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Diverse conditions contribute to the cholesterol-lowering ability of different *Lactobacillus plantarum* strains

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It has been reported that *Lactobacillus* can remove cholesterol and thus might play an important role in lowering cholesterol in humans, but the underlying mechanism is still controversial. To confirm whether different strains have different cholesterol-lowering mechanisms, we explored the cholesterol-lowering abilities of different *Lactobacillus plantarum* strains, and the factors influencing their abilities. We found that all nine strains reduced the cholesterol concentration to some extent, but there were significant differences among them. In MRS broth, *L. plantarum* AR113 and AR171 showed the greatest cholesterol-lowering abilities of 27.89% and 19.90%, respectively, but AR501 and AR300 only showed reductions of 0.34% and 0.91%, respectively. Upon addition of 0.1% ox bile, the cholesterol-removal capability of most strains increased. *L. plantarum* AR511 showed the highest cholesterol removal rate, which increased from 5.8% to 37.14%, *i.e.*, by a factor of approximately 6.4, but there was no significant change in the cholesterol removal rate of AR171. These results suggested that the effect of ox bile on the cholesterol-lowering ability was strain-specific. Except for the strains AR171, AR237 and AR495, the cholesterol-removal ability of the remaining six strains was positively correlated with the amount of free bile acid released. The addition of a bile salt hydrolase inhibitor had some effect on the cholesterol-removal ability of the six strains of bacteria other than AR171, AR237 and AR495, but little influence on the latter three. The effect of BSH was strain-specific. Similarly, the effect of pH was also strain-specific. Taken together, these results suggest that different strains of *L. plantarum* have different cholesterol-lowering capacities and different influencing factors. Therefore, further research is needed to explore the exact mechanism by which different strains lower cholesterol.

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1. Introduction

With improvement in living standards and changes in the dietary structure, the human intake of high-cholesterol foods is gradually increasing, leading to an increasing incidence of cardiovascular and cerebrovascular diseases such as hypertension, coronary heart disease and atherosclerosis.[1] It has been reported that for every 1 mmol L^{-1} increase in the total cholesterol level, the chance of death from coronary heart disease increases by 35%, while a 1% reduction in serum cholesterol reduces the risk by 2% to 3%.[2] Existing medical treatment methods are not only expensive, but also have some side effects that are not conducive to long-term administration. Evidence suggests that functional foods containing probiotics can reduce the risk of coronary heart disease, and they have therefore attracted widespread attention.[3] Numerous studies have reported their beneficial effects on human health, one of which is lowering cholesterol.[4–6]

Among the bacteria used for lowering cholesterol, research has mainly focused on *Lactobacillus*.[7,8] The cholesterol-removal rate of this genus is species-specific. *L. fermentum* SM-7 cells reduced cholesterol by 66.8% in the growth medium.[9] The cholesterol removal rate of *L. plantarum* DMDL 9010 was up to 37.58% in de Man, Rogosa, and Sharpe (MRS) broth.[10] *L. johnsonii* BCRC17474 and *L. acidophilus* BCRC17010 have also shown the cholesterol-lowering ability.[11] For the studied strains, the mechanisms of their cholesterol-lowering ability are controversial, and many hypotheses about cholesterol lowering have been proposed, including the direct assimilation of cholesterol by probiotics, the adhesion of

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cholesterol on the cellular surface, the binding of cholesterol on the cellular membrane, the deconjugation of bile salts by bile salt hydrolase (BSH), the co-precipitation of cholesterol with deconjugated bile and the conversion of cholesterol into coprostanol.[12–15]

L. plantarum is one of the most widely studied species. It is believed that *L. plantarum* cells have the ability to assimilate cholesterol from the medium at the growth stage, and the resting and dead cells can also remove cholesterol.[16,17] Some factors that affect the cholesterol-lowering rates have been investigated. The addition of ox bile significantly increased the cholesterol-removal rates of *L. plantarum* EM and *L. plantarum* FB003 compared with that in the MRS medium without bile.[18,19] Another study has found that the cholesterol-lowering effects of *L. plantarum* PH04 may be due to the deconjugation of BSH to bile salts.[20] BSH catalysed the conversion of conjugated bile salts into amino acid residues and the corresponding bile acids, and removed cholesterol by co-precipitation. However, some studies have found no correlation between cholesterol removal *in vitro* and bile salt deconjugation.[21]

