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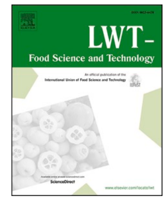

(Meta)genomics -assisted screening of novel antibacterial lactic acid bacteria strains from traditional fermented milk from Western China and their bioprotective effects on cheese

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A B S T R A C T

In this study, the bacterial composition of five traditional fermented milk samples from western China was evaluated by 16S metagenomic analysis. Forty lactic acid bacteria (LAB) strains were isolated from each sample and their antilisterial activity was tested. The inter-strain genetic diversity of the LAB isolated was investigated by randomly amplified polymorphic DNA (RAPD) analysis with reference to their antilisterial activity. The antimicrobial potential of selected LAB strains was studied by whole genome sequencing and mining, which resulted in the identification of several novel biosynthetic gene clusters (BGCs). *Lactiplantibacillus plantarum* TXZ2-35, *Limosilactobacillus fermentum* TZ-22 and *Companilactobacillus crustorum* QHS-4, carrying novel BGCs, were used as bio-protective adjunct cultures in Cheddar cheese. These three strains were able to control the proliferation of *Listeria monocytogenes* in cheese and some strains could improve cheese quality in terms of texture and sensory quality. In summary, we applied metagenomic and genomic approaches to quickly identify novel antilisterial LAB strains from traditional fermented food. Our results demonstrate the effectiveness of incorporating novel bioprotective adjunct cultures in improving the safety and quality of cheese.

1. Introduction

Fermented milk is an essential part of diets in various parts of the world. Various traditional and industrialized fermented milk products are produced worldwide, which are described by more than 400 generic names (Papadopoulou et al., 2019). Traditional fermented milk products are different in terms of milk type used, treatment of the milk, fermentation process and subsequent processing of the product (Zamfir et al., 2006). Western China has a large-scale livestock breeding activity and a convention of making fermented milk or variants thereof through natural fermentation (Liu et al., 2020). Traditional fermented milk has been consumed by local residents as a popular part of the diet for hundreds of years (Zhao et al., 2019).

Traditional fermentation techniques did not use any commercial starter cultures. The acidification and gelification depended on the native microbiota present in milk, as well as the way of inoculation (Jiang et al., 2020). The main principle in the production of concentrated fermented milk is to remove whey until the total solid in the product reaches a desired level (Hahn et al., 2012). Traditional processing techniques usually apply a special cloth bag to coagulate the fermented milk and remove whey and water content, so that the semi-solid variant can be obtained. Then the semi-solid fermented milk is further formulated into a dried yogurt variant by air drying. These procedures may contribute significantly to the composition of microorganisms in the final product (Mo et al., 2019). However, very little work has been done on deciphering the effects of the natural concentration process on the microbial composition during traditional milk fermentation.

At present, *Lactobacillus*, *Lactococcus*, *Leuconostoc*, *Pediococcus*, *Enterococcus*, and *Bifidobacterium* are among those bacteria which are

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associated with fermented milk (Ghosh et al., 2019). Some strains of these species have been highlighted as a reliable source of antimicrobial strains, so they deserve to be further characterized and exploited. Pathogenic *Listeria monocytogenes* strains have been frequently found in dairy products including soft, semi-soft, and hard cheeses, and in milk-processing plants as well. LAB strains with antilisterial activity can be used as adjunct cultures in fermented milk during storage to improve microbial safety (Panebianco et al., 2021). Previously, LAB strains with bio-protective activity against *L. monocytogenes* have been reported by various researchers, which include *Enterococcus faecium* CRL1879 from Argentina artisanal cheeses (Suarez et al., 2020), *Lactococcus lactis* subsp. lactis LABW4 from Indian fermented milk (Barman et al., 2014), and *Lactobacillus plantarum* 1QB77 from Brazilian artisanal cheeses.

In this study, we carried out a systematic study of five fermented milk samples, from Xinjiang (TZ, WXZ1, WXZ2, TXZ2) and Qinghai (QHS), by exhaustive culture-dependent and culture-independent analysis to describe their microbial composition. Randomly amplified polymorphic DNA (RAPD) analysis was used to examine the clonal identity, after which the phylogenetic distance was further calculated at the strain level among the same species based on RAPD-PCR. The bio-protective ability of a large set of LAB strains isolated was also evaluated. Finally, the bacteriocins and secondary metabolites were mined on basis of whole genome sequences of selected strains. Three strains with strong antibacterial activity were selected as the initial culture of cheese making to verify their inhibitory effect on *Listeria* and evaluate their effects on the texture and flavor of the cheese.

2. Materials and methods

2.1. Sample collection

Samples designated as TZ, WXZ1, WXZ2, TXZ2 were purchased from a local market in Xinjiang province, and QHS was purchased from Qinghai province. The milk was fermented according to traditional techniques using raw bovine milk without addition of any starter cultures. TZ and QHS were un-concentrated fermented milk. WXZ1 and WXZ2 samples were strained to a creamy status. TXZ2 were most concentrated to a semi-dry status. The samples were directly transferred into a sterilized jar and shipped to the laboratory at 4 °C. Each sample was then divided into two factions, one was prepared for total genomic DNA extraction, and another fraction was used to isolate LAB strains.

