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Original research article

The potential of species-specific tagatose-6-phosphate (T6P) pathway in *Lactobacillus casei* group for galactose reduction in fermented dairy foods

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Abstract

Residual lactose and galactose in fermented dairy foods leads to several industrial and health concerns. There is very little information pertaining to manufacture of fermented dairy foods that are low in lactose and galactose. In the present study, comparative genomic survey demonstrated the constant presence of chromosome-encoded tagatose-6-phosphate (T6P) pathway in *Lactobacillus casei* group. Lactose/galactose utilization tests and β-galactosidase assay suggest that PTS^{Gal} system, PTS^{Lac} system and T6P pathway are major contributors for lactose/galactose catabolism in this group of organisms. In addition, it was found than lactose catabolism by *Lb. casei* group accumulated very limited galactose in the MRS-lactose medium and in reconstituted skim milk, whereas *Streptococcus thermophilus* and *Lb. delbrueckii* subsp. *bulgaricus* (*Lb. bulgaricus*) strains secreted high amount of galactose extracellularly. Moreover, co-culturing *Lb. casei* group with *Str. thermophilus* showed significant reduction in galactose content, while co-culturing *Lb. casei* group with *Lb. bulgaricus* showed significant reduction in lactose content but significant increase in galactose content in milk. Overall, the present study highlighted the potential of *Lb. casei* group for reducing galactose accumulation in fermented milks due to its species-specific T6P pathway.

Keywords: comparative genomics; galactose catabolism; tagatose-6-phosphate pathway; phosphotransferase system; *Lactobacillus casei* group

1. Introduction

Lactose containing two moieties of glucose and galactose is the principal carbohydrate in mammalian milks (Adam et al., 2004). Most of the cultured dairy foods have been manufactured with starter cultures including conventional starters and functional starters for improving the shelf life, technological and nutritional aspects of milk products (Leroy and De Vuyst, 2004). Conventional starters including *Streptococcus thermophilus*, *Lactobacillus delbrueckii* subsp. *bulgaricus* (*Lb. bulgaricus*) and *Lactococcus lactis* are typical dairy starters for fermenting milk due to their capabilities of fast acidification, sufficient proteolysis, texture improvement and flavor enhancement (Buckenhuskes, 1993; Leroy and De Vuyst, 2004). Research related to probiotics has accelerated the use of probiotic bacteria as functional starters for milk fermentation (Granato et al., 2010). However, investigations in cultured dairy foods and other milk products have highlighted the occurrence of lactose and galactose accumulation in these products (Alm, 1982; Portnoi and MacDonald, 2009); although consumption of these products may not result in symptoms of lactose intolerance and galactosemia, dairy products containing high level of lactose and galactose are not recommended in diets for individuals with these problems (Silanikove et al., 2015; Van Calcar et al., 2014). Moreover, accumulated galactose in fermented dairy products has been associated with several industrial concerns including shoddier qualities of fermented product, browning in pizza and textural defects in cheese (Neves et al., 2010; Wu et al., 2015). Thus, efforts to remove or reduce lactose and galactose would be of health and industrial importance. Removal of lactose from milk generating lactose-free and lactose-reduced milk products could be achieved enzymatically or physically (Harju et al., 2012; Jelen and Tossavainen, 2003). Hydrolysis of lactose by lactase releases the moiety of galactose from lactose, however, this process is not able to eliminate galactose from milk and thus lactose-free milk products generated from this method is inhibited in diets of galactosemic individuals but is acceptable for lactose-intolerant people. The second method for lactose removal involves various chromatographic methods for the separation of lactose from other milk constitutes; this approach could eventually produce lactose-free and galactose-free milk products (Harju et al., 2012). However, these

commercialized lactose-free milk products by are sold in liquid form by dairy companies. Since fermented milk products have more advantages over non-fermented milk products with regards to nutritional and health-promoting benefits, it is necessary to rethink above technologies and develop new strategy to manufacture lactose-free or/and galactose-free fermented milk.

Some of lactic acid bacteria (LAB) could import lactose through a permease or lactose/galactose antiporter followed by hydrolysis with cytosolic β-galactosidase into glucose and galactose; these two monosaccharides are metabolized by Embden-Meyerhof-Parnas (EMP) pathway and Leloir pathway, respectively (Devos and Vaughan, 1994). Similarly, *Bifidobacterium* catabolizes lactose through β-galactosidase, Leloir pathway and bifid shunt after uptake via the membrane (Gonzalez-Rodriguez et al., 2013). Importantly, lactose-specific or galactose-specific phosphotransferase systems (PTS^{Lac} and PTS^{Gal}) have been reported in individual strains of *Lb. casei* (Bettenbrock et al., 1999; Chassy and Thompson, 1983a, b), *Lb. rhamnosus* (Tsai and Lin, 2006), *Lb. gasseri* (Francl et al., 2012) and *Lc. lactis* (Neves et al., 2010); these strains uptake lactose or galactose via membrane PTS systems resulting in the formation of lactose/galactose-6-phosphate which is further catabolized by tagatose-6-phosphate (T6P) pathway (Devos and Vaughan, 1994). Interestingly, T6P pathway does not release galactose but catabolizes galactose-6-phosphate after phosphorylation of lactose during uptake (Devos and Vaughan, 1994). This brings new insights into lactose and galactose metabolism in LAB since at least some strains having this pathway were identified and characterized; lactose-reduced and galactose-reduced fermented dairy products especially aged-cheeses could be manufactured with above unique strains. However, it is still unknown as to whether T6P pathway is strain- or species-specifically distributed in the species mentioned above, and most importantly the capacity of these T6P-positive strains for eliminating lactose and galactose. In addition, more than one species of starter culture are normally used in modern manufacture of fermented dairy products, such as *Str. thermophilus* and *Lb. bulgaricus* for yogurts, and *Lc. lactis* subsp. *cremoris* and *Lc. lactis* subsp. *lactis* for Cheddar cheese (Leroy and De Vuyst, 2004). Thus, it

would be necessary to evaluate the contents of lactose and galactose in milk when above T6P-positive *Lactobacillus* strains and conventional starters are both added as a co-culture.

Our recent comparative survey based on Kyoto Encyclopedia of Genes and Genomes (KEGG database) conducted for all the completely sequenced strains of LAB and *Bifidobacterium* indicated the presence of chromosome-encoded T6P pathway in the strains of *Lb. casei*, *Lb. rhamnosus* and *Lb. paracasei*, whereas plasmid-encoded T6P pathway was found in some strains of *Lc. lactis* (Wu et al., 2015). This suggests their unique capacity to catabolize lactose and galactose at the species level. Thus, in the present study, comparative genomic survey for T6P pathway identification in all the sequenced (complete, chromosome, scaffold and contig levels) strains of *Lb. casei* group released in GenBank database has been carried out. Further comparative characterization has been carried out to illustrate lactose and galactose catabolic capacity in *Lb. casei* group strains and dairy starters.

2. Materials and methods

2.1 Comparative genomic survey

We previously observed the strain-specific characteristic of *Lc. lactis* subsp. *cremoris* that encodes T6P pathway in plasmids (Wu et al., 2015). Only a limited number of strains have been completely sequenced and their metabolic maps were constructed and released in KEGG database. To understand whether it is species-specific or strain-specific in *Lb. casei* group, identification of genetic elements in T6P pathway in all the sequenced strains of *Lb. casei* group from GenBank database was carried out by searching enzymes involved in this pathway for each genome assembly and annotation report of 187 strains including 34 strains of *Lb. casei*, 98 strains of *Lb. rhamnosus*, 53 strains of *Lb. paracasei* and 2 strains of *Lb. zeae* dated 25 June 2016. These strains included in the survey have varying origins.