The main metabolites of *L. plantarum* are lactic acid and short-chain fatty acids, which can lead to a lower pH in the growing environment.[22] One study found that the cholesterol level was lowered by co-precipitation under acidic conditions.[13] However, it has also been reported that the final pH of the medium may not decisively influence the cholesterol removal rate.[23] These contradictory results may indicate that different cholesterol-lowering mechanisms operate in different species or even in different strains of the same species. In this study, we investigate the cholesterol-removal rates of different *L. plantarum* strains, and explore the factors affecting these rates.[25]

2. Materials and methods

2.1 Bacterial strains and culture conditions

Nine *Lactobacillus* strains used were isolated from fermented pickles and dairy products and identified as *Lactobacillus plantarum.*[24] These strains were stored in the Shanghai Engineering Research Center of Food Microbiology (Shanghai, China). The strains were as follows: AR501, AR300, AR113, AR171, AR326, AR237, AR495, AR511 and AR513. The stock strains were stored in 40% (v/v) sterile glycerol at −80 °C. When needed, these strains were cultured at 37 °C for 24 h under anaerobic conditions.

2.2 Phylogenetic tree

According to the description of Wang *et al.*,[24] the Mega software package, version 7.0 (Pennsylvania State University, USA), was used for the construction of a phylogenetic tree based on the neighbor-joining method.

2.3 Measurement of the cholesterol-removal rate

The nine strains were inoculated in MRS broth containing 100 μg mL^{-1} cholesterol micelles. The cholesterol removal rate was determined by a modified version of the *o*-phthalaldehyde (OPA) method.[25] The absorbance was measured at 550 nm (OD_{550}) using a UV spectrophotometer and it is directly proportional to the concentration of cholesterol in the sample. The concentration of cholesterol was estimated from a standard curve prepared using the cholesterol stock solution. All experiments were performed in triplicate and assayed twice to ensure accuracy and repeatability. The cholesterol removal rate can be calculated using the following formula:

$$Z\% = \frac{B - A}{B} \times 100\%,$$

where Z is the cholesterol removal rate, A is the $OD_{550\ nm}$ value of the supernatant of the fermentation broth and B is the $OD_{550\ nm}$ value of the cell-free supernatant.

2.4 Measurement of bacterial growth

The growth status of each strain in MRS medium (M) with or without cholesterol (MC)/0.1% ox bile (MB or MCB) was determined using a Bioscreen C (Labsystems, Finland), an automated optical density-monitoring system. For more conciseness, the MRS medium without cholesterol is abbreviated as M; the MRS medium with cholesterol is abbreviated as MC; the MRS medium with 0.1% ox bile is abbreviated as MB; and the MRS medium with cholesterol and 0.1% ox bile is abbreviated as MCB. A single colony was transferred from MRS agar plates into 1 mL of sterile MRS medium and incubated under anaerobic conditions at 37 °C for 24 h. The optical density (OD) at 600 nm was adjusted to 1.0. Inoculum (2%, v/v) from the adjusted culture solutions of the nine strains was inoculated into sterilized MRS broth containing 100 μg mL^{-1} cholesterol or 0.1% (v/v) ox bile. Then, each of the separately inoculated bacterial liquids was moved up to the 200 μL level of the corresponding hole of the honeycomb plate, and was placed in the instrument for monitoring at 37 °C. The measurements of the growth curve for every strain were performed in triplicate.

2.5 Fatty acid composition of cellular membranes

The fatty acid composition of cellular membranes was analyzed as previously described.[26] After saponification, methylation, extraction, and base washing were completed, the upper phase solvent was collected and transferred into GC-MS vials (Thermo Fisher Scientific, USA) for FA composition analysis. A TG-5MS capillary column (30 m × 0.25 mm × 0.25 μm, Agilent Technologies, USA) was used, the inlet temperature was 250 °C, the carrier gas was high purity helium (99.99%), the injection volume was 1 μL and the splitless injection mode was adopted. The chromatographic conditions were: a transmission line temperature of 250 °C, an ion source temperature of 280 °C, and an oven temperature that was gradually increased from 100 °C to 225 °C at a rate of 5 °C min^{-1}. The mass detector was operated in electron impact mode at an

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ionizing voltage of 70 eV. The full-range ion fragment scanning (SCAN) mode was selected. The mass scanning range was 20–400 amu, and the solvent delay was 3 min. The extraction and analysis of the FA composition of each strain were performed in triplicate.

2.6 Measurement of free cholic acid

The concentration of free cholic acid released was measured according to the method described by Irvin *et al.*[27] All experiments were replicated three times.

