Occurrence and probiotic properties of *Lactobacillus* spp. of maternal breast milk in the Uyghur, Xinjiang China, at different lactation stages

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Research article

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Abstract

Background

As a secure vehicle of probiotics, breast milk can provide babies with the *Lactobacillus* which can not only colonize and inhibit the pathogenic bacterial infection in infant's intestines. The purpose of this study was to assess the occurrence and probiotic properties of *Lactobacillus* spp. of maternal breast milk in the Uyghur population, Xinjiang China, by using culture method.

Results

Based on repetitive genomic fingerprinting PCR (rep-PCR), a total of 198 isolates of L actobacill us from 31 different lactation of breast milk samples of Uighur in kashi region of Xinjiang were classified into 11 genotypes, which were identified as *Lactobacillus fermentum* (82 isolates, prevalence: 61.3%, mean relative abundance: 45.4%, genotype: 3), *Lactobacillus brevis* (75, 41.9%, 38.1%, 4), *Lactobacillus oris* (37, 32.3%, 15.1%, 3) and rare *Lactobacillus vaginalis* (4, 9.7%, 1.4%, 1). From colostrum to mature milk, the number and species of *Lactobacillus* showed an uptrend, from mature milk to late milk, a downside was found in *Lactobacillus*. The relative abundance of *L. fermentum* decreased throughout lactation, while *L . brevis* showed an opposite trend. Three isolates were randomly selected for each genotype to estimate antimicrobial activity (33 isolates in total). Among the isolates, 20 isolates exhibited broad antibacterial spectrum with inhibition halos >10 mm against most indicator pathogens, which were selected for assessing probiotic properties. Nineteen isolates showed the resistance to vancomycin and co-trimoxazole, and KM66 (*L . brevis*) was resistant up to 5/8 antibiotics. KM147 (*L. brevis*) and Y3 (*L. fermentum*) strains were selected as potential probiotics by combining lysozyme, acid and bile salt tolerance.

Conclusion

Species composition and prevalence of *Lactobacillus* varied with the study subjects throughout lactation phase. The deliberately selected *Lactobacillus* strains from breast milk may have a great potential as probiotics to inhibit pathogen infection in infants.

Background

With the improvement of people's living quality and the constant pursuit of healthy food, there is a growing interest in probiotics, especially the lactic acid bacteria (LAB) viz. Lactobacillus spp., Bifidobacterium spp., etc (1). It is well known that Lactobacillus is a fabulous probiotic LAB, a gram-positive, non-spore forming, facultative anaerobic bacteria which plays a significant role not only in fermented dairy products, but also in beverages and silages (2). Particularly, the Lactobacillus has been demonstrated to provide many health benefits including inhibition against the infection of pathogenic bacteria, enhancing the body's immunity as well as preventing the occurrence of bacterial flora imbalance and bacterial displacement (3–5).

It has been found that probiotics in infants gut primarily come from breast milk and the mother's gut and vagina. Given the risk of antibiotic resistance, breast milk is deemed to be a more secure source of probiotics relative to other environments (6). As a vehicle of probiotics, breast milk has the mother-to-infant transfer of some bacteria that can colonize in the infant's intestines (7). Probiotics such as Lactobacillus and Bifidobacterium in breast milk have profound effects on the development of intestinal flora and immune system of infants (8). Additionally, the microbiota of breast milk is generally complex and variable: it reaches the most complex level at the mature milk, and then it stays constant and drops sharply at late milk (9). Lactobacillus is abundant in breast milk, among which the most commonly reported are L. fermentum, L. rhamnosus, L. salivarius, etc (7). To date, the occurrence of Lactobacillus in different lactation stages of breast milk is not clear. Whether the prevalence of Lactobacillus in breast milk is related to different lactation periods is of great significance for understanding the occurrence of Lactobacillus in breast milk and the intestinal metastasis of these bacteria in infants. Since most babies are breastfed after birth, and it's confirmed that it can prevent neonatal allergy, malnutrition and infections from breastfeeding (10). Based on it's potential probiotic properties, the antimicrobial activity of the Lactobacillus isolated from breast milk has become a hotter topic to scientists around the world. Some studies have found that Lactobacillus isolated from breast milk has probiotic properties, such as improving intestinal tract, therapeutic effect of diarrhea and adjuvant therapy of some diseases (11). For example, the L. gasseri CECT5714 and L. fermentum CECT5716, originated from breast milk have been shown to play a crucial role in inhibiting pathogen infection based on the mechanism of producing antimicrobial bacteriocins to compete with pathogens in intestinal tract (12,13).

In addition, breast milk microflora is closely related to human lifestyle, dietary habits, regional differences, delivery mode (14,15). Xinjiang is a large and multi-ethnic residential area with unique Uighur living habits and food culture. Thus, the breast milk of Uighur in Xinjiang provide a rich source of unique host for the exploiture and utilization of Lactobacillus. In this study, the aim was to assess the occurrence of Lactobacillus spp. of maternal breast milk in the Uyghur population, northwestern China, by using culture method, and then evaluate their key probiotic characteristics including antimicrobial activity, antibiotic resistance, lysozyme, acid and bile salt resistance. We hope to screen out lactobacilli probiotics with significant antibacterial properties, and finally apply them to health food after in vivo experiments.

Result

Molecular identification and occurrence of Lactobacillus in breast milk

Combined with morphology and partial gene sequencing of 16S rRNA, 198 lactobacilli from 31 breast milk samples of different lactation of the Uighur in kashi region of Xinjiang were identified, which were classified into 11 genotypes based on rep-PCR genotyping. Through BLAST homology analysis (above 97% homology), all the lactobacilli were identified as L. fermentum (82 isolates, genotype: 3), L. brevis (75, 4), L. oris (37, 3) and L. vaginalis (4, 1). Meanwhile, L. fermentum occurred in 19 out of 31 breast milk samples (prevalence: 61.3%). L. brevis, L. oris and L. vaginalis were isolated from 13/31 (41.9%), 10/31 (32.3%) and 3/3 (9.7%) samples respectively.

