT agar with 10 μg/mL oxacillin, including *Bifidobacterium longum* subsp. *longum* (products no. 1 and no. 13) (Fig. 2C and E), *L. brevis* (product no. 12) (Fig. 2D), and *L. plantarum* subsp. *plantarum* (products no. 14 and no. 15) (Fig. 2F and G).

Strains of *L. reuteri* isolated from 12 products displayed colonies measuring approximately 3 mm or larger, characterized by a central red gradient transitioning to an outer ring of pale pink or white hue. In contrast, strains from products numbered 6, 7, and 13 showed irregular, flat colonies around 1.0 mm in diameter, mostly red with a white periphery. Both colony variants exhibited a metallic (golden) sheen

Table 3. The load and colony counts of L. reuteri and non-*L. reuteri on MRS agar and MRS-GSOT agar.*

Spiking model	Spiking		MRS-GSOT agar (10 μg/mL oxacillin)[f]				
	L. reuteri (Log_{10} CFU/g)	non-*L. reuteri* (log_{10} CFU/g)	Total counts (Log_{10} CFU/g)	*L. reuteri* Counts (Log_{10} CFU/g)	*L. reuteri* Recovery (%)	non-*L. reuteri* Species identified	non-*L. reuteri* Counts (Log_{10} CFU/g)
L. reuteri BCRC14625^T	3.31 ± 0.03	–	3.23 ± 0.03	3.23 ± 0.03	82.6 ± 2.9	–	–
L. plantarum subsp. *plantarum* BCRC10069^T	–	3.10 ± 0.01	0	0	–	N.D.[g]	–
L. salivarius BCRC14759^T	–	3.10 ± 0.07	0	0	–	N.D.[g]	–
L. fermentum BCRC12190^T	–	3.24 ± 0.02	0	0	–	N.D.[g]	–
Mixed model A (1:1)[a]	3.31 ± 0.03	3.63 ± 0.03	3.31 ± 0.03	3.24 ± 0.02	68.4 ± 2.0	N.D.[g]	–
Mixed model B (1:10)[b]	3.31 ± 0.03	4.63 ± 0.03	3.24 ± 0.02	3.19 ± 0.05	70.4 ± 2.4	N.D.[g]	–
Mixed model C (1:100)[c]	3.31 ± 0.03	5.63 ± 0.03	TNTC[e]	3.13 ± 0.02	65.8 ± 7.6	*L. plantarum* subsp. *plantarum*	-[h]
Mixed model D (1:1000)[d]	3.31 ± 0.03	6.63 ± 0.03	TNTC[e]	3.11 ± 0.05	63.4 ± 2.9	*L. plantarum* subsp. *plantarum*	2.47 ± 0.09

[a] Spiking of 1.5×10^3 CFU/g for each strain tested.
[b] 10-fold spiking of each non-*L. reuteri* strain compared to that of *L. reuteri* BCRC14625^T.
[c] 100-fold spiking of each non-*L. reuteri* strain compared to that of *L. reuteri* BCRC14625^T.
[d] 1000-fold spiking of each non-*L. reuteri* strain compared to that of *L. reuteri* BCRC14625^T.
[e] TNTC: too numerous to count.
[f] Species identification for the colonies larger than 0.5 mm in diameter.
[g] N.D.: non-detected.
[h] In triplicate, two colonies of *L. plantarum* subsp. *plantarum* were identified on only one plate.

Table 4. Viable counts of L. reuteri and other probiotic species on MRS-GSOT agar with 10 μg/mL oxacillin.