2.7 Effect of a bile salt hydrolase (BSH) inhibitor on cholesterol removal

Following the method of Wang *et al.*,[28] the strains were inoculated with 5 mL of MRS broth containing 100 μg mL^{-1} cholesterol, 0.1% (v/v) ox bile and 0.5 mM $NaIO_4$ at a dose of 2%, and incubated at 37 °C for 24 h. The optical density at 600 nm was about 1.0. The removal rate of cholesterol was determined in the same way as that described in section 2.3.

2.8 Effect of pH-controlled conditions on cholesterol removal

The activated strains were inoculated at a dose of 2% (v/v) in MCB (MRS broth containing 100 μg mL^{-1} cholesterol and 0.1% (v/v) ox bile) medium at natural pH (changing with the incubation time), constant pH (about 5.5) and neutral pH (about 7.0), and then incubated at 37 °C for 24 h. After culturing, the cholesterol removal rate was measured according to the method described in section 2.3.

2.9 Statistical analysis

All results are presented as mean ± standard error of the mean (SEM) of the replicates. The data were analyzed using SPSS statistics software version 17.0 (SPSS Inc., Chicago, USA). Statistical significance was analyzed by using Duncan's Multiple Range test. $P < 0.05$ was considered to be statistically significant.

3. Results

3.1 The ability of *Lactobacillus plantarum* strains to remove cholesterol *in vitro*

Phylogenetic trees of the nine strains of *L. plantarum* were constructed based on 16S rRNA by the neighbor-joining method (Fig. 1). The strains were separated from each other, confirming that they were different strains. *L. plantarum* AR113 and AR300 were located a very short distance apart, which indicated that these two strains were closely related. Conversely, *L. plantarum* AR511 at the bottom was distantly related to *L. plantarum* AR113 and AR300 at the top, and these strains had the most pronounced intraspecific diversity. *L. plantarum* AR501 and the other five strains were located between the above-mentioned three strains in the phylogenetic tree and showed a range of evolutionary distances.

Fig. 2 shows the cholesterol removal rates of the nine strains at 24 h of growth in MRS with or without 0.1% ox bile.

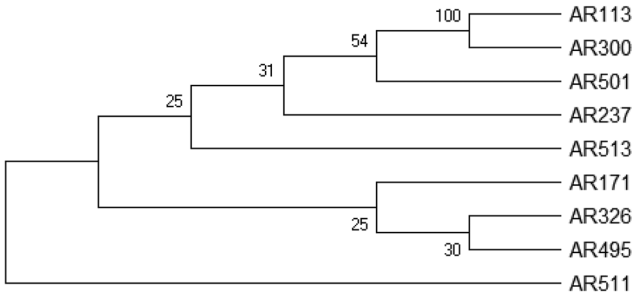

Fig. 1 Neighbor-joining phylogenetic tree of the nine examined *L. plantarum* strains based on 16S rRNA gene sequences.

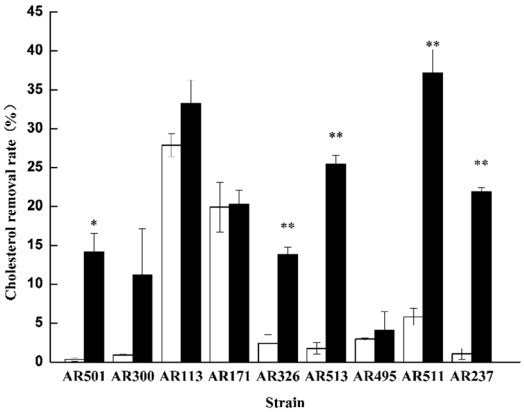

Fig. 2 Cholesterol removal rates of *L. plantarum* AR501, AR300, AR113, AR171, AR326, AR513, AR495, AR511 and AR237 (from left to right) incubated in MRS supplemented with 100 μg mL^{-1} cholesterol and with or without 0.1% ox bile for 24 h at 37 °C. Each data point is the average of three repeated measurements, $n = 3$. *$P < 0.05$, **$P < 0.01$. White bars: cholesterol. Black bars: cholesterol + 0.1% ox bile.