2.2. Total DNA isolation from fermented milk samples and metagenomic analysis

About 2 g samples were used for total genomic DNA extraction using CTAB method. DNA concentration and purity was measured on 1% agarose gels. The V4 region of 16S rRNA gene was amplified using specific primer pair 515F/806R with the barcode. All PCR reactions were carried out with 15 μL of Phusion® High-Fidelity PCR Master Mix (New England Biolabs). The PCR products were purified with Qiagen Gel Extraction Kit (Qiagen, Germany).

Sequencing of the 16S rDNA amplicon was performed at Novogene (Tianjin, China) with an Illumina NovaSeq platform and 250 bp paired-end reads were generated. Sequences were trimmed to remove barcodes/primers and paired-end reads were merged by FLASH (Magoč and Salzberg, 2011). Quality filtration were performed under specific conditions according to the QIIME (V1.9.1) quality-controlled process (Caporaso et al., 2010). Unique sequences were then analyzed by Uparse (V7.0.1001) (Edgar, 2013) with 97% similarity after removing the chimeric sequences. Representative sequence for each OTU was screened for further annotation using Silva database (Quast et al., 2012). OTUs abundance information was normalized using a standard of sequence number corresponding to the sample with the least sequences. Alpha Diversity was calculated using Qiime (Version 1.7.0, http://qiime.org/) to reflect the richness and diversity of fermented milk samples.

2.3. LAB isolation and identification

About 1 g of the solid sample TXZ2 was homogenized in 2 mL sterilizede saline (0.85%). The semi-solid samples TZ, WXZ1, WXZ2, and QHS were directly used for serial dilution. Then, samples were serially diluted with sterile saline (0.85%) and 0.1 mL of each diluted suspensions were spread on De Man, Rogosa and Sharpe (MRS) agar plates. After incubation at 30 °C for 48 h, single colonies were re-streaked on MRS agar plates twice and the purified strains were stored in 15% (w/v) glycerol at −80 °C.

For bacterial identification, the LAB isolates were added into MRS broth for overnight growth at 30 °C and bacterial cells were harvested. Pellets were suspended in TE buffer with lysozyme and further homogenized in a JXFSTPRP-CLN-24 automatic homogenizer (Shanghai Jingxin Technology Ltd., China). DNA was extracted from the lysate by phenol-chloroform treatment and recovered by isopropanol precipitation. The 16S rRNA gene was amplified from the chromosomal DNA by PCR with primers 27F (5′-3′: AGAGTTTGATCCTGGCTCAG) and 1492R (5′-3′: CGGTTACCTTGTTACGACTT). The PCR product was gel-purified and sequenced on both strands (Shenggong Biotechnological Ltd., China). The obtained nucleotide sequence was then used for an NCBI BLAST to perform the similarity search.

2.4. Antilisterial assay

Antibacterial activity of LAB strains against *Listeria monocytogenes* 19115 was checked by agar well diffusion method (Barman et al., 2014). Briefly, *L. monocytogenes* cells were mixed with Luria-Bertani (LB) agar media (pre-cooled to around 55 °C) at a final concentration of 1×10^6 cfu/mL. Then the mixed media was poured into Petri dishes to obtain pathogen-fusion agar plates. LAB strain growing in MRS after 30 °C for 48h was centrifuged at 10,000×*g* for 10 min to collect the cell free supernatant, the pH of which was then adjusted to 7 for further analyze. Fifty microliter cell free supernatant of each strain was added to the wells of nutrient agar plates containing *L. monocytogenes*. Uninoculated MRS broth (pH 7) was used as control. All the plates were incubated at 37 °C for 24 h, following by measurment of the clear halo surrounding each single well.

2.5. RAPD-PCR analysis

The RAPD (Random Amplified Polymorphic DNA) was carried out according to previously described method (Dong et al., 2017). RAPD-PCR reactions were performed with oligonucleotide primers M13 (5′-3′: GAGGGTGGCGGTTCT) (Ruiz et al., 2008). The 25 μL total reaction volume contains 12.5 μL of 2 × Rapid Tap Master Mix (Nanjing Vazyme Biotech Co., Ltd.), 2 μL of random primer (10 μM), 9.5 μL water and 1 μL of bacterial DNA template (10 ng). Thermal cycling consisted of initial denaturation at 95 °C for 5 min; 30 cycles: 94 °C/30 s, 42 °C/30 s and 72 °C/2 min; a final extension step of 72 °C/10 min was performed.

Amplified products were resolved by electrophoresis (100 V for 2 h) on 1% (w/vol) agarose in 1 × TAE buffer gels. Trans2K® DNA Marker was used as a DNA molecular weight marker and as a normalization reference. The obtained images from gel were subjected to Molecular Evolutionary Genetics Analysis software (MEGA) for further analyzing and the tree diagram was generated (Santos et al., 2019).

2.6. Whole genome sequencing and mining for antimicrobial gene clusters

Whole-genome sequencing (WGS) of isolated LAB strains was performed at the Beijing Novogene Bioinformatics Technology Co., Ltd. Briefly, purified genomic DNA was used as input material for the DNA library preparations. NEBNext® DNA Library Prep Master Mix Set for Illumina® (NEB, USA) was used to prepare sequencing libraries

following the manufacturer's recommendations. The library preparations were sequenced using the Illumina PE150 platform and 150 bp paired-end reads were generated. De novo assembly was performed using Velvet (Zerbino and Birney, 2008). Prediction of protein-encoding regions and automatic functional annotation was performed using the Rapid Annotations using Subsystem Technology (RAST) server (Aziz et al., 2008). Bacteriocins and secondary metabolites were predicted by BAGEL4 (van Heel et al., 2018) and antiSMASH (Blin et al., 2021).

