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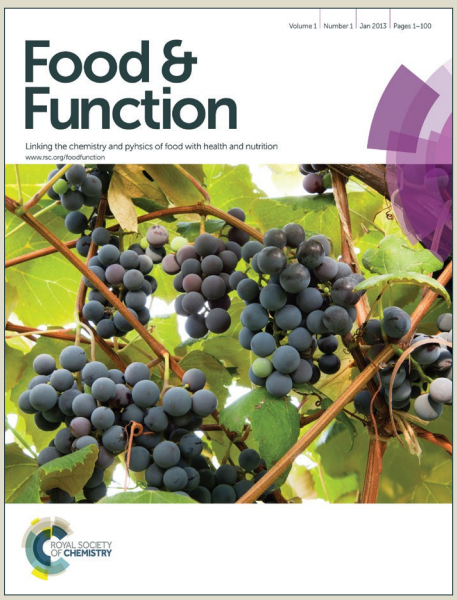

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In vitro Assessment of Antimicrobial Potentials of Lactobacillus helveticus Strains Isolated from Traditional Cheese in Sinkiang China against Food-borne Pathogens

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Abstract

The *Lactobacillus helveticus*, an obligatory hetero-fermentative LAB, as it is Generally Recognized as Safe (GRAS) and gaining popularity for application in dairy products. Lactic acid bacteria (LAB) plays remarkable role to inhibit the growth of pathogenic bacteria in food products, without disturbing the sensory attributes of food. In this study, Screening of antimicrobial potential of *Lactobacillus helveticus* KLDS1.8701 against four food-borne pathogens including *Listeria monocytogenes* ATCC 19115, *Salmonella Typhimurium* ATCC 14028, *Staphylococcus aureus* ATCC 25923, *Escherichia coli.*O157:H7 ATCC 43889 in vitro was inspected by using Oxford cup method and mixed culture inhibition assays. The organic acids productions and antimicrobial potential of cell-free supernatants (CFS) have been evaluated via different treatments and analysis by high performance liquid chromatography (HPLC). Analysis results revealed that KLDS1.8701 exhibited highest antimicrobial potential as compared to other antimicrobial strains. The antimicrobial activity of KLDS1.8701 resulted from organic acids in culture and CFS. From the study, it was depicted that carbon sources as well as organic acids production accelerate the antimicrobial activity of KLDS1.8701 and the fructooligosaccharides (FOS) were considered best to improve proliferation of KLDS1.8701 support its the antimicrobial action. Results of mixed culture inhibition assay showed part of antimicrobial activity resulted from inhibitory action of bacteria itself in culture, and this action required cellular contact between food-borne pathogens and KLDS1.8701. Conversely, results of antimicrobial spectrum assay revealed that Some *Lactobacilli* were remained unaffected by KLDS1.8701. KLDS1.8701 might be favorable to be also used as a supplementary starter in fermented dairy productions. Furthermore, KLDS1.8701 could survive well under GI tract conditions. Further studies on in vivo inhibition assays and probiotic effects are recommended.

Key words: Antimicrobial potentials, Assay, Food-borne diseases, *Lactobacillus helveticus,* food-borne pathogens, Tolerance to GI tract

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1 Introduction

Food-borne diseases (FBDs), defined by the World Health Organization was the ingestion of foodstuffs contaminated with microorganisms or chemicals. The adverse effects of contaminated foods on human health have been reported all over the world. It has been published that, in the United States, millions of people have been died due to FBDs.[1,2,3,4] Multi-organ failure, gastrointestinal, complications in immunological, gynaecological and neurological are common symptoms of attack of different food-borne pathogens, such as *Listeria monocytogenes*, *Salmonella typhimurium, Staphylococcus aureus*, *Escherichia coli.*O157. Consequently, the investigation and control of FBDs are multi-disciplinary tasks requiring skills in the area of clinical medicine, epidemiology, laboratory medicine, food microbiology and chemistry, food safety, food control, risk communication as well as food management.[1,5,6]

Lactic acid bacteria (LAB) plays remarkable role to inhibit the growth of pathogenic bacteria in food products, without disturbing the sensory attributes of food.[7,8] Strong evidence has been reported about the use of probiotic LABs for the prevention of antibiotic associated diarrhea.[9] Probiotic LABs have ability to produce substances organics acids (mainly lactic and acetic acids) and bacteriocins *etc.* which are considered as antimicrobial compounds. Probiotic bacteria adhere to intestinal epithelial cells prevent the colonization of pathogenic bacteria as well as stimulate immunity in the host.[10,11,12] Some *in vitro* studies have confirmed the ability of probiotics to obstruct the growth of pathogens like, *Escherichia coli* and *Campylobacter jejuni*,[13] to compete for adhesion to Caco-2 cells and prevent the enteropathogens from $CaCO_2$ cell surface layer.[14] However, many studies have demonstrated that, the use of LABs in different food products to increase its food chain value, ultimate enhancement in the food security, which

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yielded positive results in line with IDF's requirements for probiotic bacteria content in dairy products.[15,16]

The *Lactobacillus helveticus,* an obligatory hetero-fermentative LAB, as it is Generally Recognized as Safe (GRAS) gaining popularity for application in dairy products. *Lactobacillus helveticus,* used as a starter culture in the manufacture of semi-hard cheeses and fermented milk products.[17,18,19] It has been revealed that this microorganism produces substantially higher amounts (549 mg/mL) of exo-polysaccharides (EPS) under acidic culture conditions when grown in milk at 37°C.[20] Traditionally, *L. helveticus* used for the manufacture of Swiss-type cheeses and long-ripened Italian cheeses *i.e.* Emmental, Gruyere, Grana Padano and Parmigiano Reggiano. It has the potential to produce bioactive peptides or bacteriocins, and exerts symbiotic effect, when associated with prebiotics in fermented dairy products.[20] Thus, this multifunctional LAB strain holds promising potentials for the food and dairy industries.