2.2 Genomic islands analysis

The position of *lac-gal* cluster in the chromosomes of completely sequenced representative strains of *Lb. casei* group was identified prior to genomic island analysis in IslandView3 integrating three different genomic island prediction methods including IslandPick, IslandPath-DIMOB and SIGI-HMM (Dhillon et al., 2015). This analysis is able to differentiate the horizontal origins in their chromosomes.

2.3 Bacterial strains and cultivation conditions

Bacterial strains used in this study are listed in Table 1. *Lactobacillus* strains were cultivated in lactobacilli MRS medium (BD Company, Franklin Lakes, NJ, USA) containing glucose as the carbon source, while strains of *S. thermophilus* were inoculated in M17 medium containing lactose as the carbon source.

2.4 Phenol-red carbohydrate utilization assay

A phenol-red carbohydrate broth (pH 7.4) consisted of 10 g/L of proteose peptone, 5 g/L of sodium chloride, 1 g/L of beef extract, 0.018 g/L of phenol red and 10 g/L of lactose or galactose was used to test the utilization ability of lactose and galactose by selected LAB strains with an inoculation size of 1% (v/v). Fresh cultures of selected LAB strains (18 h) were used to inoculate into the phenol-red carbohydrate broth. The changes in color of the broth were monitored for 24 h at 37℃. A yellow color indicated a positive fermentation, whereas a reddish color indicated a negative utilization of carbohydrate.

2.5 Bacterial growth in modified MRS broth containing glucose, lactose or galactose

MRS broth is an ideal medium for growing LAB due to rich nutrients. In this study, substitution of glucose with lactose or galactose in MRS broth was carried out to prepare MRS-lactose and MRS-galactose broth, which was used to test the ability of lactose and galactose utilization, respectively, by the selected *Lb. casei* group strains. MRS-glucose was used as the control fermentation. The recipe of the modified MRS is as follows: 10 g Bacto™ peptone, 10 g beef extract, 5 g yeast extract, 1 g Tween-80, 20

g α-D-glucose (for MRS-glucose broth) or 20 g β-D-galactose-(1→4)-α-D-glucose (for MRS-lactose broth) or 10 g β-D-galactose (for MRS-galactose broth), 2 g trisodium citrate dihydrate, 5 g sodium acetate trihydrate, 0.1 g magnesium sulfate heptahydrate, 0.05 g manganese sulphate monohydrate, 2 g potassium monohydrogen phosphate and 1 L of distilled water. The pH of the three broth media was adjusted to 6.5 with 2 M hydrogen chloride and autoclaved at 121℃ for 15 min.

All the selected *Lb. casei* group strains and other LAB strains (Table 1) were firstly activated in MRS-glucose broth three times at 37℃ for 18 h before inoculating (2%; v/v) the cultures to the MRS-no sugar, MRS-glucose, MRS-galactose and MRS-lactose broths indivivisually. Optical density at the absorbance of 600 nm was used to assess the growth of each bacterial strain cultivated in MRS-no sugar, MRS-glucose, MRS-galactose and MRS-lactose broth as previously described (Wu et al., 2016). Briefly, a volume of 200 μL of each broth after inoculated with each culture was loaded into triplicate wells of 96-well microplates, followed by addition of 50 μL of sterile mineral oil to cover the surface of the broth. Bacterial growth was monitored in Multiskan™ GO microplate spectrophotometer (Thermo Scientific) at 37℃ within 24 h. For sugar catabolic capacity tests, long-term (72-h) cultivation was carried out to minimize the effect of inoculation size of different strains on their sugar utilization, and the volume of for incubation in each broth was 10 mL in 14-mL tube.

2.6 Reconstituted skim milk fermentation

Skimmed milk powder was reconstituted with distilled water and was sterilized at 121℃ for 15 min to kill all the bacteria including thermophiles and spore-forming *Bacillus* generally found in the milk powder. Bacterial strains were cultivated in MRS-glucose medium at 37℃ for 18 h three times prior to inoculation into the 8 mL of 10% (w/v) reconstituted skim milk (RSM; Nestlé® Carnation® Skimmed Milk Powder consisting of 27 g protein and 42 g lactose per 100 g of the powder). The conditions for milk fermentation were at 37℃ for 72 h without shaking. Independent experiments were performed in triplicates, and samples were collected for analysis after fermentation.

2.7 Determination of sugars and acids

For the culture broths, MRS-lactose and MRS-galactose, centrifugation was carried out at 12,000 × g and 4℃ for 10 min to obtain the cell-free supernatants; which were 10-fold diluted for HPLC analysis. For the fermented milk, 1 g of fermented milk was diluted in 9 mL of distilled water and further centrifuged at 5,000 ×g and 4℃ for 30 min to obtain the supernatants and 20 µL of 20% (w/w) acetic acid was added to the diluted suspension if the milk was not coagulated with or that without bacterial fermentation. All the clear supernatants containing sugars and acids were filtered through 0.20 µm filter membrane.

Supernatants containing lactic and acetic acids were separated and quantified using HPLC (Model Shimadzu LC-2010A, Shimadzu Corporation, Kyoto, Japan) equipped with Aminex HPX-87H HPLC column (Bio-Rad) as previously described (Wu et al., 2016).

Reducing sugars including lactose, glucose and galactose in the diluted samples were first derivatized with 1-phenyl-3-methyl-5-pyrazolone (PMP) as previously described (Li and Shah, 2016). Briefly, 200 µL of sample was mixed with 200 µL of 0.6 M sodium hydroxide, followed by the addition of 400 µL of 0.5 M PMP-methanol solution, and was vortexed thoroughly for 5 seconds. The mixture was then kept in an oven at 70℃ for 90 min. After incubation, the mixture was placed in ice for 5 min and 400 µL of 0.3 M hydrogen chloride was added to neutralize the mixture. Chloroform (400 µL) was used to extract residual PMP from the mixture and this process was repeated four times. The PMP-sugar derivatives in the aqueous layer were passed through a 0.20 µm filter membrane, and were further separated and quantified by HPLC (Model Shimadzu LC-2010A, Shimadzu Corporation, Kyoto, Japan) equipped with Kromasil 5u 100A C18 column (250 mm × 4.6 mm; Phenomenex). The PMP-sugar derivatives were eluted by a mixture of phosphate buffered saline (1X; pH 6.7) and acetonitrile in a ratio of 83:17 (v/v) at a flow rate of 1 mL/min. The column temperature was maintained at 30 ℃ and the

absorbance was set to 245 nm. Standards (lactose, galactose, glucose) prepared in the range of 100 mg/L to 4,000 mg/L were used to generate standard curves for quantitation.

2.8 Measurement of β-galactosidase activity in the whole cell

The β-galactosidase activity in the whole cell was measured with 2-nitrophenyl-β-D-galactopyranoside (ONPG) as previously described with minor modifications (Yu and O'Sullivan, 2014). Briefly, selected bacterial strains were first activated in MRS-glucose broth, and later inoculated in MRS-lactose broth at the size of 1% (v/v). Fresh bacterial cells at the log phase stage were used for this assay. An aliquot of 100 µL of fresh cultures (OD_{600}) or medium (as blank) was added to 900 µL of Z buffer (60 mM Na_2HPO_4, 40 mM NaH_2PO_4, 10 mM KCl, 1 mM $MgSO_4$), followed by the addition of 10 µL of chloroform and vortexed for 10 seconds. Then, 200 µL of a substrate solution (60 mM Na_2HPO_4, 40 mM NaH_2PO_4, 4 mg/mL ONPG) was added and the mixture was incubated at 30℃ in a water bath for yellow color development. The duration of reaction was determined by the OD_{420} value reaching in the range of 0.6 to 0.9. If there was no yellow color development, the maximum incubation time was 90 min. The reaction was terminated by adding 500 µL of the stop solution (1 M Na_2CO_3) to the mixture. The cell debris was removed by centrifugation at 10,000 ×g and 25 for 5 min. The supernatants were collected for measurement of OD at 420 nm. The enzymatic activity (Miller units) was calculated as follows: $(1000\times OD_{420})/[OD_{600}\times$volume of cultures (mL) × reaction time (min)].