2.7. Manufacture of cheddar cheese

One litre of raw milk (purchased from local dairy market) was heated at 70 °C for 15 min to eliminate possible contaminants. Milk was cooled to 35 °C and 100 μL commercial starter (*Streptococcus thermophilus*) was added (about 10^9 cfu/mL) and mixed by stirring (Suarez et al., 2020). Ten milliliter (20 g/L) $CaCl_2$ and 900 μL rennet was slowly added to milk while stirring. Then, selected antilisterial LAB strains were added as adjunct culture (Margalho et al., 2021). Different combinations of cultures were used, blank control, no additional inoculation of adjunct culture and *L. monocytogenes*; negative control, inoculate 20 μL *L. monocytogenes* at 10^8 cfu/mL; test group 1, inoculate 200 μL LAB at 10^9 cfu/mL; test group 2, inoculate 200 μL LAB and 20 μL *L. monocytogenes* (Fig. 5A). After strains were inoculated, samples were standing for 40 min to curd the milk. Curds were cut and stirred slowly (stir for 20s in every 10 min) and leave it for 30 min. The curds and whey were further separated by heating to 43 °C. Curds were then filtered with a filter cloth and the whey were discarded. The cheese wrapped in filter cloth are left to drain for 48 h in a cheese draining box with a heavy weight on top. After 48 h, a certain amount of sterile NaCl solution with a concentration of 250 g/L was added and soaked for 30 min. Drain, wrap the cheese with filter cloth and put it in the cheese box. Mature at 4 °C for 30 days.

2.8. Effects of LAB strains on listerial survivor in cheddar cheese

In order to test the antibacterial performance of isolated and screened lactic acid bacteria against *L. monocytogenes*, samples were collected and measured on the 0, 10, 20 and 30 days respectively. 0.10 ± 0.05 g cheese was dissolved in sterile water and diluted in each group, and then coated on MRS plate (For LAB counting, 37 °C, 48 h) and PALCAM plate (For *L. monocytogenes* counting, 37 °C, 48 h).

2.9. Effects of LAB strains on quality of cheddar cheese

The blank control group was sampled and measured on the 30th day, and the cheese was placed at room temperature for 2 h before TPA analysis. TPA was tested using the P5 probe of Texture analyzer (Stable Micro Systems). The pre-test speed, test speed and post-test speed were all set to 1.0 mm/s 30% compression ratio, 5 s compression time and 5g trigger force were applied, and the whole test process was compressed twice (Chavhan et al., 2014).

As sensory evaluation of cheese, the sensory analysis is carried out by trained evaluators after 30 days of maturity. The evaluator is composed of 10 members of the College of Food Science and Engineering (NWAFU) and provides them with relevant descriptive terms (Table S1). Each sample was served at 4 °C in cups with a clean spoon. Cheese samples were evaluated based on color (whiteness, creamy, surface glossiness), basic flavor (milk aroma, musty, acidic) and texture (under-rind consistency, section flatness, core hardness). The quantitative descriptive analysis was conducted based on a 10-point scale ranging from poor (1–4), medium (5–7) and strong (8-10) (Gebreyowhans et al., 2020; Rakhmanova et al., 2021).

3. Results

3.1. Microbial composition of traditional fermented milk

In this study, high-throughput sequencing of the V4 region of the 16S rRNA gene was used to analyze the microbial diversity of Xinjiang traditionally fermented yogurt. As shown in Fig. 1A, more than 1,000,000 raw reads were obtained for each sample. The raw reads were trimmed. After trimming, more than 80% of the raw reads with good quality (qualified reads) were used further analysis.

The un-concentrated yogurt sample QHS and TZ shows higher OTUs and microbial diversity. The microbial diversity was quantified using pelou_e and Shannon indices, which suggested that the concentration process reduced the microbial diversity of fermented milk (Fig. 1A). Fig. 1B shows the relative abundances of bacteria in 5 fermented milk samples at genus level. The microbial composition of sample QHS was most different from other samples, with *Streptococcus* genus constituting 32% of total bacterial abundance, indicating that the geographic location and environment have the most impact on bacterial diversity.

For samples from the same origin (TZ, WXZ1, WXZ2, TXZ2), the concentrating process increased the enrichment of *Lactobacillus* in the total OTUs (Fig. 1B). The functional genes of microbes and their metabolic pathways in the fermented milk were predicted by PICRUSt (Langille et al., 2013). COG profiles of predicted genes were analyzed to evaluate the major functions of the fermented milk microbial community (Fig. 1C). These results reveals that the microbiota in sample QHS has distinct metabolic patterns compared with other samples, particularly in those associated with ABC-transporters and metabolisms of amino acid (Fig. 1C).