According to different lactation period, breast milk samples were divided into five stages, they are colostrum stage A (1–5 postpartum days), mature milk B (15–90 days), late mature milk C (91–300 days), late milk D (301–450 days) and late milk E (451–570 days) (16). As shown in Table 1, during the whole lactation period, the number of lactobacilli in breast milk samples showed a trend of mature milk B > late milk E > late milk C > colostrum A > late mature milk D, and the diversity showed mature milk B = late mature milk C > late milk D > late milk E > colostrum A. At the level of species, the mean relative abundance of L. fermentum, L. brevis, L. oris and L. vaginalis in five stages was 45.4%, 38.1%,15.1% and 1.4% respectively. L. fermentum was the dominant species, and only L. fermentum was isolated from colostrum A. L. fermentum, L. oris and L. vaginalis had the highest isolation rate (22.2%, 13.6% and 1.5%) in mature milk B, and L. brevis had the highest isolation rate (16.7%) in late milk E. The 4 isolates of L. vaginalis were isolated from mature breast milk B and late mature milk C. As shown in Fig. 1, L. fermentum were identified from all 5 stages of lactation (A-E). Meanwhile, the relative abundance of L. fermentum showed a decreasing trend throughout the lactation. On the contrary, the relative abundance of L. brevis increased gradually, and reached the highest value (76.7%) in late milk E. Principal component analysis (PCA) (Fig. 2) showed that all shapes were distributed concentratedly and no significant independent population was formed, indicating that populations of Lactobacillus isolated during five different lactation periods were similar. According to the dispersion degree of the shape in the figure, the population structure of Lactobacillus isolated in later milk D was more similar than that in other stages, while that in mature milk B was vice versa. Three isolates were randomly selected for each genotype to assess probiotic properties (33 isolates in total). The strain ID, genus names and species names were shown in Fig. 3.

Table 1
Distribution of Lactobacillus in breast milk during

Age (day)	A (1–5 d)					B (15–150 d)							C (151–300 d)				
Samples	Y4	Y10	Y 12	Y6	Y7	KZ13	GL30	GL34	KZ19	GL37	GL28	GL26	KZ14	KZ15	GL27	KZ30	GL2
L.fermentum	1	1	14	—	—	2	6	27	5	4	—	—	4	—	1	2	1
L. brevis	—	—	—	—	—	9	4	—	3	9	—	—	7	3	—	—	—
L. oris	—	—	—	—	—	3	16	3	1	3	1	—	2	4	1	—	—
L. vaginalis	—	—	—	—	—	—	—	1	—	—	2	—	—	1	—	—	—
Total	16					99							26				

L.fermentum: Lactobacillus fermentum, L. brevis: Lactobacillus brevis, L. oris: Lactobacillus oris, L. vaginalis: Lactobacillus vaginalis.

Assessment of the antimicrobial activity

The antibacterial property is one of the significant criteria of Lactobacillus for screening potential probiotics. It was reported that some Lactobacillus strains play a pivotal role in the inhibition of the infection caused by pathogenic bacteria in infants (6,17). In our study, 20 of 33 Lactobacillus isolates had broad antibacterial spectrum with inhibition zones > 10 mm against most indicator pathogens, all of which can inhibit Escherichia coli (EPEC) O127:K63, Salmonella enterica subsp. enterica serovar Typhimurium and E. coli (ETEC). The isolate KM66 (L. brevis) exhibited the broadest antibacterial spectrum with an inhibition zone diameter (mm) of 21.36 ± 0.22, 19.82 ± 0.08, 18.00 ± 0.06 and 20.41 ± 0.10 against E. coli (EPEC), S. Typhimurium, E. coli (ETEC) and Listeria monocytogenes (CGMCC 1.9136) respectively. The KM147 (L. brevis) exhibited the largest inhibition zone (15.20 ± 0.28 mm) against E. coli (EHEC). The broadest antibacterial spectrum (inhibition zone: 19.87 ± 0.25 mm) against Salmonella enterica subsp. Enterica (CGMCC 1.10754) was observed with the Y3 (L. fermentum). Among L. oris strains, GM12-2 exhibited relatively obvious inhibition on all six indicator pathogens (Table 2). Based on broad antibacterial spectrum on indicator pathogens, 20 isolates were selected for further test.

Table 2

In vitro antimicrobial activity of Lactobacillus isolates against pathogenic bacteria with modified agar sandwich overlay method after incubating 24 h at 37 ℃.