Although many researches have been conducted to disclose the use of *Lactobacillus rhamnosus*,[21] *Lactobacillus plantarum*[22] as a probiotic, but less literature is available about the multiplications of *Lactobacillus helveticus*. In present study, we explored the antimicrobial properties of five strains of *Lactobacillus helveticus* isolated from traditional cheese in Sinkiang and preserved in KLDS (KLDS 1.0203, KLDS 1.8701, KLDS 1.9202, KLDS 1.9204 and KLDS 1.9207) against four food-borne pathogens: *Listeria monocytogenes* ATCC 19115, *Salmonella Typhimurium* ATCC 14028*, Staphylococcus aureus* ATCC 25923, *Escherichia coli.*O157:H7 ATCC 43889. We also examined the antimicrobial spectrum of selected *Lactobacillus helveticus* strains used in this study, as well as the effects of carbon source on the antimicrobial properties of selected *Lactobacillus helveticus* and part of antibacterial mechanism were also studied. It is

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104 expected that these findings will upturn the use of *L. helveticus* in the dairy and allied-industries.

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120 **2 Materials and methods**

121 *2.1 Bacteria Strains and Growth Conditions*

122 *Lactobacillus helveticus* KLDS 1.9202, 1.9204, 1.9207, 1.0203, 1.8701 and other
123 *Lactobacilli* used for antimicrobial spectrum were isolated from traditional cheese in Sinkiang
124 and stored in Key Lab Dairy Science (KLDS), Ministry of Education, China. They were
125 identified by 16S rDNA sequence analysis[23] and were anaerobically incubated in modified

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126 deMan, Rogosa, and Sharpe (mMRS) broth at 37°C. The components of 100 mL mMRS were
127 shown in **Table 1**. *Listeria monocytogenes* ATCC 19115, *Salmonella Typhimurium* ATCC 14028,
128 *Staphylococcus aureus* ATCC 25923, *Escherichia coli.*O157:H7 ATCC 43889 (provided by
129 Heilongjiang entry-exit Inspection and Quarantine Bureau, China) used as the indicator
130 bacterium for antimicrobial assays were incubated in brain heart infusion broth (BHI; Beijing
131 China) in aerobic condition at 37°C. *Aspergillus oryzae 3.800*, *Aspergillus niger 3.1858*, *3.4309*,
132 *Rhizopus 3.866* (obtained from Institute of Microbiology Heilongjiang Academy of Sciences,
133 China) and *Bacillus subtilis* used for antimicrobial spectrum were cultured in Potato Dextrose
134 Agar Medium (PDA) or Czapek–Dox Medium at 28°C or BHI at 37°C.

135 *2.2 Preparation of cell-free supernatants (CFS)*

136 2 mL *Lactobacillus helveticus* strain (10^8 CFU mL^{-1}) was inoculated into 100 mL of mMRS
137 broth and incubated for 24 h at 37 °C. Then culture was centrifuged at 10,000×g for 10 min at 4 °C.
138 The bacteria precipitate was discarded and the CFSs were treated with 2 M NaOH for different pH
139 levels (pH=3.5, 4, 5, 6, 6.5 and 7). The remaining CFSs after adjusting at pH=6.5 were treated
140 with 1 mg mL^{-1} proteinaseK, 1 mg mL^{-1} papain and 5 mg mL^{-1} catalase for 2 h, respectively
141 according to the method of Ghanbari, et al.[24]. Then they were readjusted at the initial pH by 2 M
142 hydrochloric acid in order to recover to original conditions and exclude effects of proteases. The
143 last three samples were used as control. All of supernatants were filter-sterilized, through a sterile
144 0.22 μm-pore-size filter by an injector. CFS were obtained and preserved in a refrigerator at 4°C.

145 *2.3 Screening of antimicrobial potential of Lactobacillus helveticus using Oxford cup method*

146 Antimicrobial potential of *Lactobacillus helveticus* against food-borne pathogens were
147 investigated by Oxford cup method according to Wang, et al., and Wang, et al,[25,26] with some

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modifications. Firstly, 15 mL of 1.5% (w/v) agar medium was poured into plate and allowed to solidify. Then 1% of indicator pathogen strain in the stationary phase (10^7-10^9 CFU • mL^{-1}) was inoculated into 15 mL of 1.2% (w/v) of BHI agar at 45°C. The mixtures were poured onto agar medium and allowed to solidify. Three Oxford Cups were put on BHI agar surface and pressed lightly so that there is no interspace between cups and agar surface. Afterwards, 200 μL of culture and the same volume of cell-free supernatants (CFS) by different process were poured into two cups, respectively. 50 μL of sterile water added into the rest cup as a control. The plates were incubated under anaerobic conditions at 37°C for 24 or 48 h and antimicrobial activity reflected by growth-free inhibition zones around the Oxford Cups. Inhibition zones were measured in mm from the edge of the cups. This experiment was carried out in triplicate.

2.4 Antimicrobial spectrum of Lactobacillus helveticus KLDS1.8701

The antimicrobial spectrum of *Lactobacillus helveticus* KLDS1.8701 was assessed against 18 indicator strains including food-borne pathogens, fungi, *Bacillus subtilis* and *Lactobacillus* (shown in **Table 3**) using Oxford Cups method. Firstly, 15 mL of 1.5% (w/v) agar medium poured intoplate and waited for becoming solid. 1% of indicator strain in the stationary phase was inoculated into 15 mL of 1.2% (w/v) of appropriate agar medium at 45°C. The mixture was poured onto agar medium and allowed to solidify. The remaining procedure was carried out following the description as outlined in section 2.3. The plates were incubated under anaerobic conditions at appropriate temperature for 24 or 48 h and measured inhibition zones in mm around the Oxford Cups. This experiment was carried out in triplicate.