3.2. Isolation and identification of antilisterial LAB strains

To isolate antilisterial LAB strains, forty colonies were randomly picked for each sample from MRS agar plates. All the strains were tested for Gram-positive and catalase-negative bacteria. Nucleotide sequence comparison based on 16S ribosomal RNA gene identified nine different species of LAB. In accordance with the meta-genomic results, the concentration process increased the diversity of isolated LAB, with the only exception of sample WXZ1 (Fig. 2A). Remarkably, *Companilactobacillus crustorum* was identified in all samples and dominates samples QHS and WXZ1. The anti-*Listeria* activity of the 200 isolated LAB strains was tested. Strains that displayed strong antagonist activity were found in samples QHS, TZ, WXZ2, and TXZ2 (Fig. 2B).

3.3. RAPD-PCR analysis

The electrophoretic patterns for 200 LAB strains were analyzed to provide DNA fingerprints for each strain and genetic distances between strains based on RAPD-PCR (Fig. 3). The DNA fingerprint produced from RAPD-PCR distinguishes all strains tested. The results were also able to distinguish strains isolated from different samples and exhibiting various levels of antilisterial activity. The results show the ability of the RAPD method to identify LAB isolated and purified from traditional fermented milk (Fig. S1).

Genetic diversity among strains was determined with banding patterns from the RAPD reaction. By pairwise comparison of patterns, the phenogram in Fig. 3 was constructed. As expected, the tree illustrates the similarity of RAPD patterns seen on the gels (Fig. S1, Fig. 3). For *C. crustorum*, the analysis of the normalized PCR fingerprinting patterns of 103 strains allowed us to separate profiles into six homogeneous clades. The heterogeneity coefficient was relatively high in clade I (comprising strains from all five samples) and comparably low in some of the clades. e.g., clade IV and VI contain strains from only TXZ2 or WXZ1 group, respectively. Ten *L. plantarum* strains were isolated from samples WXZ2 and TXZ2. The RAPD-clade I includes only strains from WXZ2, while clade II comprises strains from TXZ2. For the 43

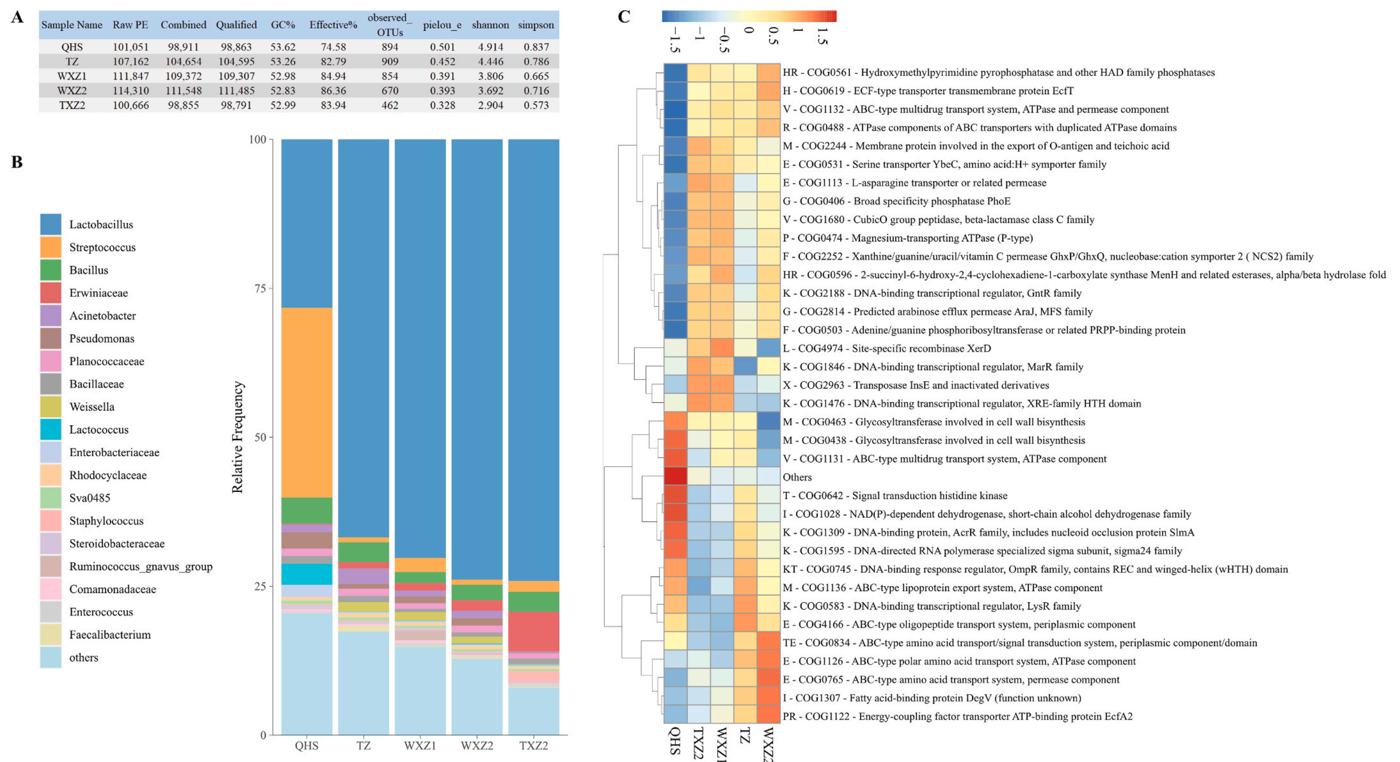

Fig. 1. The microbial composition of traditional fermented milk samples revealed by metagenomics.