All nine strains of *L. plantarum* showed different cholesterol-removal rates ranging from 0.34% to 37.14% in MRS broth containing cholesterol (MC medium). *L. plantarum* AR113 and AR171 had the highest cholesterol removal rates of 27.89% and 19.90%, respectively. Meanwhile, *L. plantarum* AR501 and AR300 had the lowest cholesterol removal rates of 0.34% and 0.91%, respectively. After addition of 0.1% ox bile (MCB medium), the cholesterol-removal rates of all strains were improved, but to varying extents and the rates of some strains did not increase significantly. The cholesterol removal rate of *L. plantarum* AR113 increased from 27.89% to 33.27%, and that of *L. plantarum* AR511 increased from 5.8% to 37.14%, *i.e.*, by a factor of approximately 6.4. *L. plantarum* AR495 had the lowest cholesterol-removal rate in MCB, which was only 4.09%, while *L. plantarum* AR171 showed only a slight increase from 19.90% to 20.24%. These results showed that different strains of the same species had different cholesterol-removal rates. After addition of 0.1% ox bile, *L. plantarum* AR300, AR113, AR171 and AR495 showed no significant increase in their cholesterol-removal rates, but significant improvements were noted in the remaining five strains. This indicated that ox

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bile was an important promoting factor for the cholesterol-removal rates of some strains, but not the others.

3.2 Effect of cholesterol on growth characteristics

The growth status of the nine strains in MRS broth (M) with or without cholesterol (MC) or 0.1% (v/v) ox bile (MB or MCB) was monitored using a growth curve meter. As can be seen from Fig. 3, these strains reached a stable stage at 15 h in MRS medium. After cholesterol was added to MRS broth (MC), their growth rates and trends remained essentially unchanged, indicating that cholesterol had no significant effect on their growth. However, although most strains showed similar growth in MRS broth with or without cholesterol, their cholesterol-removal rates were completely different, suggesting that the biomass was not the main factor influencing the cholesterol-removal rates. When 0.1% ox bile was added to MRS broth (MB), *L. plantarum* AR237 and AR326 were slightly inhibited, but the growth rate of the other strains was consistent with that in MRS, indicating that the concentration of added ox bile was appropriate and had no significant effect on the growth of the strains. In MCB medium, however, the growth rate and biomass of *L. plantarum* AR113, AR326, AR495 and AR511 were significantly suppressed, while the effects on the remaining five strains were not prominent. However, the cholesterol-lowering ability of most of the strains was increased, showing that changes to the biomass were not the mechanism by which ox bile promoted the cholesterol-removal rates and further illustrating that physical adsorption could not be the main factor for cholesterol removal. For example, the biomass of *L. plantarum* AR113 decreased from 1.4 to 1.1, while the cholesterol removal rate increased from 27.89% to 33.27%. The biomass of *L. plantarum* AR511 decreased from 1.5 to 1.2, while the cholesterol removal rate significantly increased from 5.80% to 37.14%. Thus, although both strains experienced similar changes in their biomass, the changes in their ability to lower cholesterol were extremely different.

3.3 Effect of cholesterol on the composition of fatty acids in cellular membranes

The fatty acid composition of the cellular membranes of the nine *Lactobacillus* strains grown with or without cholesterol was analyzed by GC-MS. Three saturated fatty acids (myristic, C14; palmitic, C16; and stearic, C18) and five unsaturated fatty acids (nutmeg oleic, C14:1; myristoleic, C16:1; oleic, C18:1; linoleic, C18:2; and nonadecenoic, C19:1) were detected in all nine strains, and they were used as indicators to track the differences in the fatty acid composition of these strains in the presence or absence of cholesterol (Table 1). The fatty acid percentages differed between strains in the presence or absence of cholesterol, with C16:0 and C18:1 accounting for the largest proportional changes. Thus, for all strains except *L. plantarum* AR300, the percentage of C18:2 and total unsaturated fatty acids (UFAs) increased significantly after cholesterol was added. The proportion of C18:2 in AR113 increased significantly from 7.83% to 15.52%, and that in AR501 from 6.59% to 14.58%. Despite showing similar changes in the proportion of C18:2, these two strains had completely different cholesterol-removal rates of 27.89% and 0.34%, respectively. The proportion of total UFAs in five strains increased by more than 15%, but this increasing trend was not related to their cholesterol-removal rates. In *L. plantarum* AR300, the addition of cholesterol had a slight effect on the composition of fatty acids