2.9 Statistical analysis

All presented data in the bar charts correspond to means ± standard deviation (SD). Significant difference ($p < 0.05$ or $p < 0.01$) among the groups was carried out by one-way analysis of variance (ANOVA) using IBM SPSS Statistics 20.0 version.

3. Results

3.1 T6P pathway is an ancient, species-specific pathway in *Lb. casei* group

The phylogenetic tree of lactic acid bacteria demonstrated a close genetic relationship among above three species which were classified into *Lb. casei* group (Fig. S1). We observed the constant presence of T6P pathway in all of the 187 sequenced strains of *Lb. casei* group of varying origin (Table 2). Based on this survey, it was generally concluded that T6P pathway is species-specific in *Lb. casei* group. This suggests that T6P pathway could catabolize lactose without accumulating galactose extracellularly (Fig. 1). For conventional dairy starters such as *Str. thermophilus* and *Lb. bulgaricus*, lactose was hydrolyzed by their β-galactosidases while secreting a large amount of galactose released from lactose to milk medium; reducing sugars including galactose contribute to browning reaction efficiently (Dattatreya et al., 2010), thus *Lb. casei* group has the potential to reduce galactose content in Mozzarella cheese for the manufacture of pizza without reduced browning reaction.

To understand the origin of T6P pathway in this group, we identified the location of *lac-gal* cluster in chromosomes of completely sequenced representative strains of *Lb. casei* group (Fig. S2). Further horizontal origin analysis generated from IslandViewer3 indicated that *lac-gal* cluster was not close to genomic islands in these representative strains (Fig. S3). This illustrates that T6P pathway and Leloir pathway may be of ancient origins in *Lb. casei* group but not through gain-of-function events such as horizontal gene transfer.

3.2 Effects of lactose and galactose on the growth of *Lb. casei* group strains

Very limited information is available in regards to the lactose utilization capacity in *Lb. casei* group strains compared to other LAB strains. A number of 12 strains of *Lb. casei* group of varying origins and 9 strains of other LAB strains of dairy origin were included in this study (Table 1). Phenol-red lactose and galactose utilization tests were carried out prior to fermentation studies. It is clear that the truncation of *lacG* in *lac-gal* cluster in *Lb. rhamnosus* GG leads to the failure of its lactose catabolism (Fig. S2 and

Table 1). Three strains (GG, WQ2 and W14) of *Lb. casei* group were identified as lactose-utilization-negative strains (Table 1). However, these strains could ferment galactose indicating their T6P pathway (*lacA*, *lacB*, *lacC* and *lacD*) is functional or at least not deficient. Thus, the failure of lactose utilization in strains WQ2 and W14 may be due to the truncation of *lacG* or deficient in function of PTS^{Lac} (*lacE* and *lacF*).

Bacterial growth of 22 strains inoculated in MRS broth supplemented with individual sugar including lactose, galactose and glucose is indicated in Fig. S4. A shown in this figure, *Str. thermophilus* and *Lb. bulgaricus* strains were not able to survive in the MRS medium where galactose was the sole carbon source. Notably, *Lb. acidophilus* group strains (511, 953 and 2413) could utilize galactose suggesting their unique galactose permease and Leloir pathway that were different than that of *Str. thermophilus* and *Lb. bulgaricus* (Fig. S4). Since three strains (GG, WQ2 and W14) of *Lb. casei* group were negative for phenol-red lactose utilization test (Table 1), it was also observed that their growth in MRS-lactose broth was limited compared to that in MRS-no sugar broth. However, most of selected lactose-utilization-positive strains of *Lb. casei* group had very similar curves of bacterial growth in MRS-lactose, MRS-galactose and MRS-glucose broths (Fig. S4). In general, it is observed that the optical density for *Lb. casei* group strains was higher than those of other selected strains grown in MRS-glucose broth. Even in the MRS-no sugar broth, *Lb. casei* group strains could still replicate themselves (Fig. S4); this may be due to their unique metabolisms of nitrogen such as catabolizable amino acids (Sezonov et al., 2007).

3.3 *Lb. casei* group catabolize lactose with less galactose accumulation

As shown in Fig. 2A, lactose-utilization-positive *Lb. casei* group strains could produce high amount of lactic acid, which is significantly ($p < 0.05$) higher than those from other 9 LAB strains grown in MRS-lactose broth. The production of acetic acid from heterofermentative *Lb. casei* group strains was very limited (Fig. 2B). This suggests that the activity of pentose phosphate pathway was low in these strains

241 whereas most of substrate – glucose-6-phosphate was metabolized via EMP pathway (Fig. 1C).
242 Importantly, it was observed that dairy starter strains utilized less lactose but accumulated more galactose
243 than that of *Lb. casei* group strains (Fig. 2C-D). It was noted that *Lb. acidophilus* group strains also
244 accumulated less galactose than *Str. thermophilus* and *Lb. bulgaricus* strains (Fig. 2D). Moreover, there
245 was very limited amount of galactose accumulated in the medium inoculated with *Lb. casei* group strains
246 (Fig. 2D). Overall, it was demonstrated that *Lb. casei* group strains catabolized lactose with less galactose
247 secretion.

248 **3.4 β-Galactosidase activity was not detected *Lb. casei* group strains**

249 As mentioned above, lactose-utilization-negative *Lb. casei* group strains (GG, WQ2 and W14)
250 failed in lactose utilization could be due to either the truncation of *lacG* or deficient in function of PTS^{Lac}
251 (*lacE* and *lacF*). The autoclave temperature for MRS-lactose medium was 121℃ which partially broke
252 down lactose. Although the above three strains survived in the MRS-no sugar and MRS-galactose media
253 (Fig. S4), the production of acids and reduction in galactose content by these organisms were still
254 observed (Fig. 3). However, a significant ($p < 0.05$) reduction in lactose content by these strains (Fig. 3B)
255 was still observed. To understand the reason for this reduction, β-galactosidase activities of whole cells of
256 above strains and other selected strains were measured and the result is shown in Fig. 4. Although these
257 strains were cultivated in MRS-lactose for cytosolic β-galactosidase induction, the β-galactosidase
258 activities of *Lb. casei* group strains were not detected within 90 min in the ONPG assay (Fig. 4). This
259 indicates that *Lb. casei* group may not express β-galactosidase to hydrolyze lactose, but just applied T6P
260 pathway for lactose and galactose catabolism.

261 **3.5 *Lb. casei* group catabolize galactose efficiently**

262 *Lb. casei* group strains showed very positive indication for galactose utilization (Table 1 and Fig.
263 S4). To understand its capacity to utilize galactose, we cultivated these strains and dairy starters in MRS-

galactose broth. As shown in Fig. 5, *Lb. casei* group strains including lactose-utilization-positive and -negative strains could utilize almost all of the galactose in the medium after 72 hour of incubation. It is noted that *Lb. acidophilus* group strains also utilized certain amount of galactose (Fig. 5C). This indicates that their Leloir pathway was more active than those of *Str. thermophilus* and *Lb. bulgaricus* strains, but was still less effective than T6P pathway and Leloir pathway in *Lb. casei* group. In general, combination of T6P pathway and Leloir pathway in *Lb. casei* group was very efficient in galactose catabolism.