Product No.	MRS agar		MRS-GSOT agar with 10 μg/mL oxacillin
	Species identified	Total counts (Log_{10} CFU/g)	Species identified (Log_{10} CFU/g)
1	*Bacillus coagulans*, *Bifidobacterium animalis* subsp. *lactis*, *L. rhamnosus*, *L. fermentum*, *Bifidobacterium breve*, *L. casei*,	9.11	*L. reuteri* (2.48) *Bifidobacterium longum* subsp. *longum* (5.20)
2	*B. animalis* subsp. *lactis*, *L. plantarum* subsp. *plantarum*	8.79	*L. reuteri* (2.95)
3	*B. animalis* subsp. *lactis*, *L. fermentum*, *B. coagulans*, *L. rhamnosus*, *L. plantarum* subsp. *plantarum*, *L. paracasei* subsp. *paracasei*	7.94	*L. reuteri* (4.49)
4	*B. coagulans*, *B. animalis* subsp. *lactis*, *L. plantarum* subsp. *plantarum*	9.04	*L. reuteri* (2.60)
5	*L. plantarum* subsp. *plantarum*, *L. fermentum*, *L. paracasei* subsp. *paracasei*, *B. animalis* subsp. *lactis*, *L. rhamnosus*, *L. acidophilus*, *B. coagulans*	10.76	*L. reuteri* (5.69)
6	*L. gasseri*, *L. rhamnosus*, *L. acidophilus*, *L. plantarum* subsp. *plantarum*	10.92	*L. reuteri* (5.98)
7	*L. salivarius*, *L. rhamnosus*, *L. reuteri*, *L. plantarum* subsp. *plantarum*	9.11	*L. reuteri* (3.68)
8	*Pediococcus acidilaciti*, *L. plantarum* subsp. *plantarum*, *L. fermentum*, *L. paracasei* subsp. *paracasei*, *L. brevis*, *B. animalis* subsp. *lactis*, *Bifidobacterium longum* subsp. *longum*	8.63	*L. reuteri* (5.58)
9	*L. fermentum*, *L. plantarum* subsp. *plantarum*, *L. rhamnosus*, *B. animalis* subsp. *lactis*, *B. breve*, *B. longum* subsp. *longum*, *B. coagulans*	9.11	*L. reuteri* (5.08)
10	*L. fermentum*, *B. animalis* subsp. *lactis*, *B. coagulans*, *L. paracasei* subsp. *paracasei*	8.08	*L. reuteri* (5.40)

(*continued on next page*)

Table 4. (continued)

Product No.	MRS agar		MRS-GSOT agar with 10 μg/mL oxacillin
	Species identified	Total counts (Log_{10} CFU/g)	Species identified (Log_{10} CFU/g)
11	*L. fermentum*, *L. rhamnosus*, *B. animalis* subsp. *lactis*, *L. plantarum* subsp. *plantarum*, *B. coagulans*, *L. paracasei* subsp. *paracasei*	9.70	*L. reuteri* (5.30)
12	*L. plantarum* subsp. *plantarum*, *B. animalis* subsp. *lactis*, *L. brevis*, *Streptococcus thermophilus*	10.41	*L. reuteri* (8.64) *L. brevis* (11.30)
13	*L. paracasei* subsp. *paracasei*, *B. animalis* subsp. *lactis*, *L. acidophilus*	8.43	*L. reuteri* (4.61) *B. longum* subsp. *longum* (4.43)
14	*L. plantarum* subsp. *plantarum*, *L. rhamnosus*, *L. casei*, *L. acidophilus*, *B. animalis* subsp. *lactis*	9.97	*L. reuteri* (3.00) *L. plantarum* subsp. *plantarum* (4.32)
15	*L. plantarum* subsp. *plantarum*, *P. acidilactici*	9.52	*L. reuteri* (2.60) *L. plantarum* subsp. *plantarum* (3.23)

when the MRS-GSOT agar medium was tilted (Fig. 2 and Supplementary Fig. 11 [https://doi.org/10.38212/2224-6614.3507]). Notably, previously isolated *L. reuteri* strains from retail products showed similar morphological traits (Supplementary Fig. 12 [https://doi.org/10.38212/2224-6614.3507]). Conversely, colonies of *B. longum* subsp. *longum* and *L. brevis* were round and had diameters of 0.5−1 mm and around 2 mm, respectively, with raised formations displaying a metallic sheen (Fig. 2C−E). Additionally, colonies of *L. plantarum* subsp. *plantarum* were circular, measuring 1−3 mm in diameter, featuring a dull surface texture without a metallic sheen, but showing either clear or no reflection, allowing for differentiation from *L. reuteri* (Fig. 2F and G). Upon microscopic examination, colonies of *L. plantarum* subsp. *plantarum* had less defined edges, with some colonies displaying a fragmented structure (Fig. 2H). In summary, the application of MRS-GSOT agar could effectively enhance the isolation of *L. reuteri* in complex probiotic formulations, and the distinct colony morphologies of *L. reuteri* could facilitate differentiation from other species commonly found in retail dietary supplements.