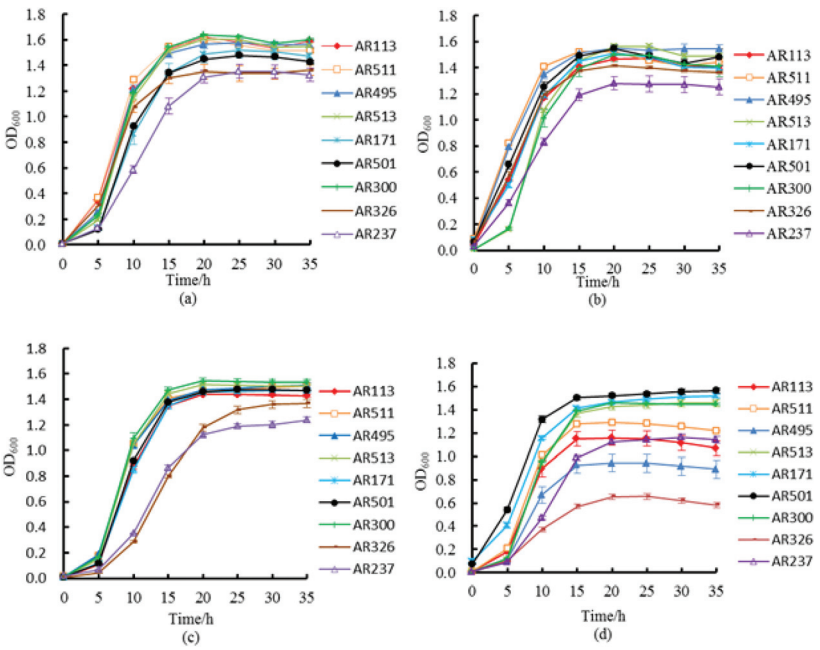

Fig. 3 Growth curve of the nine examined *L. plantarum* strains. (a) MRS, (b) MC: MRS + cholesterol, (c) MB: MRS + ox bile, (d) MCB: MRS + cholesterol + ox bile. Error bars represent SEM; each data point is the average of three repeated measurements, $n = 3$.

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Table 1 Fatty acid composition of the nine strains cultured separately in MRS with or without cholesterol[a]

Strains	Medium	Fatty acids[b] (%)								SFA[c]	UFA[d]
		C14:0	C14:1	C16:0	C16:1	C18:0	C18:1	C18:2	C19:1		
AR501	Without cholesterol	10.37 ± 0.39	14.33 ± 0.37	27.49 ± 0.84	1.71 ± 0.18	16.25 ± 0.52	18.07 ± 1.04	6.59 ± 0.38	5.19 ± 0.12	54.11 ± 1.11	39.30 ± 0.80
	With cholesterol	7.01 ± 0.81	9.21 ± 1.15	24.75 ± 2.43	1.59 ± 0.12	13.50 ± 0.71	24.90 ± 1.16	14.58 ± 1.02**	6.22 ± 0.42	43.05 ± 0.18	56.95 ± 0.18**
AR300	Without cholesterol	7.36 ± 1.04	10.50 ± 1.13	28.05 ± 3.30	1.69 ± 0.52	14.93 ± 0.47	21.67 ± 1.04	10.25 ± 3.48	5.55 ± 0.64	50.34 ± 2.67	49.66 ± 3.52
	With cholesterol	1.89 ± 0.02	4.71 ± 0.14	29.75 ± 0.21	2.77 ± 0.87	17.16 ± 1.81	21.21 ± 1.20	13.82 ± 0.60	6.71 ± 0.22	49.59 ± 0.45	50.41 ± 0.45
AR113	Without cholesterol	9.19 ± 0.83	13.60 ± 1.94	24.41 ± 2.68	1.45 ± 0.02	14.72 ± 0.84	21.77 ± 1.38	7.83 ± 0.30	4.87 ± 0.56	48.38 ± 4.70	51.71 ± 3.33
	With cholesterol	5.68 ± 0.72	7.58 ± 0.96	21.95 ± 1.62	2.51 ± 0.58	13.31 ± 0.46	24.23 ± 2.92	15.52 ± 1.68**	6.24 ± 0.55	41.09 ± 0.82	58.91 ± 0.82*
AR171	Without cholesterol	8.71 ± 1.54	13.03 ± 0.81	26.00 ± 0.99	2.25 ± 0.82	15.55 ± 1.20	21.72 ± 1.91	7.80 ± 1.67	4.94 ± 0.64	50.27 ± 1.72	41.94 ± 1.40
	With cholesterol	5.01 ± 0.27	6.20 ± 0.63	22.77 ± 0.34	2.05 ± 0.56	12.31 ± 1.16	26.42 ± 1.55	17.26 ± 1.27**	7.99 ± 0.74	40.09 ± 1.30	59.91 ± 1.30**
AR326	Without cholesterol	7.05 ± 0.67	4.96 ± 0.71	36.84 ± 1.07	0.43 ± 0.07	17.03 ± 2.38	23.14 ± 1.28	4.79 ± 0.05	5.90 ± 0.35	60.92 ± 0.25	39.08 ± 1.23
	With cholesterol	1.68 ± 0.01	4.80 ± 0.10	28.83 ± 1.58	1.44 ± 0.99	10.11 ± 1.30	20.88 ± 0.88	12.10 ± 1.24**	14.45 ± 1.61	47.73 ± 0.16	52.27 ± 0.16**
AR513	Without cholesterol	7.84 ± 0.69	5.97 ± 0.66	36.46 ± 0.36	1.23 ± 0.43	13.71 ± 0.35	23.39 ± 0.42	5.02 ± 0.18	6.73 ± 0.25	57.96 ± 0.29	42.04 ± 0.29
	With cholesterol	2.64 ± 1.19	4.05 ± 3.32	29.13 ± 0.95	2.56 ± 1.10	19.16 ± 1.57	23.26 ± 2.21	13.55 ± 1.02**	5.66 ± 0.73	50.93 ± 0.30	49.07 ± 0.30*
AR495	Without cholesterol	8.77 ± 1.83	6.90 ± 1.81	38.41 ± 1.16	1.26 ± 0.61	15.89 ± 0.96	20.26 ± 1.32	4.47 ± 0.30	4.05 ± 0.15	63.07 ± 0.96	36.93 ± 0.96
	With cholesterol	5.00 ± 0.21	6.07 ± 0.71	25.85 ± 1.81	2.15 ± 0.32	15.02 ± 1.28	26.55 ± 3.45	15.30 ± 1.60**	6.95 ± 1.50	42.54 ± 1.34	57.46 ± 1.34**
AR511	Without cholesterol	5.14 ± 0.48	3.02 ± 0.27	41.52 ± 0.53	0.95 ± 0.09	26.58 ± 0.10	14.03 ± 1.20	7.33 ± 0.89	2.18 ± 0.05	73.08 ± 0.94	26.92 ± 0.94
	With cholesterol	3.90 ± 0.36	2.19 ± 0.23	27.77 ± 0.83	3.87 ± 0.47	16.29 ± 1.47	22.89 ± 0.82	16.13 ± 4.05**	6.96 ± 0.62	47.96 ± 1.00	52.04 ± 1.00**
AR237	Without cholesterol	9.62 ± 0.90	6.02 ± 1.68	37.21 ± 0.88	ND[e]	17.15 ± 0.63	20.67 ± 1.08	4.10 ± 0.68	5.22 ± 0.39	63.98 ± 0.69	36.02 ± 0.69
	With cholesterol	10.84 ± 1.35	14.43 ± 2.32	25.64 ± 3.15	1.62 ± 0.46	14.58 ± 0.59	22.72 ± 1.65	14.10 ± 0.90**	1.34 ± 0.08	48.91 ± 1.16	51.09 ± 1.16**