3.6 Lactose depletion by *Lb. casei* group was lower than those by conventional dairy starters

MRS medium is an ideal medium for culturing *Lb. casei* group strains. To evaluate the capacity of lactose utilization in the milk environment, these strains were inoculated in RSM and the results are shown in Fig. 6. Although a reasonable amount of lactic acid production from lactose-utilization-positive *Lb. casei* group strains was detected, it was observed that less lactose was utilized by these strains than those by dairy starters (Fig. 6A-B). However, very limited amount (below 250 mg per 1 kg of fermented milk) of galactose was detected in milk fermented by *Lb. casei* group strains, whereas high amount of galactose was accumulated in milk by dairy starter fermentations (Fig. 6C).

Since *Lb. casei* group strains were not typical starters for milk fermentation, co-cultures of these strains with dairy starters including *Str. thermophilus* and *Lb. bulgaricus* was carried out for milk fermentation. Co-culturing increased lactic acid production significantly ($p < 0.01$) from these organisms compared to the fermentation using mono-culture of *Str. thermophilus* ASCC 1275 or *Lb. bulgaricus* ASCC 756 alone (Fig. 6D). Importantly, co-culturing of *Str. thermophilus* ASCC 1275 with *Lb. casei* group (strains 290 or Shirota) reduced galactose content significantly ($p < 0.01$) compared to the fermentation by the former strain alone, while lactose content was not significantly changed between mono-culturing and co-culturing (Fig. 6E). However, co-culturing of *Lb. bulgaricus* ASCC 756 with *Lb. casei* group (strains 290 or Shirota) reduced lactose content significantly ($p < 0.01$) compared to the fermentation by the former strain alone, while galactose content was also significantly ($p < 0.01$) changed

after co-culturing (Fig. 6F). Thus, it appears that the interactions between dairy starters and *Lb. casei* group strains vary from case to case.

The behavior of lactose-utilization-negative strains of *Lb. casei* group in milk is associated with their Leloir pathway. Lactic acid production and galactose reduction were still observed in these organisms (Fig. 6G-H); this suggests that their T6P pathway was responsible for galactose reduction in milk.

4. Discussion

Residual lactose and galactose in fermented milk products can lead to several concerns in the quality of dairy foods and health risk (Wu et al., 2015). The present study aimed to evaluate the efficiency of conventional dairy starters and other novel LAB strains for lactose and galactose reduction. Firstly, we found the species-specific presence of T6P pathway in *Lb. casei* group (Table 2). There have been several reports on the identification or characterization of T6P pathway and lactose- or galactose-specific PTS systems in individual LAB strains (Bettenbrock et al., 1999; Chassy and Thompson, 1983a, b; Francl et al., 2012; Tsai and Lin, 2006). To our knowledge, this is the first global review on the identification of T6P pathway in all the sequenced strains of *Lb. casei* group offering novel insights for lactose and galactose catabolism in LAB at the species level (Fig. 1).

Prior to the catabolism of lactose and galactose by T6P pathway, uptake of lactose and galactose through PTS systems is the first step. Although transport assay and isotopic labeling-assisted metabolite analysis have identified the presence of two specific PTS^{Gal} and PTS^{Lac} systems in *Lb. casei* group (Bettenbrock et al., 1999; Chassy and Thompson, 1983a), genetic elements encoding a PTS^{Gal} system have never been identified among this group. In addition, genes encoding *lacZ* and lactose permease or lactose/galactose antiporter have also not been identified in the genome of this group strains (data not shown). In a previous report, β-galactosidase activity in *Lb. rhamnosus* TCELL-1 was detected by

311 growing it in medium agar plate spreading 5-bromo-4-chloro-3-indolyl-β-d-galactopyranoside (X-Gal) on
312 its surface though the data or photo was not shown (Tsai and Lin, 2006). This method generated blue
313 colonies if the bacteria exhibited β-galactosidase (β-gal) activity after incubation for at least 12 hours.
314 However, our ONPG assay, a standard method for measuring β-gal activity of whole bacterial cell, failed
315 to detect the β-gal activity of *Lb. casei* group strains within 90 min, though these organisms were pre-
316 incubated in MRS-lactose broth. This suggests that β-gal activity of *Lb. casei* group was very limited; this
317 could be evidenced by the fact that *lacG*-deficient *Lb. rhamnosus* GG strain grown in MRS-lactose
318 medium could not utilize lactose efficiently (Fig. 3B). A significant ($p < 0.05$) increase in the acetic acid
319 production and a significant ($p < 0.05$) decrease in the lactose content indicate that there was galactose
320 moiety after lactose hydrolysis metabolized through Leloir pathway (Fig. 3). Hence, PTS^{Lac} system and
321 T6P pathway contributes largely to lactose catabolism in *Lb. casei* group.

322 The presence of Leloir pathway in individual strains of *Lb. casei* group has been identified
323 previously (Bettenbrock and Alpert, 1998; Tsai and Lin, 2006). In addition, we found that Leloir pathway
324 is common in all the completely sequenced strains of LAB and *Bifidobacterium* in our previous
325 comparative KEGG survey (Wu et al., 2015). Although gene encoding galactose permease was not
326 identified, the uptake activity by this permease was confirmed by transport assay for *ptsH* mutant strain of
327 *Lb. casei* 64H (Bettenbrock et al., 1999). In addition, UDP-galactose and UDP-glucose generated from
328 Leloir pathway were largely less abundant than galactose-6-phosphate and tagatose-1,6-diP produced
329 from T6P pathway in *Lb. casei* 64H as revealed by thin layer chromatography. Similarly, mutants of this
330 organism with defects in T6P pathway contained very limited UDP-galactose and UDP-glucose
331 intracellularly (Bettenbrock et al., 1999). This clearly confirms that T6P pathway contributes more to
332 galactose catabolism than Leloir pathway in *Lb. casei* group. Moreover, our sugar analysis also suggested
333 that *Lb. casei* group strains could utilize galactose efficiently (Fig. 5). Overall, T6P pathway and PTS^{Gal}
334 system are the major contributors to efficient galactose utilization in *Lb. casei* group.

335 Compared to conventional dairy starters, *Lb. casei* group strains catabolize lactose and galactose
336 with very limited galactose accumulated in the MRS-lactose medium, MRS-galactose medium and
337 skimmed milk (Fig. 2, Fig. 5 and Fig. 6). Cheeses normally contain less lactose and galactose than those
338 in yogurts or yogurt-like drinks because liquid whey from curds containing large amount of sugars is
339 drained off; further long-term ripening of cheeses allow bacterial metabolism for residual sugars (Hickey
340 et al., 2015). Hence, *Lb. casei* group could be added to the curds before ripening process to manufacture
341 galactose/lactose-low cheeses that are suitable for lactose intolerant and galactosemic people. Unlike the
342 domesticated dairy starters, cell viability and metabolic activity of *Lb. casei* group cells in milk
343 environment are the important concerns for dairy industry before adopting as dairy starter (Buckenhuskes,
344 1993). However, it is possible to apply this group of strains as adjunct dairy starters since they have
345 dominated and classified as non-starter LAB in cheeses (Peterson and Marshall, 1990; Settanni and
346 Moschetti, 2010). Galactose-rich Mozzarella cheese leads to undesirable browning of the final pizza
347 product made with such cheese after baking process (Wu et al., 2015). Our results showed that a
348 combination of *Str. thermophilus* and *Lb. casei* group is very promising for manufacturing galactose-
349 reduced Mozzarella cheese (Fig. 6E). Notably, it appears that the interacting behavior between *Str.*
350 *thermophilus* and *Lb. casei* group was different to that between *Lb. bulgaricus* and *Lb. casei* group; this is
351 evidenced by the sugar profiles (Fig. 6E-F). Although the co-culture experiment of *Lc. lactis* and *Lb.*
352 *casei* group strain was not carried out in this study, it was observed that certain strains of *Lc. lactis* subsp.
353 *cremoris* but not *Lc. lactis* subsp. *lactis* also possesses T6P pathway for galactose reduction (Wu et al.,
354 2015). Above two subspecies of *Lc. lactis* are normally used as cheese starters, the T6P-positive *Lc. lactis*
355 subsp. *cremoris* may help galactose reduction during the ripening process of cheese, i.e., aged Cheddar
356 cheese. This type of cheese with low galactose content has been recommended for consumption by
357 galactosemic people (Portnoi and MacDonald, 2009; Van Calcar et al., 2014). In addition, *Lb. casei* has
358 been found as dominant non-starter lactobacilli in Cheddar cheese (Peterson and Marshall, 1990; Settanni
359 and Moschetti, 2010); this suggests that cooperation between *Lb. casei* and *Lc. lactis* in milk may
360 contribute to galactose depletion. Further understanding about these interactions between *Lb. casei* group