4. Discussion

In this study, we developed a selective medium, MRS-GSOT agar, to specifically support the growth of *L. reuteri* by modifying the MRS agar formula. By incorporating oxacillin at appropriate concentrations, we applied selective pressure on other species commonly found in retail dietary supplements. Meanwhile, D-gluconic acid and peptone from soymeal served as the sole carbon and peptide sources, providing growth advantages for *L. reuteri*. The data of retail dietary supplements demonstrated the optimal performance of MRS-GSOT agar in isolating *L. reuteri* from the diverse and complex composition of probiotic bacteria. Our findings support the development of a selective medium tailored to enhance the growth of specific probiotic species, which could be extended to other species typically challenging to isolate from probiotic products.

Lactobacilli, known for their demanding growth requirements, are traditionally cultured in complex media like MRS media, which are rich in proteins and have a high buffering capacity, ideal for optimal cell growth [39]. Modifying the ingredients of MRS media, such as carbon sources, nitrogen sources, or ion environments, is common for studying lactobacilli metabolic traits and developing tailored selective culture media. Tabasco et al. replaced glucose and meat extract in MRS media with fructose to create an antibiotic-free medium promoting growth of *L. delbrueckii* subsp. *bulgaricus* in fermented milk [40]. In a 2010 study, researchers replaced D-glucose with L-rhamnose, adjusted ion composition, and added vancomycin and metronidazole, resulting in the M-RTLV medium which distinguishes *L. casei*, *L. paracasei* subsp. *paracasei*, and *L. rhamnosus* [41]. In 2015, Lena et al. proposed the MMV medium, which removed glucose and meat extract, added maltose and vancomycin, and selectively promoted the growth of bacteria from the *L. casei* group in dairy products [42]. Modified MRS media have been used in various studies on *L. reuteri* strains, exploring metabolic pathways, enhancing biomass, and increasing production of functional food ingredients and antimicrobial substances [35,43−45]. However, few studies have focused on applying modified MRS media to develop selective culture media