[a] Results are presented as mean ± SEM; each data point is the average of 3 repeated measurements, $n = 3$. *$p < 0.05$, **$p < 0.01$. [b] C14:0 = myristic acid; C16:0 = palmitic acid; C18:0 = stearic acid; C14:1 = nutmeg oleic acid; C16:1 = myristoleic acid; C18:1 = oleic acid; C18:2 = linoleic acid; C19:1 = nonadecenoic acid. [c] SFA, saturated fatty acids. [d] UFA, unsaturated fatty acids. [e] ND = not determined.

in the cell membrane, and its cholesterol-removal rate was only 0.91%. These results showed that except for *L. plantarum* AR300, the presence of cholesterol significantly changed the composition of fatty acids in the cell membrane, especially linoleic acid (C18:2) and total UFAs, but these changes were not significantly correlated with the cholesterol-removal rate.

3.4 Effect of the deconjugation ability of bile salts on cholesterol removal

The deconjugation activities of the nine strains of *L. plantarum* are shown in Fig. 4. All strains were able to deconjugate bile salts (sodium glycocholate and sodium taurocholate) to varying extents. As can be seen from Fig. 4, the concentration of released cholic acid ranged from 0.065 to 1.664 mM for sodium taurocholate and from 1.829 to 3.601 mM for sodium glycocholate, and the difference between these was significant. Consistent with the results of other studies,[29,30] the deconjugation of sodium glycocholate was more efficient than that of sodium taurocholate in all nine strains examined in our study. However, the deconjugation abilities were significantly different among the strains. A comparison of the deconjugation abilities and cholesterol-removal rates showed that *L. plantarum* AR113, AR513 and AR511 deconjugated sodium taurocholate with the release of more than 0.8 mM cholic acid and their deconjugation ability was significantly higher than that of the other strains. But, there were no correlations between the BSH activity and cholesterol-removal rates in the strains AR171, AR237 and AR495. Thus, BSH may be just a key factor for lowering cholesterol by some strains. To confirm this hypothesis, we added a BSH inhibitor to MCB for further investigation.