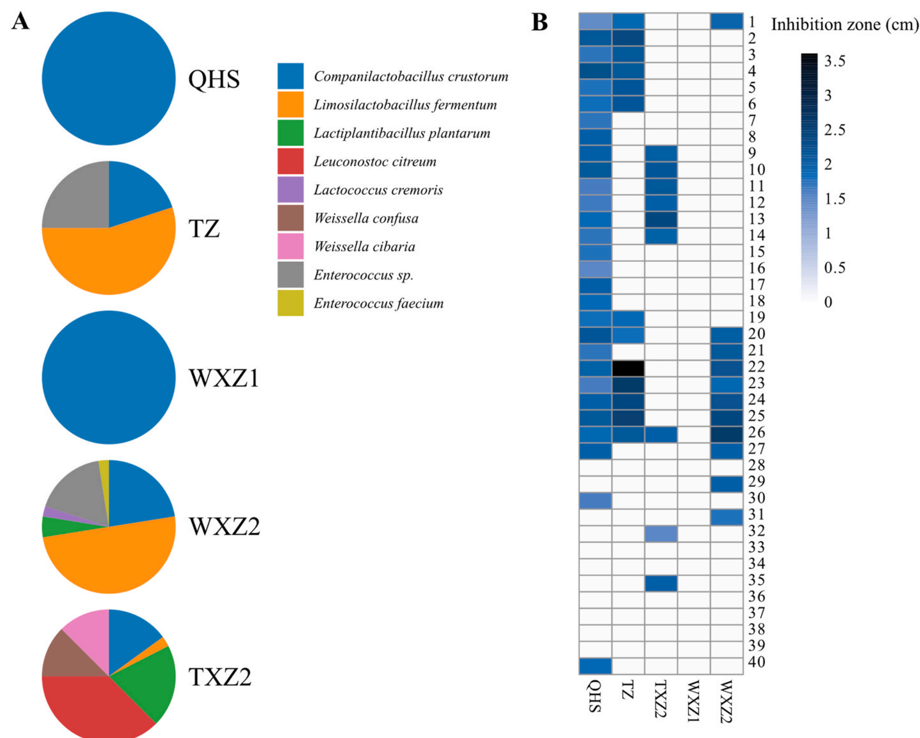

Fig. 2. (A) Identification of isolated lactic acid bacteria from traditional fermented milk and (B) evaluation of their antilisterial activity.

L. fermentum strains, the DNA fingerprints resulted in the placement of the strains in four clades in the presented phenogram and all RAPD clades contain strains from different samples (Fig. 3).

3.4. Mining bacteriocin genes from anti-listerial strains

Ten anti-listrerial strain from different samples and RAPD-clade were selected for whole genome sequencing. In order to evaluate the biosynthetic gene clusters (BGCs) of antimicrobial compounds of the

Fig. 3. Phylogenetic tree of (A) *Companilactobacillus crustorum*, (B) *Lactiplantibacillus plantarum*, and (C) *Limosilactobacillus fermentum* isolated from five samples based on RAPD-PCR.