strain ID	Test organism with Inhibition Zone Diameter mean (mm) ± S.D.						
	E. coli (EPEC) O127:K63 (CICC 10411)	E. coli (ETEC) O78:K80(CICC 10421)	E. coli (EHEC) O157:H7 (CICC 21530)	Listeria monocytogenes (CGMCC 1.9136)	Salmonella enterica subsp. enterica serovar Typhimurium (CICC 10420)	Salmonella enterica subsp. Enterica (CGMCC 1.10754)	average value
KM147	18.00 ± 0.08	16.80 ± 0.18	15.20 ± 0.28	16.65 ± 0.18	18.00 ± 0.16	14.62 ± 0.20	16.55
KM66	21.36 ± 0.22	18.00 ± 0.06	14.81 ± 0.10	20.41 ± 0.10	19.82 ± 0.08	19.80 ± 0.07	19.03
KM86	18.33 ± 0.08	10.20 ± 0.12	9.25 ± 0.05	14.06 ± 0.27	18.24 ± 0.09	15.45 ± 0.09	14.26
GM1-2	16.35 ± 0.04	15.65 ± 0.14	10.25 ± 0.07	10.53 ± 0.07	11.53 ± 0.15	10.00 ± 0.07	12.39
GM3-1	15.65 ± 0.12	17.53 ± 0.05	11.48 ± 0.09	8.75 ± 0.05	10.35 ± 0.05	—	12.75
KM26	11.90 ± 0.17	17.07 ± 0.05	12.43 ± 0.08	9.28 ± 0.05	14.83 ± 0.24	14.50 ± 0.22	13.34
KM165	8.94 ± 0.10	10.65 ± 0.08	16.74 ± 0.04	10.40 ± 0.06	11.90 ± 0.05	11.80 ± 0.10	11.74
KM22	14.83 ± 0.09	14.35 ± 0.28	9.28 ± 0.05	10.03 ± 0.02	11.45 ± 0.18	—	11.99
KM54	14.45 ± 0.07	15.25 ± 0.05	—	9.43 ± 0.05	11.65 ± 0.08	8.98 ± 0.01	11.95
KM15	16.30 ± 0.06	14.88 ± 0.08	9.69 ± 0.05	9.12 ± 0.04	11.45 ± 0.05	—	12.29
KM164	15.33 ± 0.05	14.58 ± 0.08	10.80 ± 0.05	11.30 ± 0.04	10.08 ± 0.08	9.05 ± 0.07	11.86
Y7	11.28 ± 0.08	8.58 ± 0.04	—	—	8.65 ± 0.04	15.08 ± 0.10	10.90
KM176	10.68 ± 0.09	16.55 ± 0.44	10.10 ± 0.01	15.95 ± 0.04	10.28 ± 0.29	9.25 ± 0.04	12.14
GM12-2	12.50 ± 0.04	13.75 ± 0.04	13.28 ± 0.04	14.58 ± 0.50	13.05 ± 0.14	11.43 ± 0.04	13.10
Y3	18.90 ± 0.16	12.73 ± 0.16	9.83 ± 0.07	16.80 ± 0.42	10.41 ± 0.12	19.87 ± 0.25	14.75
KM4	10.78 ± 0.08	12.30 ± 0.04	11.70 ± 0.04	15.31 ± 0.13	13.18 ± 0.08	11.62 ± 0.04	12.46
GM68-2	12.34 ± 0.13	11.00 ± 0.10	13.60 ± 0.04	11.85 ± 0.13	11.62 ± 0.04	11.00 ± 0.01	11.90
KM68	14.43 ± 0.26	14.90 ± 0.19	9.61 ± 0.08	17.95 ± 0.13	9.71 ± 0.07	12.43 ± 0.16	13.17
KM11	8.80 ± 0.06	12.58 ± 0.04	14.43 ± 0.20	13.48 ± 0.16	13.53 ± 0.32	—	12.56
KM50	11.05 ± 0.01	10.90 ± 0.02	—	—	11.00 ± 0.11	—	10.98
KM1	8.53 ± 0.05	9.23 ± 0.04	8.75 ± 0.03	8.58 ± 0.04	8.58 ± 0.05	9.05 ± 0.10	8.79
GM42-2	10.33 ± 0.05	—	—	—	—	9.08 ± 0.09	9.71
GM37-2	—	—	9.95 ± 0.01	—	8.78 ± 0.07	9.13 ± 0.05	9.29
GM59-2	9.80 ± 0.01	9.43 ± 0.06	—	—	—	9.83 ± 0.16	9.68
KM97	9.83 ± 0.06	—	8.60 ± 0.05	—	9.85 ± 0.01	—	9.43
GM33-1	9.98 ± 0.12	—	—	8.58 ± 0.05	8.40 ± 0.04	—	8.99
Y9-1	11.25 ± 0.05	—	8.55 ± 0.06	—	8.73 ± 0.05	11.03 ± 0.09	9.89
GM61-1	8.68 ± 0.04	—	9.10 ± 0.07	—	10.25 ± 0.04	—	9.34
KM122	—	12.00 ± 0.13	8.98 ± 0.03	—	8.93 ± 0.10	—	9.97
KM93	9.30 ± 0.09	—	—	—	—	—	9.30
KM121	8.10 ± 0.07	—	—	—	—	8.15 ± 0.26	8.13
GM42-1	—	—	—	—	8.45 ± 0.30	8.93 ± 0.15	8.69

The diameter of AGAR wafer are 7 mm, — = no activity, ± = standard deviation (S.D), assays were carried out in quadruplication.

strain ID	Test organism with Inhibition Zone Diameter mean (mm) ± S.D.						
	E. coli (EPEC) O127:K63 (CICC 10411)	E. coli (ETEC) O78:K80(CICC 10421)	E. coli (EHEC) O157:H7 (CICC 21530)	Listeria monocytogenes (CGMCC 1.9136)	Salmonella enterica subsp. enterica serovar Typhimurium (CICC 10420)	Salmonella enterica subsp. Enterica (CGMCC 1.10754)	average value
KM13	8.06	—	—	—	—	—	8.06
The diameter of AGAR wafer are 7 mm, — = no activity, ± = standard deviation (S.D), assays were carried out in quadruplication.							

Antibiotic resistance profile

Since the overuse of antibiotics, antibiotic resistance has become one of the important criteria for selecting potential probiotics (18). The antibiotic susceptibility of breast milk Lactobacillus was evaluated by the K-B paper method and results are shown in Table 3. All 20 isolates were found sensitive to ampicillin, rifampicin and tetracycline, and only KM66 was resistant to chloramphenicol. All isolates exhibited antibiotic resistance to vancomycin and co-trimoxazole except KM4 and KM11 respectively. Fifty-five percent of isolates showed resistance to gentamicin (Fig. 4). In particular, KM66 is the most obvious multidrug-resistant isolate with resistance up to five antibiotics (chloramphenicol, gentamicin, co-trimoxazole, penicillin-G and vancomycin).

Table 3
Antibiotic susceptibility of lactobacilli using the K-B paper method after 24 incubation at 37 ℃.

antibiotic strain ID	KM147	KM66	KM86	GM1-2	GM3-1	KM26	KM165	KM22	KM54	KM15	KM164	Y7	KM176	GM12-2	Y3
Chloramphenicol (30 μg)	S	R	S	S	S	S	S	S	S	S	S	S	S	S	S
Gentamicin (10 μg)	S	R	S	R	R	S	S	R	R	S	R	R	S	S	S
Co-trimoxazole (25 μg)	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R
Ampicillin (10 μg)	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S
Rifampicin (5 μg)	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S
Penicillin-G (10 μg)	I	R	R	I	S	I	I	S	S	I	R	S	I	R	S
Tetracycline (30 μg)	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S
Vancomycin (30 μg)	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R
S = sensitive, I = mediating, R = resistant.															