3.5 Effect of a BSH inhibitor on cholesterol removal

Wang *et al.* reported that NaIO4 showed a 96.4% inhibitory effect on the BSH activity.[28] Herein, the effect of this BSH inhibitor on the cholesterol removal rate was observed. As shown in Fig. 5, when $NaIO_4$ was added, the cholesterol-removal rates of six of the nine strains decreased. The cholesterol-lowering ability of *L. plantarum* AR113, AR513 and AR511 was significantly inhibited, for example, by 26.33% (from 37.14% to 10.81%) for the last of these, and the concentration of cholic acid released was positively correlated with the cholesterol-removal rate, indicating that BSH had a great effect on the cholesterol-lowering ability. The addition of the BSH inhibitor had little effect on the cholesterol-lowering ability of *L. plantarum* AR171, AR495 and AR237, and there was no correlation between the concentration of cholic acid released and the cholesterol-lowering ability. In summary, the addition of the BSH inhibitor had significant effects on *L. plantarum* AR113, AR513 and AR511 and slight effects on *L. plantarum* AR501, AR300 and AR326, but no significant effect on *L. plantarum* AR171, AR495 and AR237, indicating that BSH had a strain-specific effect on cholesterol removal.

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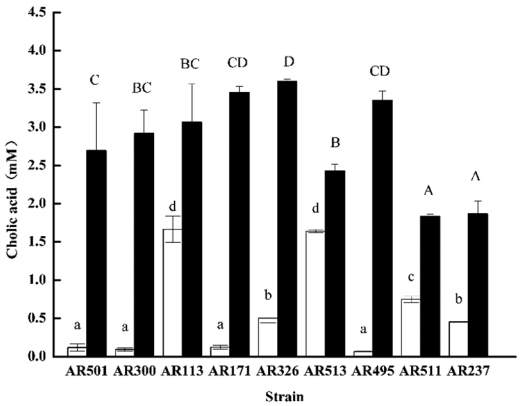

Fig. 4 Bile deconjugation activities of the nine strains incubated in MRS supplemented with 6 mM sodium glycocholate and 6 mM sodium taurocholate at 37 °C for 24 h. Error bars represent SEM; each data point is the average of three measurements, n = 3; abcd means significantly different sodium taurocholate activities between groups, as indicated by different lowercase letters, $P < 0.05$; ABCD means significantly different sodium glycocholate activities between groups, as indicated by different uppercase letters, $P < 0.05$. White bars: sodium taurocholate. Black bars: sodium glycocholate.

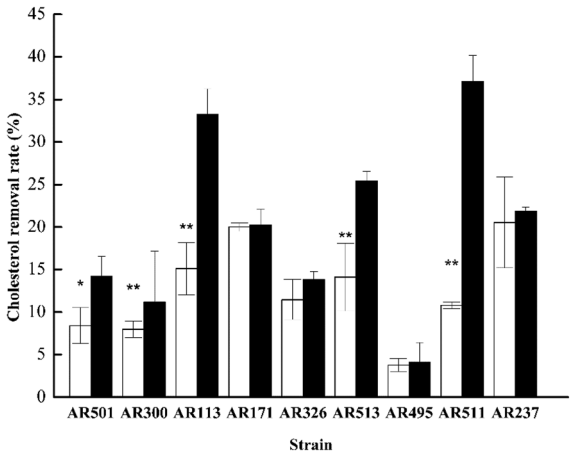

Fig. 5 The removal of cholesterol in the MRS medium containing cholesterol (100 μg mL^{-1}), ox bile (0.1%, v/v) and $NaIO_4$ (0.5 mM) as determined by the OPA method. Error bars represent SEM; each data point is the average of three measurements, n = 3,*$P < 0.05$, **$P < 0.01$. White bars: MCB + $NaIO_4$. Black bars: MCB.