2.5 Determination of antimicrobial substances of Lactobacillus helveticus KLDS1.8701

In order to determine the antimicrobial substances of KLDS1.8701, CFS were processed by

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adding different component including 2 M NaOH, 5 mg mL^{-1} catalase and 1mg mL^{-1} proteinase K and 1mg mL^{-1} papain according to preparation methods of CFSs in section 2.2. CFS with no process, was as control. The remaining antimicrobial activities against *L. monocytogenes* ATCC 19115, *Salmonella Typhimurium* ATCC 14028 and *Escherichia coli*.O157:H7 ATCC 43889, were assessed using an indicator bacteria by Oxford Cups method follow as described in section 2.4. The data were presented as percentage (%). Each experiment was replicated three times.

2.6 Determination of organic acid production

Organic acids concentration was determined by following the procedure of Zhang et al. [14] with some modifications. The yields of organic acids produced by KLDS 1.8701 after culturing 24 h in mMRS were determined by high performance liquid chromatography (HPLC). Acid separation was using an AMINEX HPX-87H ion exchange column (BioRad Labs, Berkeley, California, USA) and organic acids were detected by differential refraction detector using 5mM H_2SO_4. Acid identification was carried out by comparing the retention times of the samples with that of the standards of organic acids.

2.7 Effect of carbon source on Antimicrobial activity of Lactobacillus helveticus KLDS1.8701

1% of fructose, glucose, lactose, sucrose and FOS as supplementation carbon sources, were added into mMRS broth with 1% glucose respectively and mMRS broth with 1% glucose was used as control. Then *Lactobacillus helveticus* KLDS1.8701 was inoculated into these MRS broths at 37℃ for 24h. part of cultures via appropriate dilutions, every four hours were spread onto mMRS agar plates and incubated at 37℃ for 24 h. Colonies of KLDS 1.8701 was counted. Then CFSs of *Lactobacillus helveticus* KLDS1.8701 were prepared by the rest part of cultures as

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previously described in section 2.2. Antimicrobial activities of CFSs were carried out as mentioned above in section 2.3. The organic acid productions of CFSs supplemented with different saccharides were measured by HPLC. Experiments were carried out every 4 hours and in triplicate.

2.8 Mixed culture inhibition assay

Antimicrobial potential against *L. monocytogenes* ATCC 19115, *Salmonella Typhimurium* ATCC 14028, *Escherichia coli.*O157:H7 ATCC 43889 of KLDS1.8701 cell was carried out by the use of mixed culture method. Equivoluminal of pathogens above (10^3 CFU mL^{-1}) and KLDS1.8701 (10^6 CFU mL^{-1}) were co-inoculated into BHI broth and a diffusion chamber separated with a filter size of 0.22 μm with BHI broth according to Saraoui, et al.[27] and incubated at 37℃ for 24 h. 1% pathogens above (10^3 CFU mL^{-1}) and 1% KLDS1.8701 (10^6 CFU mL^{-1}) monocultured into BHI broth under the same conditions were used as control. All of cultures via appropriate dilutions, every four hours were spread onto BHI and mMRS agar plates and incubated at 37°C for 24 h. Colonies of pathogens and KLDS 1.8701 were counted. The pHs of above mentioned cultures was measured by PHS-3C electrode pH meter (METTLER TOLEDO, Switzerland), after every four hours. Each experiment was carried out in triplicate.

2.9 Tolerance of KLDS 1.8701 under conditions simulating the human GI tract

Tolerance of the KLDS 1.8701 to simulated gastric juice was tested according to the method of Charteris et al.[28] with some modifications. KLDS 1.8701was cultured in mMRS at 37℃ for 24h, and its cells were collected at 4℃ for 5 min (10000 × g), washed twice with PBS buffer (pH 7.3) and suspended in PBS. 0.3 mg/mL pepsin was added into PBS (pH=2, 2.5, 3, 3.5 and 4) as simulated gastric juice. 3% (w/w, nearly 10^8 CFU mL^{-1}) of washed cell suspensions were

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inoculated into 1 mL simulated gastric juice and 0.3 mL NaCl (0.5%, w/v), mixed and incubated at 37 ^{o}C. Viable counts were calculated at 0, 1, 2 and 3h for testing the tolerance of gastric juice during the digest food in the stomach. Tolerance to small intestine juice was tested in PBS solution (pH 8.0) with 0.1 mg/mL pancreatin (Sigma). 3% (w/w, nearly 10^{8} CFU mL^{-1}) of washed cell suspensions were added into and incubated at 37℃. Viable counts were calculated at 0, 1, 2 and 3h for testing the tolerance of gastric juice during the digest food in small intestinal.[29] Tolerance to bile salt was tested in PBS with 1% (w/v) Oxgall (Sigma). 4% (w/w, nearly 10^{8} CFU mL^{-1}) of washed cell suspensions were added into and incubated at 37℃. Viable counts were calculated at 0, 1, 2 and 3h for testing the tolerance of bile salt during the digest food in small intestinal. All of experiments above were carried out in triplicate.

2.10 Statistical analysis

All the analysis done in triplicate manner and collected the data. Statistical treatment of data was conducted by analysis of variances (ANOVA) of SPSS PASW Statistics v18.0 and Duncans Test was used to compare the means when the overall P value of the experiment was below the value of significance ($P < 0.05$). Mean values and the standard errors were calculated and presented in chart as coordinate pairs with error bars.