Resistance of lysozyme

Assessing their resistance to extreme conditions in the digestive tract is an important criterion for selecting potential probiotics (19,20). The obstacles to overcome, in turn, are high concentration of salivary lysozyme in the mouth, low pH in the stomach and bile in the intestinal fluid (21). After 30, 90 and 120 minutes of lysozyme treatment, bacterial survival activity was shown in Fig. 5. Interestingly, among 20 isolates, only the KM50 and GM3-1 were sensitive (survival: 34.7% and 44.4%) to the lysozyme treatment after 120 min respectively. The rest of isolates exhibited resistance to lysozyme with resistance > 50%, among which 8 isolates showed severe resistance to the treatment of lysozyme (with resistance > 80%), which proved that Lactobacillus had high lysozyme tolerance. These eight isolates were selected for further research.

Resistance of acid and bile salt

The survival of Lactobacillus under low acid condition and high concentration of bile salt is of great significance to withstand gastrointestinal environment stress (22). Among 8, 5 isolates (KM66, KM86, KM147, Y3 and GM12-2) were resistant at pH 3.0 with resistance > 50%, and the maximum resistance were observed with KM66 (61.078%) and KM147 (61.077%), followed by the Y3 (55.205%) at pH 3.0. The above 3 isolates were also resistant to 0.3% of bile salt (survival: 75.291%, 62.940% and 65.049%) (Fig. 6).

Discussion

The 198 isolates of the isolated Lactobacillus belong to four species: L. fermentum, L. brevis, L. oris, and L. vaginalis. It has been reported that L. fermentum and L. rhamnosus were the most frequent species from breast milk at the level of species (23). In the present study, L. fermentum was the dominant species, which was the same as the results obtained by Soto et al. (24). Moreover, the present study also found that L. fermentum had 100% of relative abundance in colostrum. However, Ozgun et al. revealed that L. brevis was frequently found from colostrum samples from Turkey (25). This may be related to differences in region, the lifestyle and dietary habits of the mother. For colostrum A to mature milk B, the number and species of Lactobacillus isolated showed an uptrend,

mature milk B reached the highest level, and showed a downside from mature milk B to late milk E. Similar results were reported by Solis et al., who also found that the Lactobacillus spp. in mature milk (10–90 postpartum days) has higher isolation ratio and more species than colostrum (1 day) (26). It has been reported that the breast milk composition influence microbiota (27). Therefore, we can speculate that the growth of some Lactobacillus may be related to changes of nutrient composition in breast milk during different lactation periods. And mature milk is more suitable for Lactobacillus multiplication. In the present study, four isolates of L. vaginalis were rarely isolated from breast milk of stages B and C, which is seldom reported in other studies. Ana et al. detected L. vaginalis in only 2 of the 27 breast milk samples (24). This may due to its harsh survival requirement or the introduction of new technologies, such as metagenomics and sequencing, which allowed the rapid development of Lactobacillus isolation. Lactobacillus are notoriously widespread in the skin, mouth cavity, gastrointestinal and vaginal cavities of humans (28). As previously reported, L. vaginalis was usually isolated from the vaginas of women (29). As demonstrated by Ramsay et al., a degree of reflux into the mammary duct during breast-feeding may facilitate the exchange of bacteria between mammary glands and the oral cavity of infants who acquire bacteria from the vaginal microbiome at birth (27). This shows that the presence of L. vaginalis in breast milk may be associated with vertical transmission during vaginal birth and breastfeeding.

Since the probiotic characteristics of Lactobacillus are specific to strains, the feasibility of exploring new strains is determined by these probiotic characteristics (30). The authors found that the isolates had strong antimicrobial activity. Notably, the KM66 exhibited a broad antimicrobial spectrum against all indicator pathogens and exhibited the broadest antimicrobial spectrum against E. coli (EPEC), E. coli (ETEC), L. monocytogenes, and S. Typhimurium. Mojgani et al. reported that L. brevis LB32 isolated from ewes' milk in Iran also exhibited a wide spectrum of inhibition against L. monocytogenes and S. Typhimurium (31). However, the KM15 with the same genotype as KM66 failed to inhibit S. Enterica. The Y3 (L. fermentum) exerted inhibition against all indicator pathogens. This result differs with the report done by Olivares et al., which indicated that L. fermentum CECT5716 isolated from human milk exhibited the suppression of L. monocytogenes and E. coli (ETEC), but failed to inhibit the growth of E. coli (EHEC) (32). The mechanism of the different antibacterial activities of these strains is largely determined by their different secreted antibacterial substances and the competition with pathogenic bacteria on nutrition and adhesion of intestinal epithelial cells (33–35).

The risk of Lactobacillus transmitting antibiotic-resistant genes to intestinal pathogens cannot be ignored. With regard to antibiotic resistance, 95% of isolates exhibited antibiotic resistance to vancomycin and co-trimoxazole, though the majority of isolates were found susceptible to other antibiotics. These results are similar with other research (36,37). Mohammadi et al also showed that 94% of the Lactobacillus isolated from human milk were resistant to vancomycin (38). It has been reported that resistance to vancomycin and co-trimoxazole in certain Lactobacillus strains is not a safety issue since it is encoded by chromosomes rather than acquired, and therefore not transmissible (39). For safety assessment, the resistance to vancomycin can be used as a useful criterion for screening safe probiotic strains of Lactobacillus (40).

Ninety percent of candidates were tolerant to the lysozyme, suggesting that Lactobacillus had high lysozyme tolerance, which was confirmed by other authors (16, 41). However, only 3 isolates were substantially resistant to acid pH 3.0 and 0.3% of bile salt, respectively: KM66 (survival: 61.078%, 75.291%), KM147 (61.077%, 62.940%), and Y3 (55.205%, 65.049%). It indicate that these isolates could survive in digestive tract. However, the KM26 with the same genotype as KM66 exhibited the lowest survival rate of 31.33% and 29.74% correspondingly at acid pH 3.0 and 0.3% of bile salt. The strains of L. fermentum isolated from human gastrointestinal tracts had relatively better acid tolerance in comparison to other species of Lactobacillus (22). Shokryazdan et al. also reported that the L. fermentum HM2 from human milk showed high acid tolerance at pH 3.0 with a resistance of 72.44%, but exhibited low tolerance to 0.3% of bile salt with a resistance of 20.42% (35), proving the specificity of the tolerance of strains. As reported, the L. fermentum CECT5716 isolated from breast milk was added as the probiotic and was found to reduce the incidence of gastrointestinal infection in infants after six months of continuous feeding (42). Additionally, Albesharat et al. demonstrated that L. fermentum, L. brevis, and L. oris were found in the mothers' milk and the faeces of the corresponding babies (43). In this study, the candidates are expected to colonize the intestine and inhibit the infection of pathogenic bacteria in infants.