and conventional dairy starters would be necessary prior to addition of *Lb. casei* group to milk fermentation and cheese ripening.

5. Conclusions

Comparative genomic survey demonstrated species-specific characteristic of T6P pathway in *Lb. casei* group for lactose and galactose catabolism. Although Leloir pathway has been confirmed for galactose metabolism in this group, the undetectable β-galactosidase activity in *Lb. casei* group strains suggests that very limited amount of lactose could be catabolized through β-galactosidase, Leloir pathway and EMP pathway. Combining the efficient galactose utilization by this group, we conclude that T6P pathway are the major contributor for utilizing lactose and galactose by *Lb. casei* group after uptake by PTS^{Lac} or PTS^{Gal} system. Importantly, this group catabolizes lactose with very limited galactose secretion in MRS-lactose and RSM. Overall, *Lb. casei* group has the potential for reducing galactose accumulation in fermented dairy foods.

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Figure legends

Fig. 1. General presentation on the Leloir and tagatose-6P pathways in lactic acid bacteria. (A) The *gal* operon. (B) The *lac* operon. (C) Diagram for two pathways. Denotation: 1, glucokinase; 2, phosphoglucomutase; 3, UDP-glucose pyrophosphorylase; 4, phosphatase. It is generally regarded that *lacA*, *lacB*, *lacC* and *lacD* encodes T6P pathway whereas Leloir pathway is encoded by *galM*, *galK*, *galT* and *galE*.

Fig. 2. *Lactobacillus casei* group strains utilize lactose with limited galactose accumulation in the MRS-lactose medium. (A) Lactic acid production. (B) Acetic acid production. (C) Residual lactose content. (D) Residual galactose content. Cultivation was carried out at 37℃ for 72 h under static condition. Denotation: ST, *Str. thermophilus*; LB, *Lb. bulgaricus*; LH, *Lb. helveticus*; LA, *Lb. acidophilus*; LC, *Lb. casei*; LR, *Lb. rhamnosus*; LP, *Lb. paracasei*. Lowercase letters (a, b, c & d) above the group bars is used to indicate the significance: values with no letters in common indicate a significance of $p < 0.05$.

Fig. 3. Genetically deficient *Lactobacillus casei* group strains was not able to utilize lactose efficiently in the MRS-lactose medium. (A) Acids production. (B) Residual lactose and galactose contents. Cultivation was carried out at 37℃ for 72 h under static condition. Denotation: LC, *Lb. casei*; LR, *Lb. rhamnosus*; LP, *Lb. paracasei*. *, $p < 0.05$; **, $p < 0.01$.

Fig. 4. *Lactobacillus casei* group is deficient in β-galactosidase activity. N.D., not detectable within 90 min of the assay. Denotation: ST, *Str. thermophilus*; LB, *Lb. bulgaricus*; LH, *Lb. helveticus*; LC, *Lb. casei*; LR, *Lb. rhamnosus*.

Fig. 5. *Lactobacillus casei* group strains utilize galactose efficiently in the MRS-galactose medium. (A) Lactic acid production. (B) Acetic acid production. (C) Residual galactose content. Incubation was carried out at 37℃ for 72 h under static condition. N.D., not detectable (value was set to zero for group statistics). Denotation: ST, *Str. thermophilus*; LB, *Lb. bulgaricus*; LH, *Lb. helveticus*; LA, *Lb. acidophilus*; LC, *Lb. casei*; LR, *Lb. rhamnosus*; LP, *Lb. paracasei*. Lowercase letters (a, b, c & d) above the group bars is used to indicate the significance: values with no letters in common indicate a significance of $p < 0.05$.

Fig. 6. *Lactobacillus casei* group is not able to utilize more lactose than conventional dairy starters, but accumulates less galactose in milk. (A) Lactic acid production. (B) Residual lactose content. (C) Residual galactose content. (D) Lactic acid production from co-cultures. (E) Residual lactose and galactose contents from co-cultures of *Str. thermophilus* and *Lb. casei* group strains. (F) Residual lactose and galactose contents from co-cultures of *Lb. bulgaricus* and *Lb. casei* group strains. (G) Lactic acid production from lactose-utilization-negative *Lb. casei* group strains in milk. (H) Residual lactose and galactose contents in milk fermented by lactose-utilization-negative *Lb. casei* group strains. Incubation was carried out at 37℃ for 72 h under static condition. N.D., not detectable (value was set to zero for group statistics). Denotation: ST, *Str. thermophilus*; LB, *Lb. bulgaricus*; LH, *Lb. helveticus*; LA, *Lb. acidophilus*; LC, *Lb. casei*; LR, *Lb. rhamnosus*; LP, *Lb. paracasei*. Lowercase letters (a, b, c, d & e) above the group bars is used to indicate the significance: values with no letters in common indicate a significance of $p < 0.05$. N.S., not significant; **, $p < 0.01$.