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3 Results and Discussion

3.1 Screening of antimicrobial potential by Oxford cup method

The results of the antimicrobial potential of *Lactobacillus helveticus* strains against all provided food-borne pathogens were studied by Oxford cup method and the results are shown in **Tables 2 and 3**. KLDS1.8701 showed the strongest antimicrobial capability of all *Lactobacillus helveticus* strains against all provided pathogens especially, inhibition zone of *L. monocytogenes* ATCC 19115 reached 12.63±0.11 mm by culture and 8.79±0.04 mm by CFS, but its CFS had no significant effect ($P<0.05$) on *Staphylococcus aureus,*. KLDS 1.0203 and its CFS only showed antimicrobial capability against *L. monocytogenes,* (7.71±0.07 mm and 5.35±0.13 mm respectively) but no significant effect ($P<0.05$) on *S.typhimurium, Staphylococcus aureus* and *Escherichia coli*. KLDS 1.9202, KLDS 1.9204 and KLDS 1.9207 all showed antimicrobial capability against *L. monocytogenes* and *Staphylococcus aureus,* but no effect ($P<0.05$) on *S.typhimurium* and *Escherichia coli*. Furthermore, inhibition zones by their cultures were larger than those by their CFSs. Results above also suggest that part of antimicrobial capability of KLDS 1.8701 might be from bacteria itself. So based on the results of the screening of antimicrobial potential, *Lactobacillus helveticus* KLDS 1.8701 were found to be most effective for the inhibition of food-borne pathogens and used in further experiments.

There has been a significant interest in the use of LAB for application to inhibit pathogens in vitro. Multiple species of the genera *Lactobacillus, Lactococcus*, have been studied for their ability to inhibit the growth of Gram-positive and Gram-negative pathogens in model systems.[30] In this study, *Lactobacillus helveticus* KLDS1.8701 showed strong antimicrobial activities against two Gram-negative pathogens (*Salmonella Typhimurium* ATCC 14028, *Escherichia*

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258 *coli.*O157:H7 ATCC 43889) and one Gram-positive pathogen (*Listeria monocytogenes* ATCC
259 19115). Result might suggest that, KLDS1.8701 was more useful to inhibit Gram-negative
260 pathogens than Gram-positive pathogens. The antimicrobial activity of *Lactobacillus helveticus*
261 PJ4 and 50P1 against pathogens has been reported.[31,32] They also showed strong antimicrobial
262 activities against *Salmonella typhimurium* and *Escherichia coli.* and large inhibition zones were
263 obtained.

264 *3.2 Antimicrobial Spectrum of KLDS1.8701*

265 The results for the antimicrobial spectrum of KDLS 1.8701 are as shown in **Table 3**.
266 KLDS1.8701 had antimicrobial capability against all food-borne pathogens, *Bacillus subtilis,*
267 fungi except for, *Aspergillus niger 3.4309* and some *Lactobacillus* strains such as *L. paracasei*
268 KLDS1.0201, *L. plantarum* KLDS 1.0344, *L. helveticus* KLDS 1.9202 and *L. helveticus KLDS*
269 1.9207 which inhibition zones less than 5mm. However, there were no significant effects
270 ($P<0.05$) on some provided LABs used as starter cultures in fermented dairy products including
271 *Lactobacillus Bulgaricus* KLDS 1.0205,*Sterptococcus Thermophilus* KLDS 3.0207, *Lactococcus*
272 *lactis* KLDS 4.0325, *Lactobacillus helveticus* KLDS 1.0203 and 1.9204. Inhibition zones of
273 KLDS1.8701 culture for most microbes were also larger than those of its CFSs. Results indicated
274 that KLDS1.8701, showed a relatively stronger and broader spectrum of antibacterial effects, but
275 did not impede growth of some LABs used as starter cultures. So this might suggest
276 KLDS1.8701 could be used as a supplementary start culture in yogurt and cheese productions.

277 Previous antimicrobial spectrum assays have been carried out in many studies to confirm
278 wide antimicrobial activities by bacteriocins of *Lactobacillus* species and the results showed
279 strong antimicrobial activities against some *Lactobacillus* strains.[33,34] However, some studies

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showed that the antimicrobial activity of *L. casei Shirota* and *L. rhamnosus GG* was solely due to the production of lactic acid.[35] And in vitro evaluation of the antimicrobial activities of *L. helveticus (PXN 45)* and other Lactobacillus strains also have found evidence for organic acids against pathogens.[10] So it is important to confirm the antimicrobial substances which showed a relatively stronger and broader inhibitory spectrum in the study.

3.3 Determination of antimicrobial substances

Table 4 showed antimicrobial activities of CFSs of KLDS1.8701 treated with NaOH at different pH, proteinase K, Papain and catalase. The CFSs remained antimicrobial activities after 2 h treatment with catalase, proteinase K, papain and adjusted pH from 3.5 to 4. When pH was increased from 4 to 6, antimicrobial activities of CFSs declined and disappeared after adjusting at pH 6.5 and 7. Results suggested that antimicrobial activity of CFS of KLDS1.8701 was primarily related to organic acids produced by this strain.