Conclusion

The breast milk of Xinjiang Uyghur provided a rich source of Lactobacillus, the change trend of Lactobacillus population in breast milk was observable throughout lactation. The antibiotic resistance of the strains is low. Due to the specificity of the strain and safety considerations, Y3 (L. fermentum MG 551079) and KM147 (L. brevis KM495913) were regarded as candidate potential probiotics to be used in the food or healthcare industries. And they were isolated from mature milk B(15–150 days) and late milk E(451–750 days) respectively.

Methods

Isolation and identification of Lactobacillus spp.

Samples of breast milk were collected with the assistance of hospital doctors, and samples of mothers with underlying diseases were then removed. Strains were isolated and purified with modified MRS (de Man, Ragosa Sharp) medium (contains 0.5 g/L of L-cysteine). Based on morphology, rod-shaped bacterial colonies were cultured anaerobically at 37 °C for 24–48 h in an anaerobic glove box (80% N_2, 10% H_2 and 10% CO_2) (Shanghai jiehan co. LTD). All isolates were stored in − 80 °C refrigerator with 20%-25% glycerine. Single colony DNA of isolates were extracted by method of CTAB (Cetyltriethylammnonium Bromide) (44). Genomic fingerprinting rep-PCR was amplified using primer BOXAIR (5´CTACGGCAAGGCGACGCTGAC3´), and the augmented system is shown in Table 4. The PCR products were electrophoresed on 1% ~ 1.2% agarose gel about 40 min 100V(2.5 V/cm), imaged on observation(CCD camera Biometra Gel DOC XR). Universal primers 27f and 1492R were used for 16S r RNA partial gene sequencing (45). The 16S r RNA gene sequences were retrieved from the National Center from Biotechnology Information (NCBI) database. Sequences with similarities above 97% in BLAST database were identified.

Method	Primer	Mix	Augmented system	Amplification procedure
BOX-PCR	BOXAIR		Mix: 12.5μL	Initial denaturation: 95℃ 5min
	(CTACGGCAAGGCGACGCTGAC)	10×buffer，Taq enzyme，dNTP	BOX: 0.5μL	Degeneration: 94℃ 1min
			Double distilled water: 9.5μL	Annealing: 52℃ 1min
			Template DNA: 1.5μL	Extention: 65℃ 8min
				Eventual extention: 65℃ 16min
				(35 Cycles: 6h 30min)

Table 4
Rep-PCR amplification system

Assessment of antimicrobial activity

Antimicrobial activity of Lactobacillus against pathogens was detected by the modified sandwich overlay method as described (46). The 100 μl bacterial suspension of each bacterium (10^7-10^8 CFU/ml) was dispensed into MRS medium well and cultured at 37 °C for 24 h under the anaerobic condition. Pathogens purchased from China Industrial Microbial Species Preservation and Management Center (including E. coli (EPEC) O127:K63, S. Typhimurium, E. coli (ETEC) O78:K80, E. coli (EHEC) O157:H7, L. monocytogenes and S. Enterica) were incubated at 37 °C for 16 h, then 100 μl of suspension was uniformly coated with sterile cotton swab. The agar covered with isolates colonies was drilled with a hole punch (diameter:7 mm) and the agar blocks were immediately placed onto each indicator pathogen plate. The inhibition zone diameter, which inhibited the growth of pathogens obviously near the colony was measured and the results were divided into < 10, 10–20 and > 20 mm. MRS agar was taken as the negative control and assays were carried out in quadruplication.

Antibiotic resistance

The antibiotic resistance profiles of isolates were determined by the K-B paper method with the following 8 different types of antibiotics tablets: penicillin-G (10 μg), gentamicin (120 μg), rifampicin (5 μg), ampicillin (10 μg), cotrimoxazole (25 μg), vancomycin (30 μg), tetracycline (30 μg) and chloramphenicol (30 μg). The 100 μl of bacterial suspension (10^7-10^8 CFU/ml) were spread over the plates, and these tablets were placed onto the agar surface. Then plates were cultivated at 37 °C anaerobically for 24 h and the diameter of its inhibition zone was measured with calibrated scale. The drug tablets were purchased from the British company OXOID. The results of drug susceptibility were determined according to CLSI standard and were identified as sensitive (S), mediating (I) and resistant (R) (47). Assays were carried out triplicate.

Resistance of lysozyme

Likewise, bacterial inoculum (10^7-10^8 CFU/ml) were centrifuged at 4000 rpm for 5 min and washed thrice with phosphate buffer (pH7, 0.1M) and resuspended. Subsequently, 0.2 ml of cell suspension was transferred to 10 ml of MRS broth containing lysozyme (0.1 g/L) separately. Meanwhile, MRS broth was taken as positive control. The optical density at 600 nm (OD_{600nm}) was measured by ELIASA after incubating anaerobically at 37 °C for 30, 90 and 120 minutes and compared with that without treatment. The % resistance = the residual quantity of OD_{600nm} in MRS broth with different concentrations of lysozyme / increment of $OD600_{nm}$ in MRS broth without lysozyme. The results were divided into three categories: the survival % > 80% after 120 min of cultivation, which could be considered a severe resistance to lysozyme, 50% ~ 80% of survival was considered moderate resistance and < 50% as sensitive to lysozyme (48). The experiments were carried out triplicate.