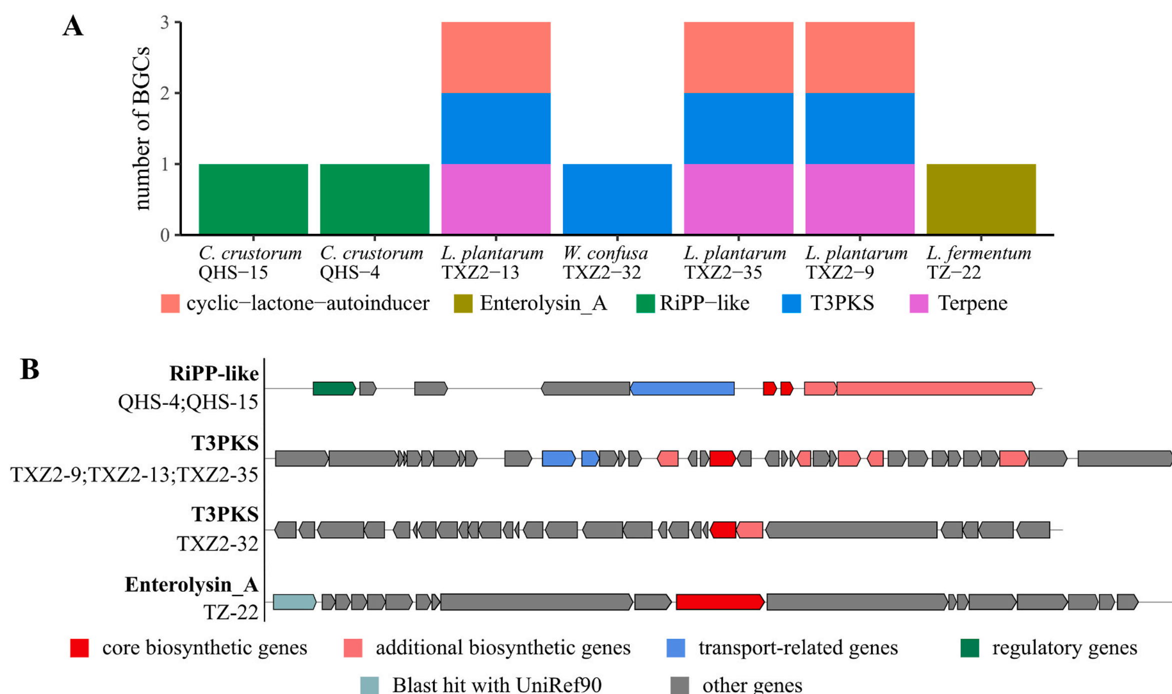

Fig. 4. Numbers of BGCs harbored by the isolated LAB strains and predicted gene cluster structures. (A) Total number of BGCs in the strains. (B) Structure of BGCs in the strains.

selected strains, their genomic sequences were analyzed by BAGEL 4 (van Heel et al., 2018) and antiSMASH 6.0 (Blin et al., 2021). A total of 13 BGCs were identified in seven LAB strains (Fig. 4A). Among them, *C. crustorum* strain QHS-4 and QHS-15 harbor the same Ripp-like BGCs, which encoding a potential two-peptide bacteriocin. T3PKS BGCs were found in *L. plantarum* TXZ2-13, TXZ2-35, and TXZ2-9 and *Weissella confuse* TXZ2-32. A enterolysin_A encoding gene cluster were identified in *L. fermentum* TZ-22 (Fig. 4B).

3.5. *In situ antilisterial activity of selected LAB strains on cheeses*

Representative strains of *L. plantarum* (TXZ2-35), *L. fermentum* (TZ-22) and *C. crustorum* (QHS-4) were selected to test their antilisterial potential in Cheddar cheese during ripening at 4 °C. Cheddar cheeses were made as shown in Fig. 5A, and different LAB strains were added as an adjunct culture with or without *L. monocytogenes*. The variable numbers of LAB and *L. monocytogenes* cells were counted every 10 days. In the absence of adjunct culture, *L. monocytogenes* grew rapidly in cheeses during ripening and reached maximum cell numbers of 2×10^9 CFU/g after 30 days. The growth rate of *L. monocytogenes* was strongly reduced in mixed culture with selected LAB strains (Fig. 5B). Among the three tested strains, *L. plantarum* TXZ2-35 grew most rapidly at 4 °C in cheese, increasing from 1×10^6 CFU/g (initial inoculation level) to maximum cell numbers of 5×10^8 CFU/g after 30 days of ripening. In comparison, *C. crustorum* QHS-4 also grew albeit at a much slower rate. The cell number of *L. fermentum* TZ-22 in cheese gradually decreased after 10 days (Fig. 5C).

3.6. *Effects of selected LAB strains on the quality of cheese*

Texture is an essential parameter of cheese quality. The addition of different LAB adjunct strains had different effects on texture profiles of Cheddar cheese. The changes in TPA of the cheese samples during the storage period are shown in Fig. 6A. *C. crustorum* QHS-4 merely changes the adhesiveness of cheese. At the end of ripening, the highest hardness and resilience value was determined in cheese samples containing *L. plantarum* TXZ2-35. Moreover, both *L. plantarum* TXZ2-35 and *L. fermentum* TZ-22 both increased chewiness and gumminess value of cheese samples (Fig. 6A).

The cheeses made from cow milk and their blends were assessed for sensory attributes using QDA (Fig. 6B) after 30 days of ripening. The QDA results showed that different additives have certain effects on the color, smell and texture of cheese. Compared with the blank group, *C. crustorum* QHS-4 changed the under-rind consistency and core hardness of the cheese with little difference. At the same time, it also improves the putrid smell of cheese. *L. plantarum* TXZ2-35 and *L. fermentum* TZ-22 greatly increased the hardness of cheese, which was consistent with the results of texture analysis. *L. plantarum* TXZ2-35 and *L. fermentum* TZ-22 changed the color and smell of cheese, affecting visual evaluation. In short, *C. crustorum* QHS-4 retains the original sensory properties of cheese, *L. plantarum* TXZ2-35 and *L. fermentum* TZ-22 not only greatly affected the texture of cheese, but also improved the flavor of cheese (Fig. 6B).

4. Discussion

Traditional fermented milk is known as one of the natural habitats of LAB. Functional LAB strains can be used in the developing of new types or improving the quality of fermented milk products (Widyastuti & Febrisiantosa, 2014). It is of increasing interest to evaluate the microbial composition of traditional fermented milk and to obtain strains with promising properties. Deep metagenomic sequencing has been proven to be a powerful tool for describing the microbial composition of fermented foods, and predicting the functional potential of the metagenome (You et al., 2022). In this study, we analyzed the bacterial composition of five traditionally fermented milk samples by 16S rRNA gene sequencing. Among them, TZ, WXZ1, WXZ2, TXZ2 were from a same market in Xinjiang province and QHS was collected from Qinghai province. Our results show that the geographical distance between samples was the primary factor of the variation in the microbial community composition of fermented milk (Fig. 1B). Previous studies showed that *Lactobacillus* and *Streptococcus* were the predominant genera at the genus level in