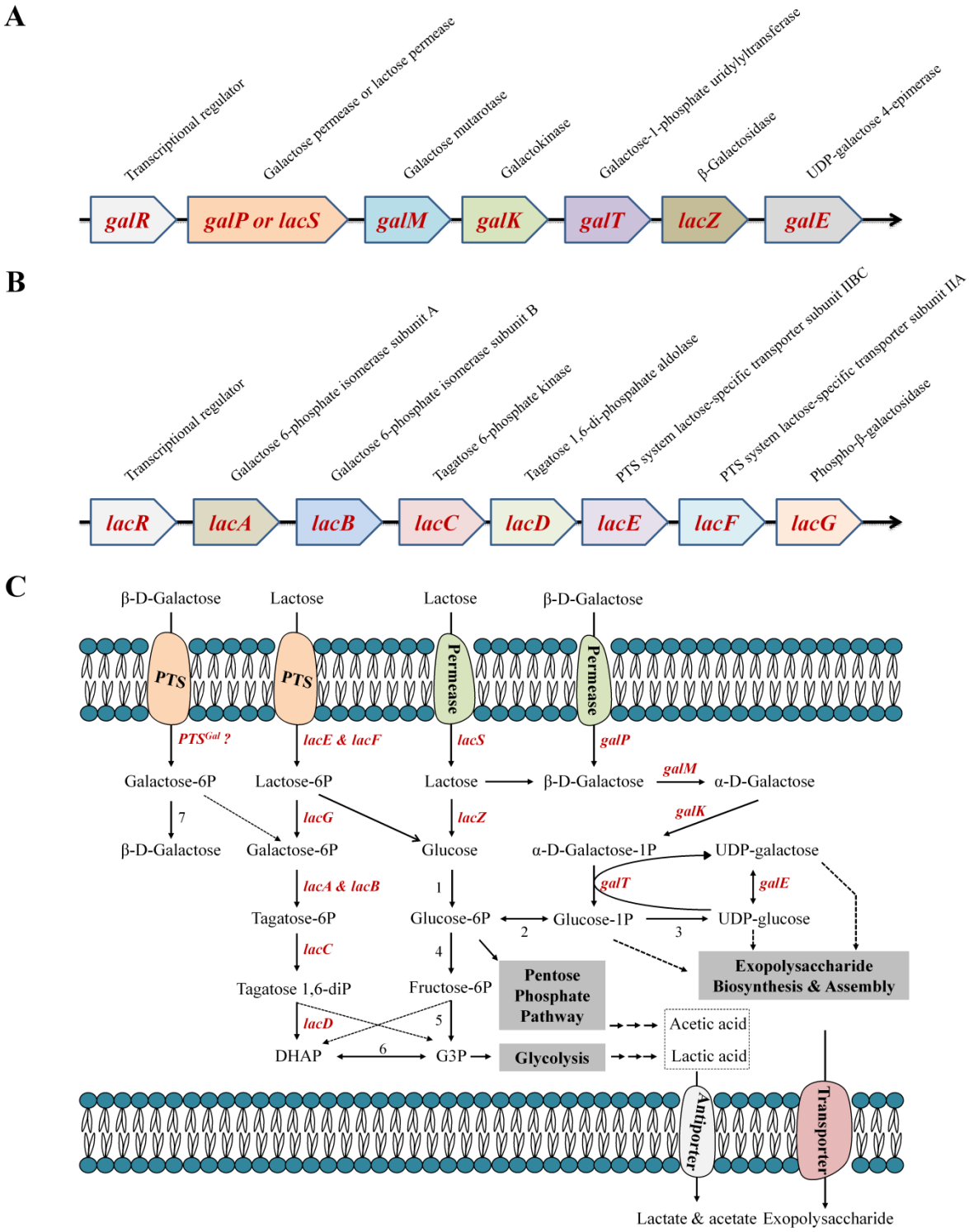

Fig.1. Wu and Shah

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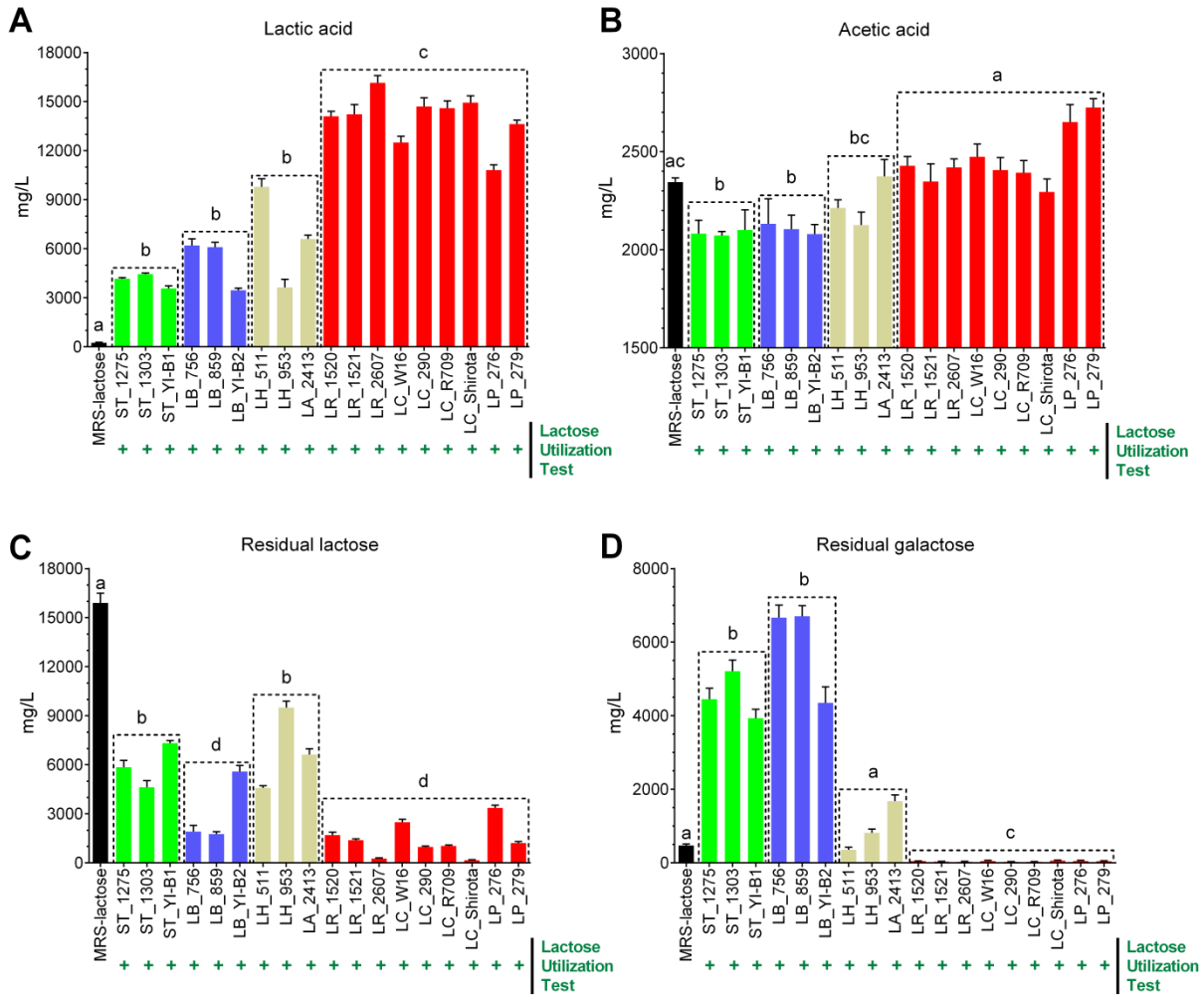

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491 **Fig. 2.** Wu and Shah

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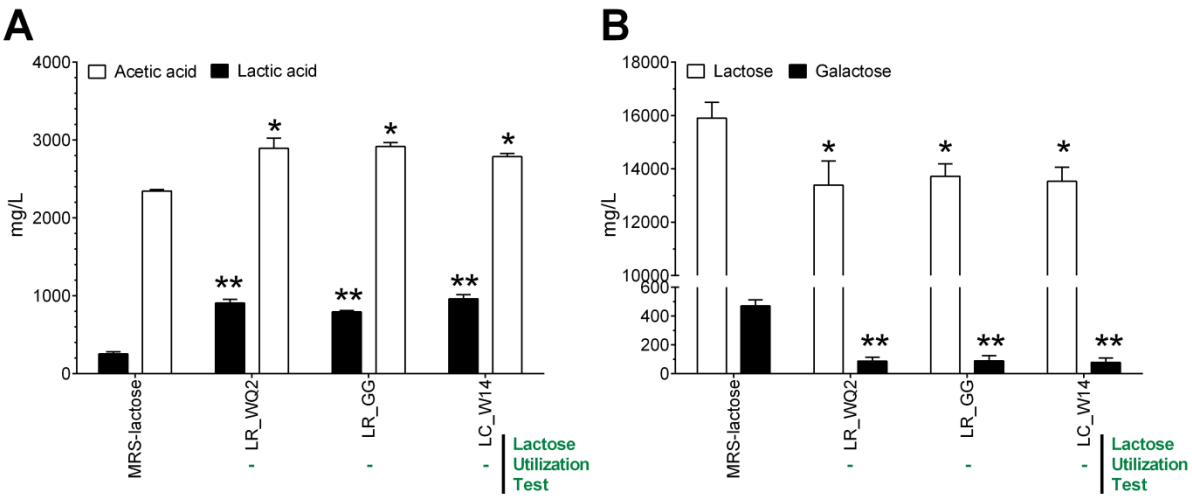