Resistance of acid and bile salt

Briefly, 0.2 ml of bacterial inoculum (10^7-10^8 CFU/ml) was added to 10 ml of MRS liquid medium adjusted to pH 2, 3, and 4 by 36% hydrochloric acid respectively. OD_{600nm} was measured after anaerobic incubation at 37 °C for 16 h against the positive control (pH 7.0). Lactobacillus isolates showing resistance more than 50% at pH 3 were considered as acid resistant (49). Similarly, 0.2 ml of each bacteria solution (10^7-10^8 CFU/ml) was transferred to 10 ml of MRS broth containing different concentrations of pig bile salt (0.3%, 0.5% and 1.0% w/v) against the control (without bile salt). The broth were incubated at 37 °C for 16 h, then the OD_{600nm} were measured. Lactobacillus isolates at the minimum resistance range of 50% at 0.3% (w/v) bile salt were considered as bile resistant (50). The % resistance = the residual quantity of OD_{600nm} in MRS broth with different concentrations of bile salt or pH (2,3,4) / increment of OD_{600nm} in MRS broth without bile salt or pH (7) × 100. Assays were carried out in triplicate.

Abbreviations

LAB
lactic acid bacteria
16SrRNA
16S ribosomal ribonucleic acid

rep-PCR
repetitive genomic fingerprinting PCR
OD
Optical density
spp
Species

Declarations

Ethics approval and consent to participate

All participants gave written informed consent in accordance with the Declaration of Helsinki. The protocol was approved by the Human Research Ethics Boards at Shihezi University, Xinjiang. Samples of breast milk were collected with the assistance of hospital doctors, and samples of mothers with suspected diseases were then removed.

Consent for publication

Not applicable

Availability of data and materials

All data and materials are available on request for academic use. No administrative permission is required but approval from a local hospital is required to obtain breast milk samples from healthy individuals. The individuals mentioned in the method had no associated disease. The 16S r RNA gene sequence can be submitted to the National Center from Biotechnology Information (NCBI) database.

Competing interests

The authors declare that they have no conflict of interests.

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Authors' contributions

YQ and FW conceived the study. YQ participated in the experimental design. Manuscript was written by XJ and edited by YQ, XL and YZ. Each author informed the consent to participate of this publication.