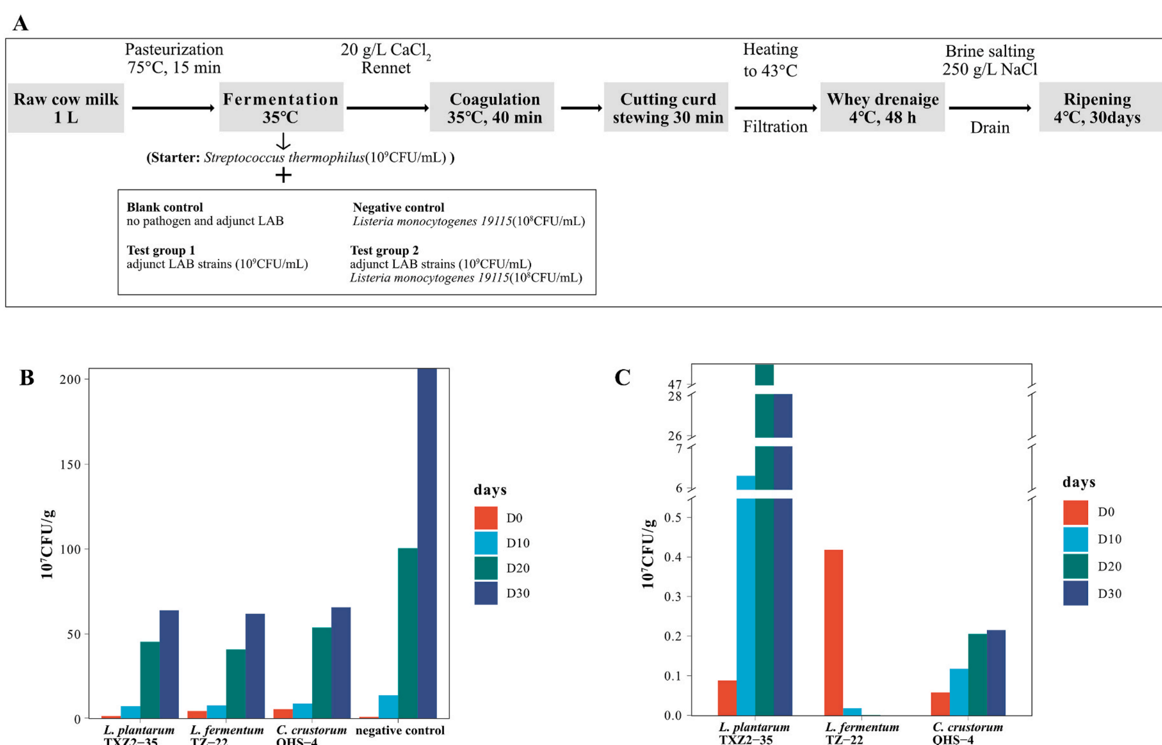

Fig. 5. Antilisterial activity of selected LAB strains in Cheddar cheese.

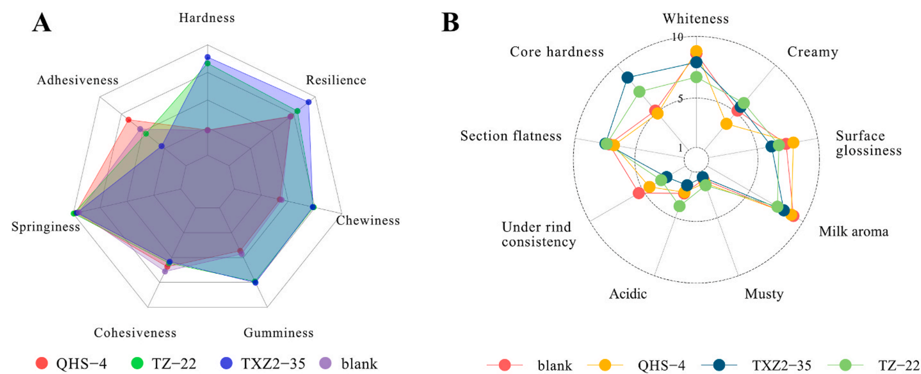

Fig. 6. Effects of selected LAB strains on (A) texture and (B) sensory of Cheddar cheese.

homemade yogurts of Xinjiang (Xu et al., 2015). Similar results were obtained from our study in sample QHS from Qinghai. Interestingly, in the four samples collected from Xinjiang, *Streptococcus* species take up only a small portion of the total microbial community. It is well established that microbial abundances are strongly associated with metabolic activities (Zhang et al., 2016). The high abundance of *Streptococcus* in sample QHS may contribute to the high glycosyl transferase, nucleoid occlusion protein, and amino acid transport metabolism potential (Fig. 1C). Herve-Jimenez et al. revealed that at late stages of milk fermentation, the expression of genes associated with amino acid metabolism and transport, glycosyl transferase and DNA metabolism were highly upregulated in *Streptococcus thermophiles* (Herve-Jimenez et al., 2008).

The changes in microbial composition by straining of fermented milk were also analyzed. It is shown that the straining process decreased the alpha-diversity but increased the composition of lactobacillus in fermented milk samples. It has been reported that the total solid content of milk whey has a positive influence on the viability of *Lactobacillus acidophilus* and *Lactobacillus delbrueckii* subsp. *bulgaricus* (Almeida et al., 2009). However, due to limited number of samples sequenced, the detailed mechanisms of how straining process of fermented milk influence LAB micro-flora needs to be studied further.