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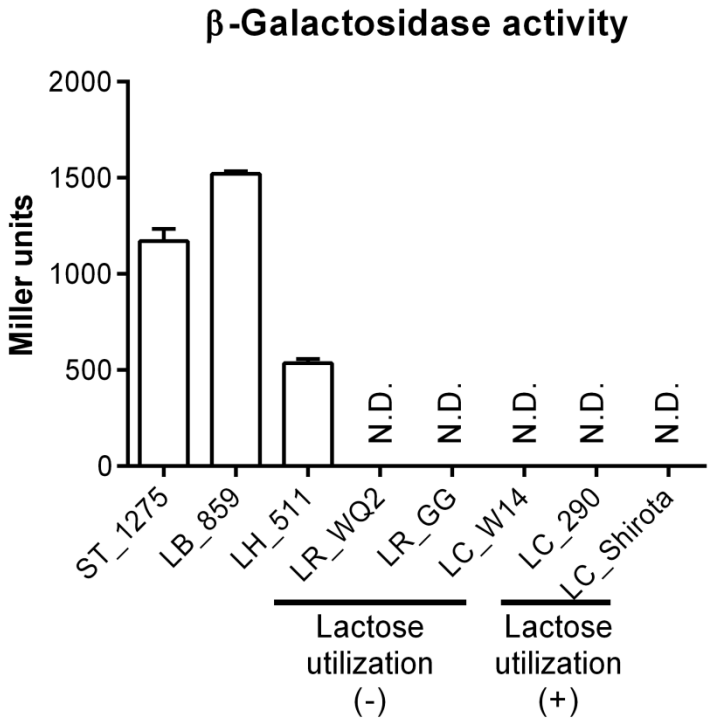
494 **Fig. 3.** Wu and Shah



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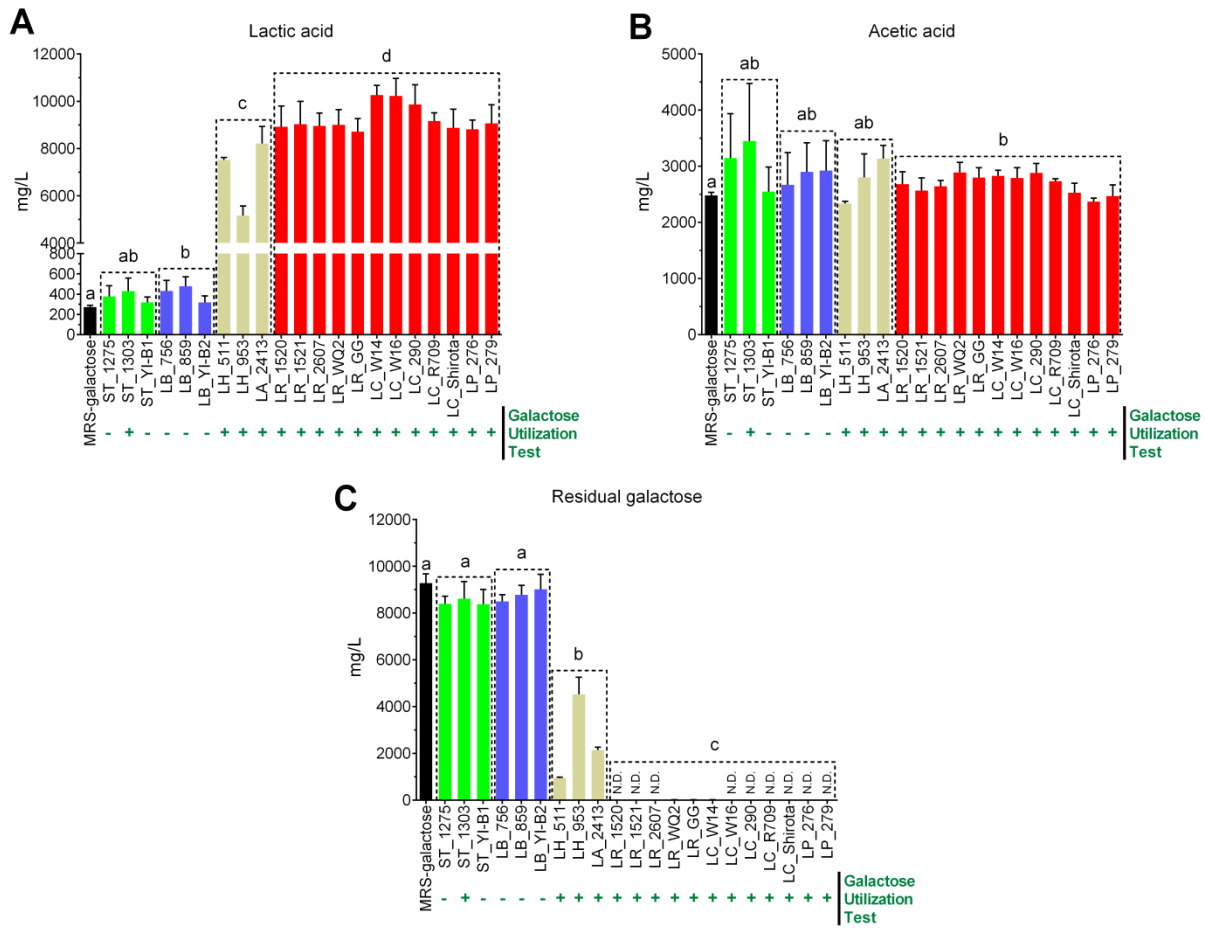

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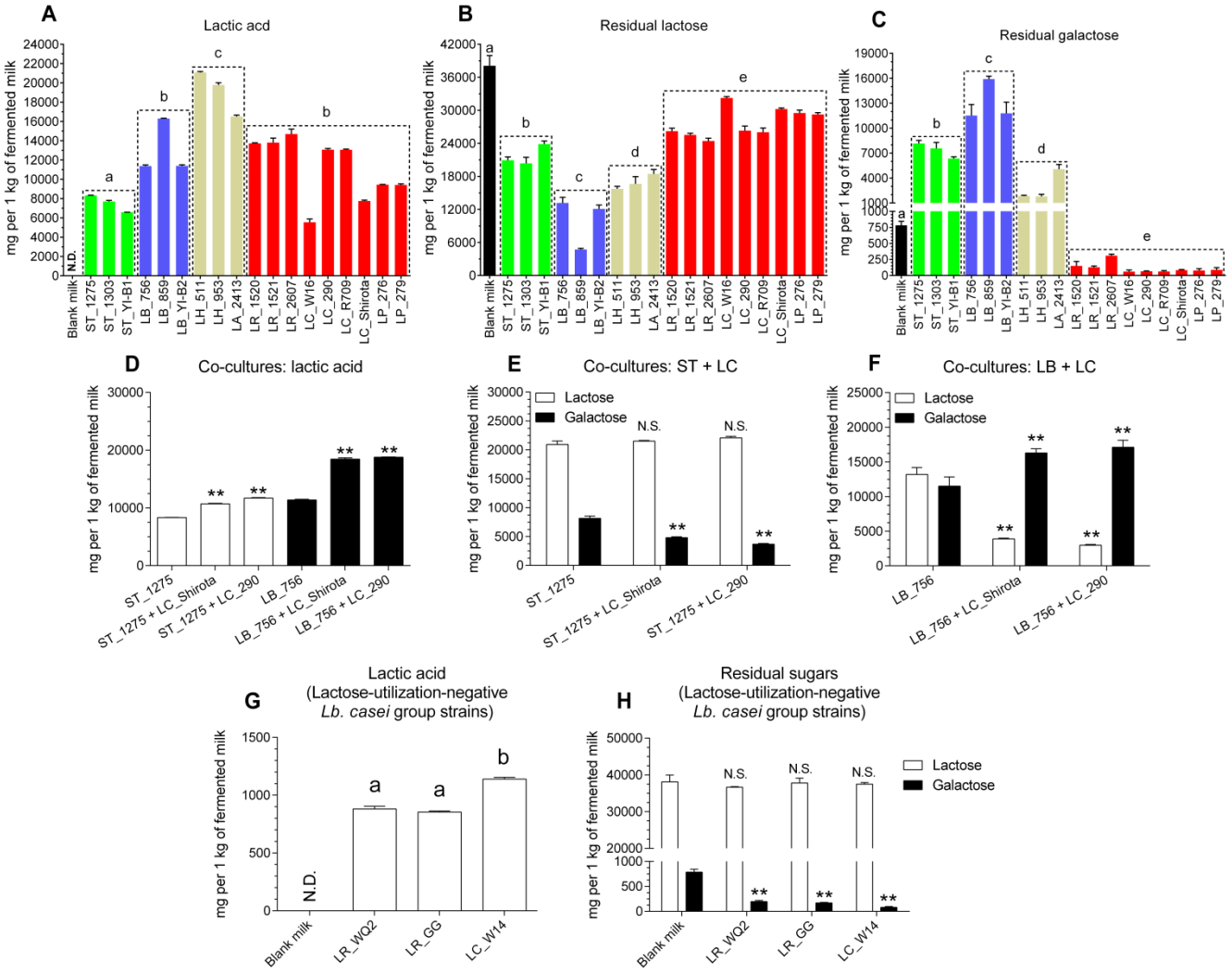