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Figures

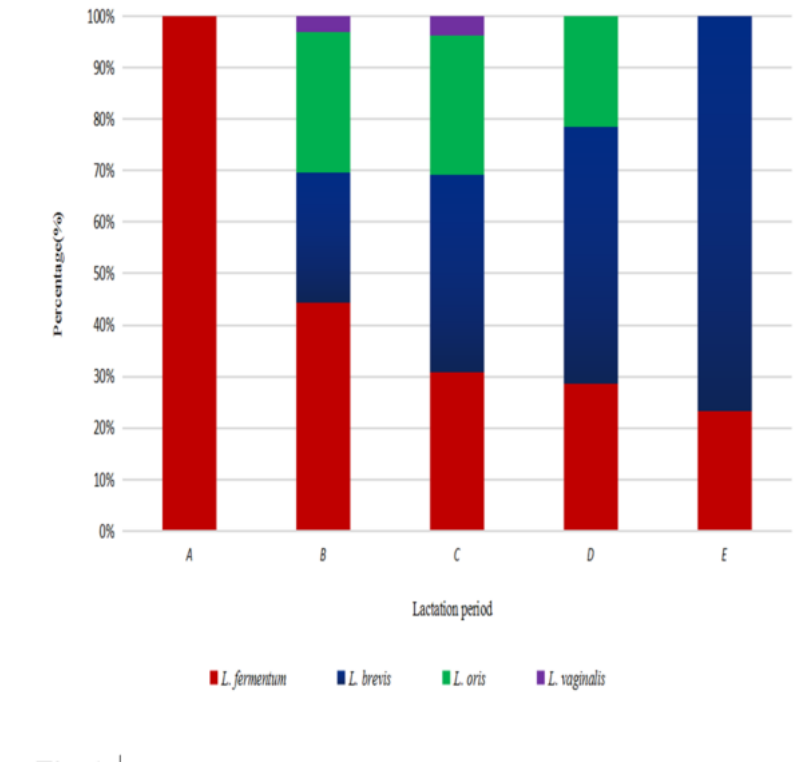


Figure 1

Relative abundance of Lactobacillus species in different lactation period

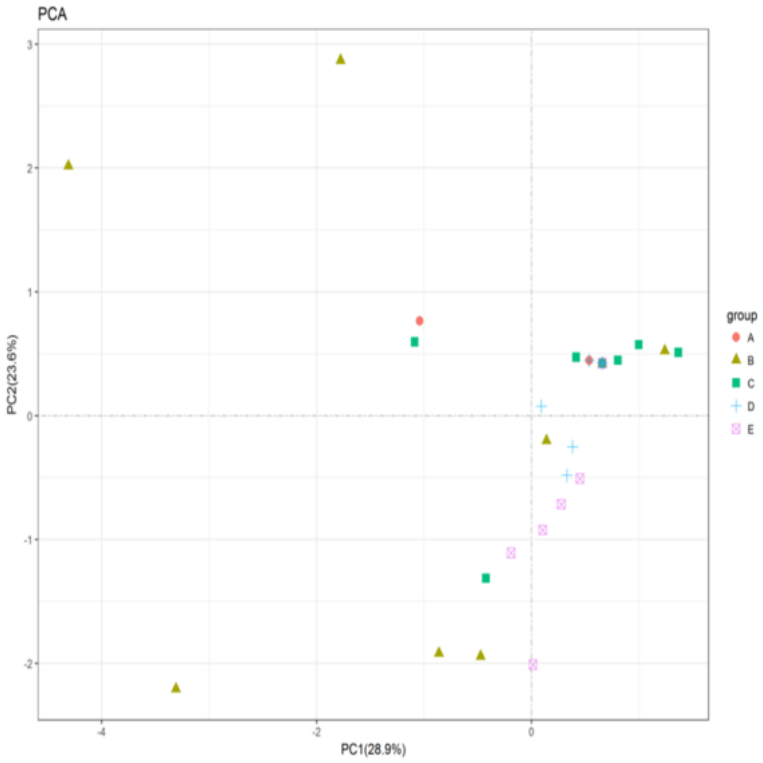
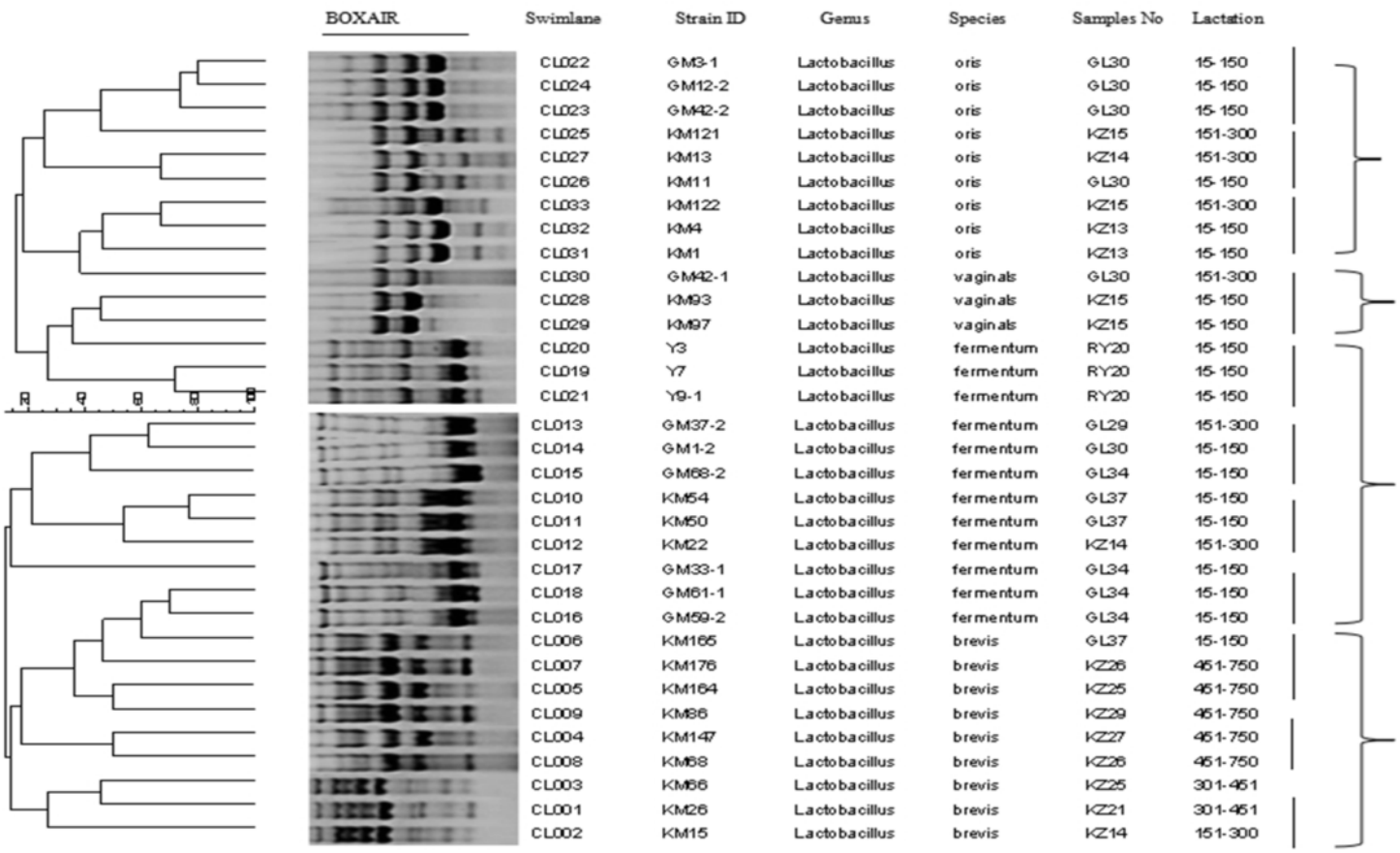


Figure 2

Principal component analysis of Lactobacillus population structure in different lactation stages



Swimlane	Strain ID	Genus	Species	Samples No	Lactation
CL022	GM3-1	Lactobacillus	oris	GL30	15-150
CL024	GM12-2	Lactobacillus	oris	GL30	15-150
CL023	GM42-2	Lactobacillus	oris	GL30	15-150
CL025	KM121	Lactobacillus	oris	KZ15	151-300
CL027	KM13	Lactobacillus	oris	KZ14	151-300
CL026	KM11	Lactobacillus	oris	GL30	15-150
CL033	KM122	Lactobacillus	oris	KZ15	151-300
CL032	KM4	Lactobacillus	oris	KZ13	15-150
CL031	KM1	Lactobacillus	oris	KZ13	15-150
CL030	GM42-1	Lactobacillus	vaginals	GL30	151-300
CL028	KM93	Lactobacillus	vaginals	KZ15	15-150
CL029	KM97	Lactobacillus	vaginals	KZ15	15-150
CL020	Y3	Lactobacillus	fermentum	RY20	15-150
CL019	Y7	Lactobacillus	fermentum	RY20	15-150
CL021	Y9-1	Lactobacillus	fermentum	RY20	15-150
CL013	GM37-2	Lactobacillus	fermentum	GL29	151-300
CL014	GM1-2	Lactobacillus	fermentum	GL30	15-150
CL015	GM68-2	Lactobacillus	fermentum	GL34	15-150
CL010	KM54	Lactobacillus	fermentum	GL37	15-150
CL011	KM50	Lactobacillus	fermentum	GL37	15-150
CL012	KM22	Lactobacillus	fermentum	KZ14	151-300
CL017	GM33-1	Lactobacillus	fermentum	GL34	15-150
CL018	GM61-1	Lactobacillus	fermentum	GL34	15-150
CL016	GM59-2	Lactobacillus	fermentum	GL34	15-150
CL006	KM165	Lactobacillus	brevis	GL37	15-150
CL007	KM176	Lactobacillus	brevis	KZ26	451-750
CL005	KM164	Lactobacillus	brevis	KZ25	451-750
CL009	KM86	Lactobacillus	brevis	KZ29	451-750
CL004	KM147	Lactobacillus	brevis	KZ27	451-750
CL008	KM88	Lactobacillus	brevis	KZ26	451-750
CL003	KM86	Lactobacillus	brevis	KZ25	301-451
CL001	KM26	Lactobacillus	brevis	KZ21	301-451
CL002	KM15	Lactobacillus	brevis	KZ14	151-300