Most of the LABs produce organic acids by the main metabolites of glucose fermentation. *Lactobacillus helveticus* is an obligatory heterofermentative LAB. So component of organic acids produced were not just lactic acid. In this study, it was confirmed that composition of organic acids produced by KLDS1.8701 were primarily acetic acid and lactic acid. Concentration of total organic acids after 24 h fermentation reached 12.63 mg mL^{-1} (**Fig. 1**). Concentrations of acetic acid and lactic acid were 10.74 mg mL^{-1} and 1.89 mg mL^{-1}, respectively (data not shown). This is consistent with the findings of Tejero-Sarinena, et al.,[10] who reported the production of lactic acid and acetic acid by Lactobacillus bacteria. Production of lactic acid and acetic acid was nearly the same as result in our study. The findings of this study also agree with earlier assertions that, the ability of probiotics to prevent gastrointestinal infections is thought to be as a result of

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their ability to produce antimicrobial properties. Such as organic acids (mainly lactic and acetic acids) as well as stimulate immune processes in the host, thus imputing health-promoting features.[36,11,12]

3.4 Antimicrobial activity of KLDS1.8701 cultured by adding different carbon source

Results in this part of study showed that concentrations of total organic acids and viable counts of KLDS1.8701 in mMRS supplemented with different carbon sources both increased during 24 cultures, and the data of FOS group was higher than other saccharides groups (**Fig 1**). It also suggests that FOS could improve growth of KLDS1.8701 and increase productions of total organic acids much better than other saccharides. It was also observed that KLDS1.8701 which cultured in mMRS supplemented with different carbon sources all showed antimicrobial activities against *L. monocytogenes* ATCC 19115, *Salmonella typhimurium* ATCC 14028 and *Escherichia coli.*O157:H7 ATCC 43889 (**Fig.2**). The antimicrobial activities observed were due to the presence of FOS, fructose, lactose, glucose and sucrose along with productions of organic acids. Overall, results not only showed that carbon source played a crucial role on enhancing the production of organic acids (lactic and acetic), thus giving rise to the corresponding antimicrobial capability of KLDS1.8701 but also that FOS was a better carbon source for antimicrobial actions than other saccharides. These observations are in line with a previous research which compared LABs cultured in mMRS with FOS to mMRS with glucose in order to study the effect of carbon source on antimicrobial activities of LABs. Results from that study showed that FOS was more useful than glucose to inhibit pathogens.[10] Prebiotics have been applied to the food industry as functional ingredients in food products.[37] Previous studies have demonstrated that FOS could stimulate the growth of probiotic such as bifidobacteria. It has also

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been shown to increase the growth of lactic acid bacteria (LAB) as well as butyrate and lactate production.given these properties, FOS may thus benefic intestinal inflammation.[38,39] These observations were also found for KLDS1.8701 in our studies. That is to say that FOS could also be useful to improve the growth and probiotic action such as antimicrobial activities of *some Lactobacillus hevelticus* strains.

3.5 Mixed culture inhibition assay

In section 3.1, it is shown that the inhibition zones of *L. monocytogenes* ATCC 19115, *Salmonella typhimurium* ATCC 14028, *Escherichia coli*.O157:H7 ATCC 43889 by KLDS1.8701 culture were larger than those obtained for its CFS. So there might be a part of antimicrobial capability related to action of KLDS1.8701 itself. Mixed culture inhibition assay were studied for antimicrobial activity verification and exploration of antimicrobial mechanism and results are shown in **Fig. 3 A, B and C**. In co-cultured with three pathogens group, viable counts of *L. helveticus* KLDS1.8701 all increased rapidly. Diffusion chamber group and monoculture group had the same increase trend, but viable counts of KLDS1.8701 have significant differences between different groups ($P<0.05$). The pH of cultures in all three groups also showed significant ($P<0.05$) decrease. Results suggested that KLDS1.8701 can survive well in BHI and produce organic acids using glucose from BHI. Viable counts of three pathogens increased in monoculture group but decreased in diffusion chamber group. This appearance showed organic acids of culture possess antimicrobial effect to impede the growth of three pathogens. Moreover, viable counts of three pathogens rapidly decreased to 0 log CFU mL^{-1} in the co-culture group whereas it decreased to 0 log CFU mL^{-1} after 24 h in the diffusion chamber group. However, pH values of BHI in three groups were not significantly different ($P>0.05$). This might suggest that

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346 the part of antimicrobial activity resulted from the action of *L. helveticus* KLDS1.8701. This
347 implies that inhibition against three pathogens in the co-culture group might resulted from both
348 organic acids in CFS and the action of bacteria itself, but only organic acids participated in
349 inhibition of three pathogens in diffusion chamber group. It can thus be hypothesized that KLDS
350 1.8701 could come in contact and thus interact with three pathogens in co-culture group, but not
351 in diffusion chamber group. This may indicate that the inhibitory action of KLDS1.8701 itself
352 against three pathogens requires contact between the two bacteria cells. In addition, results from
353 **Fig. 3 A, B and C** showed viable counts of three pathogens decreased at least 3 logs in in
354 co-culture group and diffusion chamber group. These results were similar with the study of
355 Atassi[40] which showed that *L. helveticus* strain KS300 lessened *S. typhimurium* SL1344,
356 pathogenic *E. coli* IH11128 and C1845 with a decrease of 2.0-5.5 logs in viable bacteria. We
357 must also accentuate that other factors may be responsible for these results and more promising
358 factors could further lower pathogenic growth. Previous studies have carried out on co-culture
359 inhibition and provided an antimicrobial mechanism which called Contact dependent inhibition
360 (CDI) mechanism.[41,42,43,27] In this study, it was confirmed that part of antimicrobial activity was
361 related to bacteria itself and antimicrobial activity of co-culture group was stronger than the
362 diffusion chamber group. This meant that, antimicrobial activity of bacteria itself might be
363 interrelated to above described CDI mechanism. CDI mechanism can be explicated by exchange
364 of information between bacteria. Such exchange of information included conjugation, secretion
365 systems, contact dependent inhibition, allolysis and nanotubes.[27] We therefore recommend
366 further studies on the description of CDI mechanism of *Lactobacillus* KLDS1.8701, as these will
367 be useful in increasing the applicability of this KLDS LAB strain.