Fig. 6. Wu and Shah

Table 1. Bacterial strains used in this study and their sugar utilization capability

Classification	Species	Strain ID	Abbreviation	Source	Phenol red sugar utilization test	
					Lactose	Galactose
Conventional dairy starters	*Streptococcus thermophilus*	ASCC 1275	ST_1275	ASCC	+	-
		ASCC 1303	ST_1303	ASCC	+	+
		YI-B1	ST_YI-01	Laboratory stock	+	-
	Lactobacillus delbrueckii subsp. *bulgaricus*	ASCC 756	LB_756	ASCC	+	-
		ASCC 859	LB_859	ASCC	+	-
		YI-B2	LB_YI-02	Laboratory stock	+	-
***Lactobacillus acidophilus* group**	*Lactobacillus helveticus*	ASCC 511	LH_511	ASCC	+	+
		ASCC 953	LH_953	ASCC	+	+
	Lactobacillus acidophilus	CSCC 2413	LA_2413	CSCC	+	+
***Lactobacillus casei* group**	*Lactobacillus rhamnosus*	ASCC 1520	LR_1520	ASCC	+	+
		ASCC 1521	LR_1521	ASCC	+	+
		ASCC 2607	LR_2607	ASCC	+	+
		WQ2	LR_WQ2	Laboratory stock	-	+
		GG	LR_LGG	Laboratory stock	-	+
	Lactobacillus casei	W14	LC_W14	Laboratory stock	-	+
		W16	LC_W16	Laboratory stock	+	+
		ASCC 290	LC_290	ASCC	+	+
		R709	LC_R709	Laboratory stock	+	+
		Shirota	LC_Shirota	Laboratory stock	+	+
	Lactobacillus paracasei	ASCC 276	LP_276	ASCC	+	+
		ASCC 279	LP_279	ASCC	+	+

Note: ASCC, Australian Starter Culture Research Center; CSCC, CSIRO Starter Culture Collection. The result of sugar utilization tests (n=3) was generated after 72 h of incubation at 37℃. +, positive fermentation (yellow); -, negative fermentation (reddish). Denotation: ST, *Str. thermophilus*; LB, *Lb. bulgaricus*; LH, *Lb. helveticus*; LA, *Lb. acidophilus*; LC, *Lb. casei*; LR, *Lb. rhamnosus*; LP, *Lb. paracasei*.

Table 2. Distribution of T6P pathway (genes encoding *lacA*, *lacB*, *lacC* and *lacD*) in all the sequenced strains of *Lb. casei* group

Species	Sequencing level	No. of sequenced strains	T6P-positive strains
Lb. casei	Complete	8	8 (Zhang, BL23, BD-II, LC2W, 12A, W56, LOCK919, ATCC 393)
	Chromosome	2	2 (LcY, LcA)
	Scaffold	6	6 (A2-362, KL1-Liu, DSM 20011, 844_LCAS, BM-LC14617, Lbs2)
	Contig	18	18 (21/1, 32G, A2-362, CRF28, M36, T71499, UCD174, UW1, UW4, Lc-10, Lpc-37, UW4, 12A, 5b, N87, 867_LCAS, DPC6800, Lc1542)
Lb. paracasei	Complete	7	7 (ATCC 334, 8700:2, N1115, JCM 8130, CAUH35, L9, KL1)
	Scaffold	6	6 (ATCC 25302, DSM 20258, 1316.rep1_LPAR, 1316.rep2_LPAR, 275_LPAR, 525_LPAR)
	Contig	40	40 (Lpp230, Lpl7, Lpp122, Lpp46, Lpp226, Lpp120, Lpp223, Lpp228, Lpp221, Lpp49, Lpp227, CNCM I-2877, Lpp17, Lpp22, Lpp225, Lpp219, Lpp229, Lpp74, Lpp7, CNCM I-4270, Lpp189, Lpp14, Lpl14, Lpp37, CNCM I-4649, Lpp43, Lpp125, Lpp70, COM0101, DSM 20207, NRIC1981, NRIC1917, NRIC0644, DSM 5622, Lpp123, CNCM I-4648, Lpp48, Lpp126, Lpp71, Lpp41)
Lb. rhamnosus	Complete	7	7 (GG, ATCC 53103, Lc 705, ATCC 8530, LOCK900, LOCK908, BPL5)
	Chromosome	1	1 (ASCC 290)
	Scaffold	14	14 (LMS2-1, CASL, ATCC 21052, 769_LRHA, 784_LRHA, 979_LRHA, 944_LRHA, DSM 20021, HN001, 186_LRHA, 214_LRHA, 526_LRHA, 390_LRHA, 541_LRHA)
	Contig	76	76 (R0011, LRHMDP2, LRHMDP3, CRL1505, LR231, 51B, E800, PEL5, PEL6, K32, 24, L34, L35, L31, 116, 308, CLS17, CNCM-I-3698, Lr073, Lr071, Lr108, Lr138, 40f, 313, 319_LRHA, 870_LRHA, 699_LRHA, 708_LRHA, 893_LRHA, 906_LRHA, 988_LRHA, 943_LRHA, R19-3, Lrh8, Lrh32, Lrh31, Lrh29, Lrh26, Lrh23, Lrh22, Lrh20, Lrh19, Lrh15, Lrh11, Lrh1, Lrh34, Lrh9, Lrh7, Lrh6, Lrh5, Lrh4, Lrh30, Lrh3, Lrh28, Lrh27, Lrh25, Lrh21, Lrh24, Lrh2, Lrh18, Lrh17, Lrh16, Lrh14, Lrh13, Lrh12, Lrh10, Lrh33, Lrh42, Lrh44, Lrh43, MTCC 5462, 2166, Lr140, Lr053, Lr032, Lr044)
Lb. zeae	Scaffold	1	1 (DSM 20178)
	Contig	1	1 (KCTC 3804)
Total strains		187	100 % presence

Note: GenBank databased accessed in June 2016.