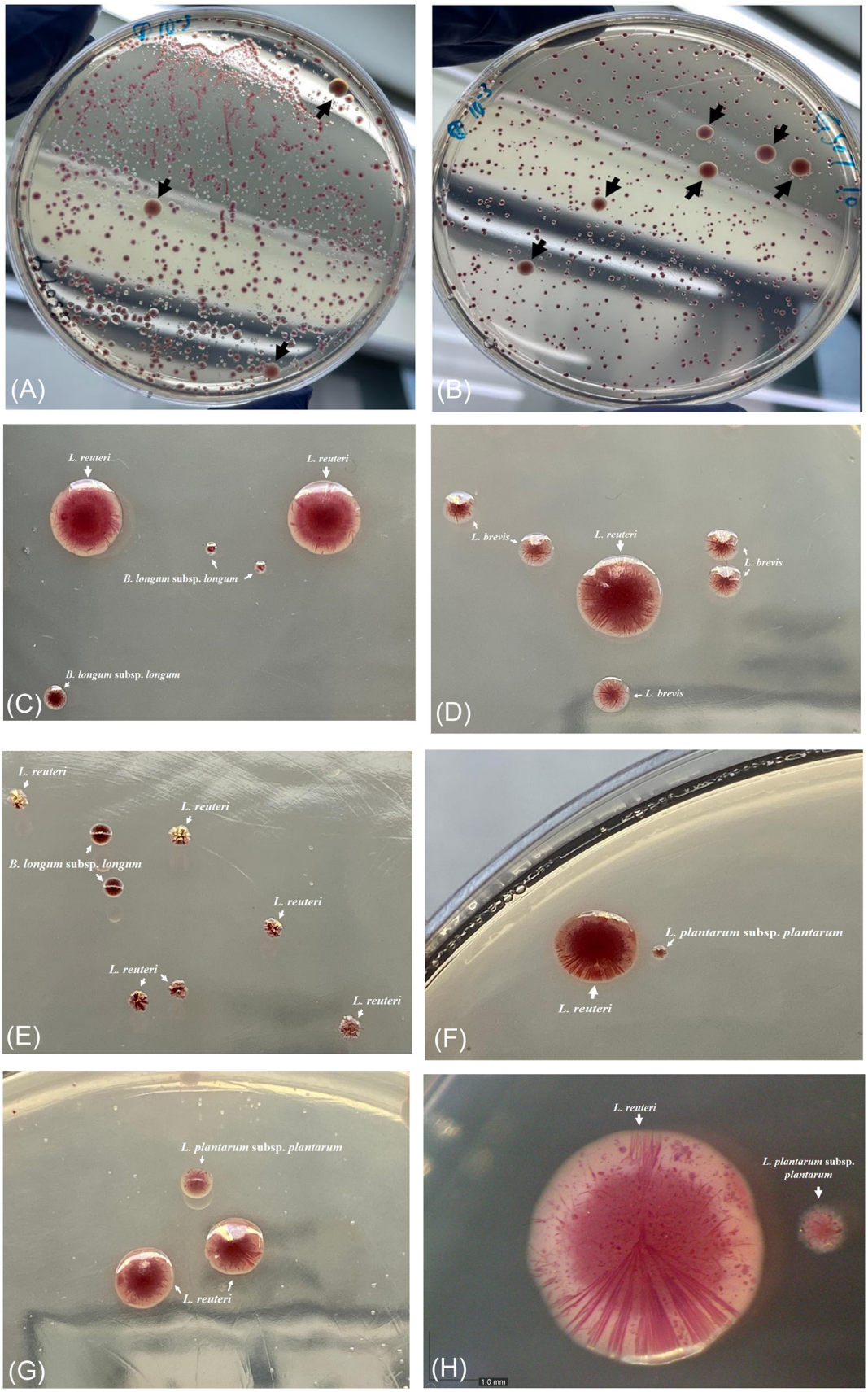

Fig. 2. ***Colonies grown from the diluted solution of retail dietary supplements grown on MRS-GSOT agar.*** *Colonies grown from product no.1 on MRS-GSOT agar supplemented with 4 μg/mL oxacillin (A) or 10 μg/mL oxacillin (B) L. reuteri was marked by black arrow. Colonies grown from product no. 1, no. 12, no. 13, no. 14 and no. 15 on MRS-GSOT agar with 10 μg/mL oxacillin were displayed in order from (C) to (G). (H) Colony morphologies of L. reuteri and L. plantarum* subsp. *plantarum from product no. 14 were observed under a stereomicroscope. Colonies of L. reuteri and L. plantarum* subsp. *plantarum were marked by white arrows, separately.*

specifically for *L. reuteri*. Therefore, this study aims to explore growth variations between *L. reuteri* and other lactobacilli strains by altering carbon and nitrogen sources in MRS media. The objective is to identify carbon and nitrogen sources that give *L. reuteri* a competitive edge, laying the foundation for developing tailored selective culture media for *L. reuteri*.

Given that D-glucose in MRS agar is utilized by nearly all LAB, it was crucial to identify carbohydrate sources advantageous for *L. reuteri* growth to develop a selective medium. D-Gluconic acid, known for its prebiotic properties and its stimulation of butyrate production, particularly by *L. reuteri* [44], emerged as a potential substitute for glucose. By employing commercially available kits, we identified D-gluconic acid as a promising single carbohydrate substitute. Additionally, prior studies showed that phytone peptone, derived from soybean meal/flour, significantly enhanced *L. reuteri* growth and density [46]. Ayad et al. [47] and Shi et al. [43] identified soymeal and phytone peptone as superior protein and peptide sources, respectively. Substituting peptone from soymeal with commercial phytone peptone in MRS-GSOT agar revealed colony morphology similarities (data not shown), suggesting the potential of phytone peptone in differentiating *L. reuteri* colonies from other probiotic species.