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368 *3.6 Tolerance of the Lactobacillus strains to simulated GI tract*

369 Although probiotics usually pass through the stomach to reach the small intestine, most
370 microorganisms cannot survival under gastric environment with complex digestive enzymes and
371 acids as well as intestinal environment. It is thus necessary to study tolerances of *Lactobacilli* to
372 artificial gastric juice, small intestine juice and bile salts. From our results, it was observed that
373 viable counts of KLDS 1.8701 could keep about 7 Logs at pH 2, 2.5, and 3 after inoculated into
374 artificial gastric juice for 3 h, but gradually decreased at pH 1.5 and reach 0 Log at 3 h **(Table 5**
375 **A)**. These results were also similar with the findings of previous study by Fernandez et al. [44] and
376 suggested that KLDS 1.8701 could be conveyed through the acidic conditions of the stomach in
377 significant quantities to the intestine where it can proliferate significantly and exert
378 health-promoting effects. KLDS 1.8701 also could survive well at pH 8.0 conditions with
379 trypsine and in 1% (w/v) bile salts solution for 3 h. Viable counts of KLDS 1.8701 after 3 h of
380 exposure under artificial small intestine juice was no loss compare to the initial **(Table 5 B)**.
381 Results showed that KLDS 1.8701 showed strong capacities against simulated GI tract. This
382 might suggest that KLDS 1.8701 could enter into gastrointestinal tract and play inhibitory roles
383 against food-borne pathogens. This result was in accordance with some previous studies which
384 showed most strains could survive well under small intestine conditions and bile salts.[45,46] The
385 results above indicated that KLDS 1.8701 could endure GI tract challenge and commendably
386 play probiotic role in intestinal systems.

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5 Conclusions

This study investigated the antimicrobial capability of the *Lactobacillus helveticus* KLDS1.8701 against food-borne pathogens, especially, *L.monocytogenes* in vitro. It was confirmed that antimicrobial activity resulted from organic acids including acetic and lactic acids, as well as action of KLDS1.8701 itself. FOS was more valuable for KLDS1.8701 to improve the proliferation of KLDS 1.8701 and inhibit pathogens than other saccharides studied. The inhibitory action of bacteria itself requires cellular contact between pathogens and KLDS1.8701. KLDS1.8701 also had high antimicrobial potential against *Salmonella typhimurium and E. coli* and could survive well under GI tract conditions. Further studies in understanding the antagonism mechanism against these two pathogens by KLDS1.8701 and probiotic properties of KLDS1.8701 in vivo are recommended.

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Tables

Table 1 The components of modified MRS (100 mL)

components	content
yeast extract	0.5 g
tryptone	1 g
beef extract	0.5 g
peptone	0.5 g
glucose	2 g
Tween-80	0.1 mL
dipotassium hydrogen phosphate	0.2 g
diammonium citrate	0.2 g
sodium acetate	0.5 g
magnesium sulfate monohydrate	0.058 g
manganese sulfate monohydrate	0.025 g
deionized water	95 mL
agar	2 g

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Table 2 Antimicrobial potential against indicator strains of *Lactobacillus helveticus KLDS 1.9202, 1.9204, 1.9207, 1.0203, 1.8701* and their CFSs

Indicator bacterium	Inhibition zones [a] (mm)									
	KLDS1.0203		KLDS1.8701		KLDS1.9202		KLDS1.9204		KLDS1.9207	
	Culture pH 4.71±0.08	CFS pH 4.66±0.02	Culture pH 3.73±0.10	CFS pH3.69±0.05	Culture pH 5.45±0.11	CFS pH 5.41±0.07	Culture pH 4.59±0.03	CFS pH 5.55±0.04	Culture pH 5.67±0.06	CFS pH5.65±0.07
L.monocytogenes ATCC19115	7.71±0.07	5.35±0.13	12.63±0.11	8.79±0.04	6.92±0.16	3.88±0.34	9.05±0.21	5.55±0.14	4.38±0.25	3.02±0.11
S.Typhimurium ATCC 14028	-	-	5.33±0.23	4.59±0.09	-	-	-	-	-	-
S. aureus, ATCC 25923	-	-	3.15±0.06	-	3.07±0.25	2.88±0.18	6.14±0.22	5.74±0.13	4.05±0.08	3.82±0.14
E. coli O157:H7 ATCC 43889	-	-	6.95±0.02	4.11±0.17	-	-	-	-	-	-

-:no antimicrobial activity

[a] Results are presented as the mean value of triplicate trials ± standard deviation (SD).

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570 **Table 3** Spectrum of antimicrobial activity of culture of *Lactobacillus helveticus KLDS 1.8701* and its CFS

indicator bacterium	Medium	temperature(℃)	Sensitivity a by culture (pH 3.73±0.10)	Sensitivity by CFS (pH 3.69±0.05)
L.monocytogenes ATCC 19115	BHI	37	+++	++
S.Typhimurium ATCC 14028	BHI	37	+	+
Staphylococcus aureus ATCC 25923	BHI	37	+	-
Escherichia. coli ATCC 43889	BHI	37	++	+
Aspergillus oryzae 3.800	PDA	28	+++	++
Aspergillus niger 3.1858	Czapek–Dox	28	+++	++
Aspergillus niger 3.4309	Czapek–Dox	28	-	-
Rhizopus 3.866	PDA	28	++	++
bacillus subtilis	BHI	37	+++	+++
Lactobacillus paracasei KLDS1.0201	mMRS	37	+	+
Lactobacillus Bulgaricus KLDS 1.0205	mMRS	43	-	-
Sterptococcus Thermophilus KLDS3.0207	M17	37	-	-
Lactobacillus plantarum KLDS 1.0344	mMRS	37	+	+
Lactococcus lactis KLDS 4.0325	M17	37	-	+
Lactobacillus helveticus KLDS 1.0203	mMRS	37	-	-
Lactobacillus helveticus KLDS 1.9202	mMRS	37	+	+
Lactobacillus helveticus KLDS 1.9204	mMRS	37	-	-
Lactobacillus helveticus KLDS 1.9207	mMRS	37	+	+