C. crustorum was isolated in all the samples and dominates the cultivable *Labctobacilli* in QHS and WXZ1 samples. Interestingly, *C. crustorum* strains isolated from sample QHS show stronger antilisterial activity than those from sample WXZ1 (Fig. 2). *C. crustorum* was widely distributed in fermented food such as traditional fermented cucumber (Cui et al., 2020) and sourdough (Comasio et al., 2019). It was also commonly isolated from dairy products including koumiss (Yi et al., 2016) and Iranian traditional dairy products (Sharafi et al., 2015). The analysis of all the isolated *C. crustorum* strains showed that the variation in antilisterial activity fits well with the phylogenetic dendrogram, where clade III containing antilisterial strains while clade VI containing strains with no antilisterial activity (Fig. 3A). The strain-dependent variation in antimicrobial activity was also reported in human-derived *Lactobacillus reuteri* (Spinler et al., 2008). Cen et al. reported that *L. plantarum* strains of different origin had distinct genetic background, and strains within the same niche tended to harbor similar functional genome profiles (Cen et al., 2020). Our results revealed considerable phenotype and genotype variations of *C. crustorum* strains within the same niche.

LAB can produce antimicrobial metabolites that exert strong antagonistic activity against many microorganisms, including pathogenic and food spoilage microorganisms (Gao et al., 2019). The current screening paradigm of antimicrobial LAB continue to follow a conventional "one-strain-one-test" process, which seems to be unproductive due to the increasing rediscovery rate (Lebedeva et al., 2021). Genome mining based on gene cluster sequences and biosynthetic pathways provides a strategy of natural product discovery. The key purpose of genome mining for antagonistic compounds is to identify novel gene clusters of their biosynthesis (BGCs). By mining genomes of 10 isolated LAB strains, we discovered 7 potential producers of antimicrobial secondary metabolites, among which, two type-III polyketides and one RiPPs are novel BGCs from LAB (Fig. 4). It should be noted that the Illumina DNA sequencing generates accurate but short reads and produce fragmented genome assemblies. BGC prediction using contig-level genome is not optimal because genes in a BGC are often predicted to be scattered through several contigs. Applying both the PacBio sequencing method generates long reads, and the Illumina sequencing method with a low error rate, could be the solution to improving the accuracy of genome mining (Lee et al., 2020).

Three strains carrying novel BGCs showed similar efficiency in controlling *L. monocytogenes* in Cheddar cheese. As in all the strains tested, *L. plantarum* TXZ2-35 exhibited good surviving ability and fermenting capability in cheese matrices. The addition of TXZ2-35 increased the hardness and resilience of cheese (Fig. 5C). *L. plantarum* has been tested successfully as adjuncts in several types of cheeses, the effects on the texture and sensory of cheese varied among strains (Ciocia et al., 2013; Milesi et al., 2008; Ortigosa et al., 2006). It has been reported that the addition of a Lactobacilli adjunct culture promoted better texture and body quality of cheeses (Madkor et al., 2000). However, some adjunct *Lactobacillus* strains may also bring off-flavour to cheese (Antonsson et al., 2003). Although *L. fermentum* TZ-22 showed antagonistic activity towards *Listeria*, the culturable cell number decreased with time during the post-ripening process of cheese (Fig. 5C). This phenomenon may be related to its metabolites or its low tolerance to the processing of cheese. Some studies showed that during cheese ripening, some LAB strains may enter a VBNC (viable but non-culturable) state that allows continuation of basic metabolic activities without reproduction and growth (Ruggirello et al., 2014). Whether there is a high population of VBNC cells exists in our strains needs further study.

5. Conclusion

In this study, we describe the bacterial diversity in 5 traditional fermented milk samples from Western China. Based on 16S metagenomic analysis, we investigated the effects of a natural concentration process on the microbial composition of fermented milk. Two hundreds LAB strains were isolated, with their antilisterial activity tested. Ten strains with strong antimicrobial activity were selected for genome sequencing and mining, resulting in the identification of several novel BGCs. Fermentation of selected strains with BGCs (*C. crustorum* QHS-4, *L. plantarum* TXZ2-35, and *L. fermentum* TZ-22) adding as an adjunct culture decreased the levels of *L. monocytogenes* in cheese, possibly due to the release of antimicrobial substances. Moreover, *L. plantarum* TXZ2-35, and *L. fermentum* TZ-22 showed the potential of improving the flavor and texture when used as adjunct cultures in processing of Cheddar

cheese. Therefore, lactobacilli with BGCs could serve as ideal adjunct cultures in cheese to prevent listeria contamination and improve cheese quality.

CRediT authorship contribution statement

Lin Li: Conceptualization, Investigation, Visualization, Writing – original draft. **Linxuan Zhang:** Data curation, Formal analysis. **Tingting Zhang:** Software, Writing – review & editing, Data curation. **Yuanfa Liu:** Software, Writing – review & editing. **Xin Lü:** Supervision. **Oscar P. Kuipers:** Writing – review & editing. **Yanglei Yi:** Conceptualization, Funding acquisition, Investigation, Resources, Supervision.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.lwt.2023.114507.

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