Acquired antibiotic resistance raises safety concerns regarding probiotic bacteria, while intrinsic resistance offers survival advantages and aids in establishing selective culture media. In lactobacilli, intrinsic resistance to specific antibiotics has been observed [48], but studies on intrinsic resistance in probiotic species are relatively limited. Conversely, varying degrees of antibiotic resistance have been reported in LAB strains, including lactobacilli resistance to β-lactam antibiotics due to the presence of β-lactamase. Certain food-isolated strains of *L. plantarum* subsp. *plantarum*, *L. rhamnosus*, *L. paracasei* subsp. *paracasei*, *L. fermentum*, and *Latilactobacillus curvatus* exhibit resistance, or low-level resistance, to oxacillin [32,36,49–52]. Various methods such as disc diffusion, E test, and dilution methods are commonly used to assess antibiotic resistance in lactobacilli, albeit with varying testing conditions [48]. We adopted the ISO method to evaluate oxacillin tolerance among lactobacilli, including *L. reuteri* and 14 other species commonly found in dietary supplements in Taiwan. The results showed varying levels of oxacillin resistance between *L. reuteri* and non-*L. reuteri* strains, indicating the potential of oxacillin to inhibit the growth of non-*L. reuteri*.

Antibiotics exert selective pressure on microorganisms with low susceptibility, as demonstrated by studies showing higher MICs of oxacillin in *L. reuteri* compared to other probiotic species [33–35,45]. Our study found varying MIC values of oxacillin among *L. reuteri* isolates from retail products (MIC: 32–>256 μg/mL) and observed two distinct colony morphologies. Isolates TFDA-Lreu-06, 07, and 09 displaying significantly high MICs (>256 μg/mL) and large, round colonies (>3 mm). Additionally, on MRS agar supplemented with TTC and oxacillin, we noted a gradual decrease in colony size of *L. reuteri* BCRC14625^T with increasing oxacillin concentration (Supplementary Fig. 13 [https://doi.org/10.38212/2224-6614.3507]). We speculate that colony morphologies on MRS-GSOT agar may be linked to oxacillin tolerance. Future research will employ scanning electron microscopy to examine colony surfaces and high-throughput sequencing to analyze metabolic characteristics, aiming to uncover factors contributing to distinct colony morphologies of *L. reuteri* on MRS-GSOT agar.

Our study focused on 14 non-*L. reuteri* species commonly found in retail dietary supplements. Examination of these supplements revealed that *B. longum* subsp. *longum* could thrive on MRS-GSOT agar, suggesting the importance of targeting *Bifidobacterium* strains for refining MRS-GSOT agar. Notably, while all products contained *L. plantarum* subsp. *plantarum*, growth of this species on MRS-GSOT agar was observed in only two products. Furthermore, understanding mechanisms of resistance, such as cell wall impermeability and presence of multidrug transporters, is crucial for elucidating strain-specific differences within species [48,53–56]. Thus, further analysis of non-*L. reuteri* strains isolated from retail products in this study will provide a foundation for refining MRS-GSOT agar.

5. Conclusion

We developed a novel selective medium, MRS-GSOT agar, containing D-gluconic acid as the carbon source, peptone from soymeal as the nitrogen source, 10 μg/mL oxacillin to provide selective pressure, and TTC as a color indicator. On MRS-GSOT agar, *L. reuteri* presented unique morphologies compared with the other LAB tested. This study provides an efficient method for isolating *L. reuteri* from a matrix with a complicated composition of probiotic bacteria. Our findings provide a route for developing a selective medium designed to facilitate the specific growth of certain probiotic species that are commonly challenging to isolate

from retail products. Further analysis investigating *L. reuteri* and non-*L. reuteri* strains isolated from retail products in this study will serve as the basis for optimizations of MRS-GSOT agar.

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Conflict of interest

None.

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