571 [a] Inhibition zone (mm): -, no inhibition +, 1-5 mm; ++, 5-10 mm; +++, >10 mm;

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572 **Table 4** Effect of catalase, protease treatment and pH on the antimicrobial activity of CFS
573 derived from KLDS1.8701

Treatment	Remaining antimicrobial activity (%)		
	L.monocytogenes ATCC 19115	*S.typhimurium* ATCC 14028	*E. coli* O157:H7 ATCC 43889
catalase	92.43±0.05	96.27±0.22	94.16±0.18
1mg/mL proteinase K	100±0	100±0	100±0
1mg/mL papain	100±0	100±0	100±0
pH 3.5	100±0	100±0	100±0
pH 4	100±0	100±0	100±0
pH 5	75.22±0.25	72.35±0.33	74.23±0.11
pH 6	12.61±0.19	12.13±0.20	10.39±0.12
pH 6.5	0±0	0±0	0±0
pH 7	0±0	0±0	0±0

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589 **Table 5 A** Viable counts under conditions simulating the human gastric juice of KLDS 1.8701

pH	0.3 mg/ml pepsin (log CFU mL^{-1})			
	0h	1h	2h	3h
1.5	7.32±0.15	5.19±0.24	3.87±0.22	0
2	7.34±0.23	7.35±0.31	7.33±0.17	7.34±0.35
2.5	7.32±0.32	7.33±0.23	7.31±0.21	7.30±0.26
3	7.36±0.18	7.35±0.14	7.35±0.19	7.31±0.32

590 [a] Data are mean ± deviation of three independent experiments.

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626 **Table 5 B** Viable counts under conditions simulating the human small intestine juice and bile salts
627 of KLDS 1.8701

Time (h)	0.1 mg/ml pancreatin, pH 8.0 (log CFU mL^{-1})	1% Oxgall (log CFU mL^{-1})
0	7.40±0.41	7.43±0.71
1	7.48±0.39	7.45±0.55
2	7.33±0.27	7.54±0.64
3	7.39±0.45	7.34±0.76

628 [a] Data are mean ± deviation of three independent experiments.
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Figure Captions

Fig. 1 Productions of total organic acids and viable counts of KLDS 1.8701 in MRS culture with different carbon sources during 24h culture. (■, ▲, ◆, ＋, ● and × with solid line) represent contents of total organic acids in MRS broth supplemented with 1 % of fructose, lactose, glucose, sucrose, fructooligosaccharides and no saccharide supplementation (control) during 24h culture. (■, ▲, ◆, ＋, ● and × with dotted line) represent viable counts of KLDS 1.8701 in MRS broth supplemented with 1 % of different saccharide and no saccharide supplementation during 24h culture. Bars represent means of duplicate ±SD.

Fig. 2 Antimicrobial activities against pathogens and total organic acids productions by KLDS 1.8701 in MRS broth with different carbon sources during 24h culture. (◆, ■, ●) represent inhibition zone diameters of plates of *L. monocytogenes* ATCC 19115, *S. typhimurium* ATCC 14028 and *E. coli* O157:H7 ATCC 43889 after treatment with CFS of KLDS 1.8701. Pillars represent total organic acids productions by KLDS 1.8701. Bars represent means of duplicate ±SD.

Fig. 3 Growth of pathogens, KLDS 1.8701 and change of pH value in contact co-culture test. (■ with solid line, dotted line and □ with solid line) represents viable counts of pathogens including *L.monocytogenes* ATCC 19115(**A**), *Salmonella Typhimurium* ATCC 14028(**B**), *E. coli* O157:H7 ATCC 43889(**C**)) in co-cultured with KLDS 1.8701 group, diffusion chamber group and monoculture group, respectively; (● with solid line, dotted line and ○ with solid line) represents viable counts of KLDS 1.8701 in co-cultured with pathogens group, diffusion chamber group and monoculture group, respectively; (▲and △ with solid line) represents pH of cultures in co-culture group and diffusion chamber group, respectively.

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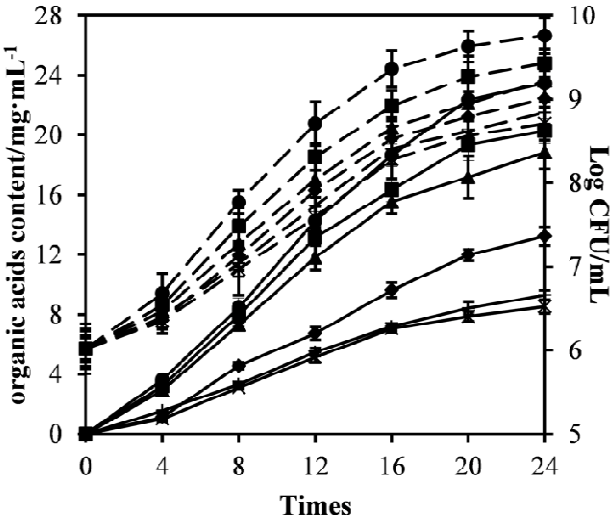