Figure 3

Dendrogram depicting of 33 Lactobacillus isolates based on rep-PCR

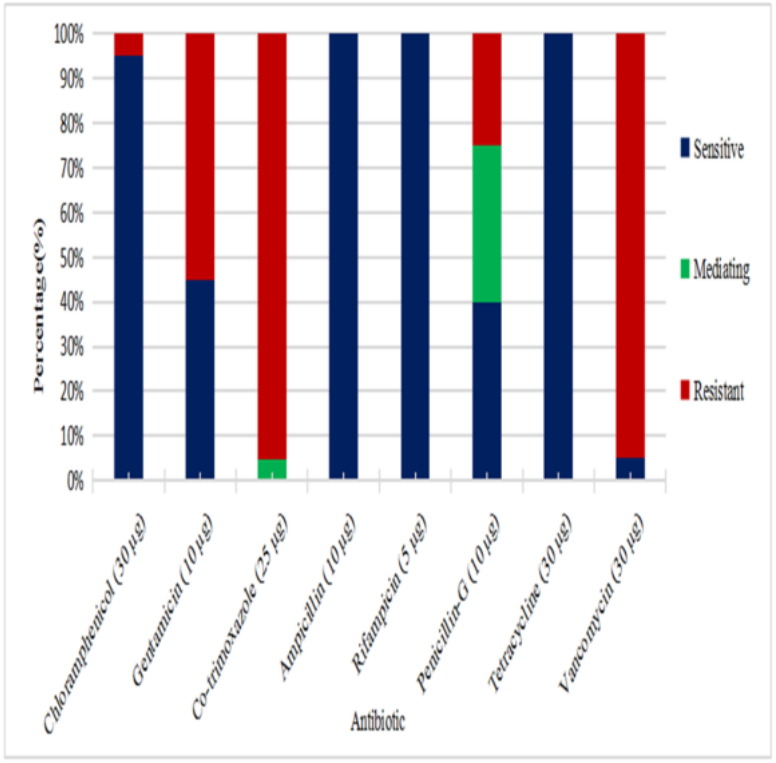


Figure 4

Antibiotic resistance rate of Lactobacillus from breast milk

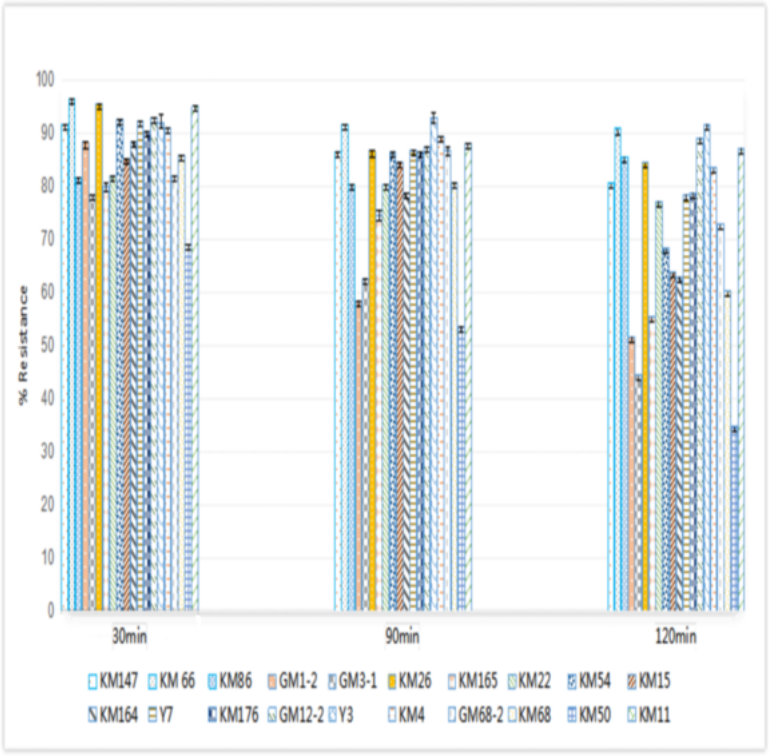


Figure 5

Effect of lysozyme treatment on the viability of Lactobacillus

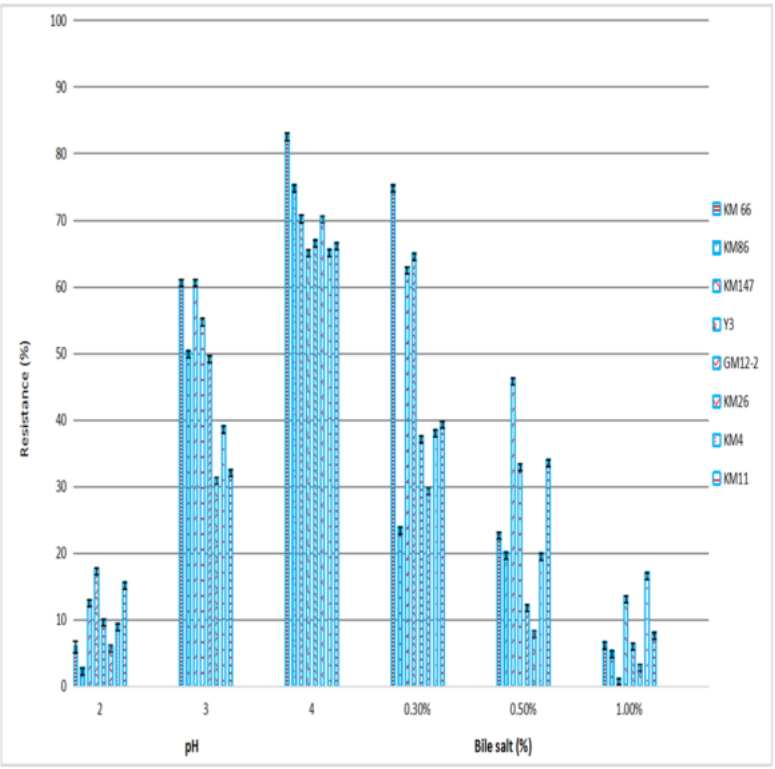


Figure 6

Effect of different acid pH values and bile salt concentration on survival of Lactobacillus isolates