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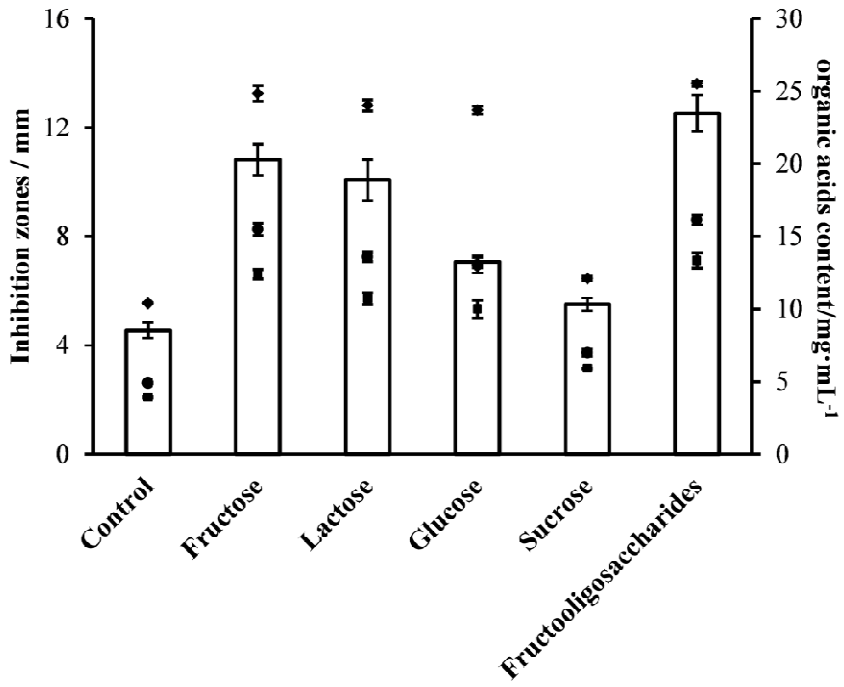

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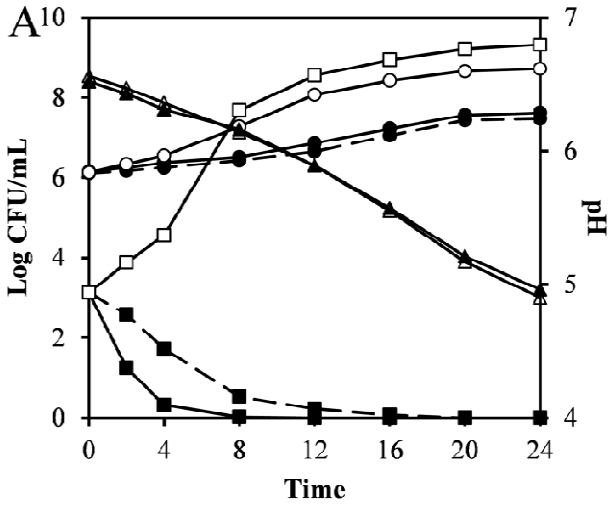

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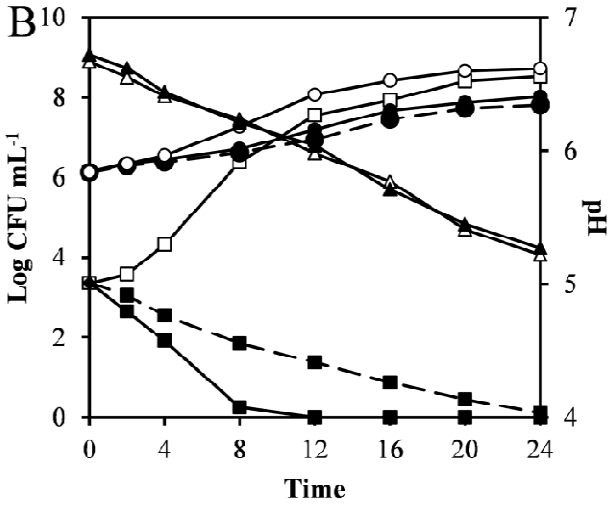

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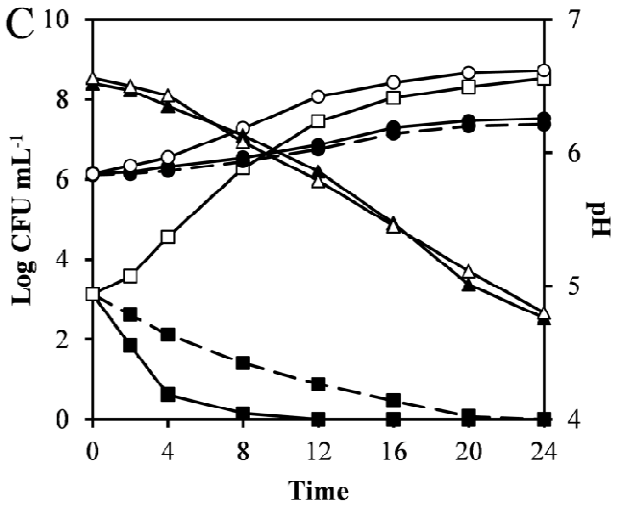

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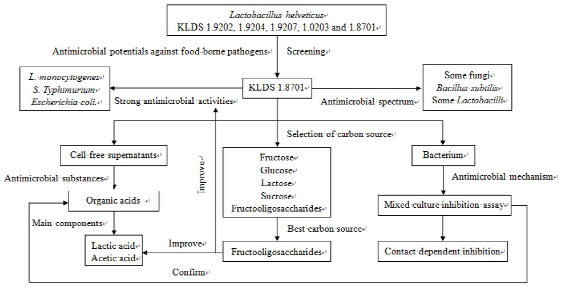

KLDS1.8701 showed wide antimicrobial spectrum especially food-borne pathogens and antimicrobial activities resulted from organic acids and contact dependent inhibition.

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