3.6 Effect of pH-controlled conditions on cholesterol removal

L. plantarum is a species of lactic acid bacteria (LAB) that produces acids during growth. After 24 h of culture, the pH value in the growing environment dropped to approximately 3–4. As shown in Fig. 6, we found that the effects of pH on the cholesterol-lowering ability of different *L. plantarum* strains were diverse. At constant pH (about 5.5), the cholesterol removal rates of six strains were enhanced, while those of *L. plantarum* AR513, AR511 and AR237 were not. AR113 remained the strain with the highest cholesterol removal rate, although *L. plantarum* AR300, with a lower overall cholesterol-removal

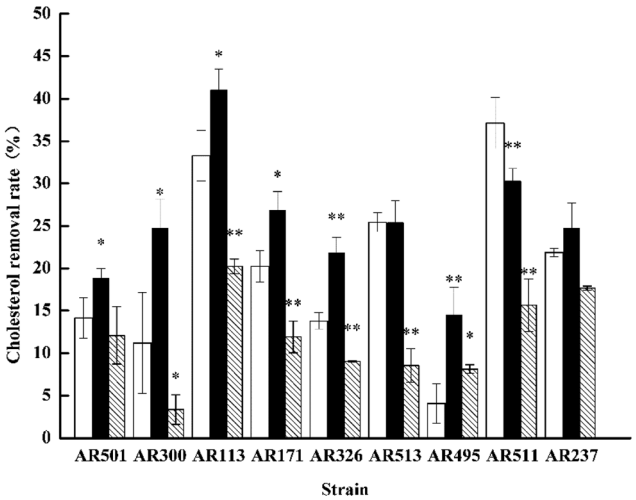

Fig. 6 The cholesterol removal rates of the nine strains at natural pH, constant pH (about 5.5) and neutral pH (7.0) as determined by the OPA method. Error bars represent SEM; each data point is the average of 3 measurements, n = 3. *$P < 0.05$, **$P < 0.01$. White bars: natural pH. Black bars: constant pH. Hatched bars: neutral pH.

rate, showed a significant increase from 11.21% to 24.72%. pH was an important factor of cholesterol removal in six strains, but had no significant effect on the three strains *L. plantarum* AR513, AR511 and AR237 (Fig. 6). However, under neutral pH conditions, the cholesterol removal rate of all strains decreased, except that of *L. plantarum* AR495, AR501 and AR237. The reductions in the cholesterol-removal rates of the other six strains were significant; for example, that of *L. plantarum* AR511 decreased from 37.14% to 15.65% and *L. plantarum* AR113 from 33.27% to 20.23%. These results showed that constant pH conditions significantly favoured the cholesterol-removal rates of the six strains AR501, AR300, AR113, AR171, AR326 and AR495, and neutral pH conditions reduced the cholesterol removal rates of the six strains *L. plantarum* AR300, AR113, AR171, AR326, AR513 and AR511.

4. Discussion

An elevated level of serum cholesterol in humans has been strongly associated with the risk of coronary heart disease. The use of *Lactobacillus* to reduce the serum cholesterol levels has attracted wide attention.[3] The strains of *L. plantarum* used in this study showed different cholesterol-removal rates. This is consistent with the earlier findings that the ability to reduce cholesterol differed among species and strains.[13] Hence, we hypothesized that different strains would be subject to different influencing factors, implying different cholesterol-lowering mechanisms.

Some reports have shown that *L. plantarum* FB003, *L. rhamnosus* GG, *L. bulgaricus* FTCC 0411, *L. bulgaricus* FTDC 1311 and *L. casei* ATCC 393 assimilated more cholesterol in the presence of 0.3% ox bile compared with that in the

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absence of bile.[13,19] In accordance with those findings, we found that *L. plantarum* AR501, AR326, AR113, AR511 and AR237 showed higher cholesterol removal rates in MRS containing 0.1% ox bile, but *L. plantarum* AR171 and AR495 showed small changes. Similarly, Liong and Shah showed that most strains had higher cholesterol removal rates than the control, except for the strains *L. acidophilus* ATCC 4357, *L. casei* ASCC 279 and *L. casei* ASCC 290.[23] These observations suggested that the effect of ox bile on the cholesterol removal rate was strain-specific. Most strains had high growth rates in the presence of cholesterol, but *L. acidophilus* ATCC 4962, *L. casei* ASCC 1520 and *L. casei* ASCC 1521 showed better growth in the absence of cholesterol, suggesting that cholesterol might hinder their growth.[23] For both *L. acidophilus* CSCC 2404 and CSCC 2410, there was a negative correlation between residual cholesterol and growth in MRS supplemented with 100 μg mL^{-1} water-soluble cholesterol and 0.3% ox bile.[30] However, as shown in Fig. 3, the growth rates and trends of most strains studied herein were essentially the same in MRS with or without cholesterol, including *L. plantarum* AR511, AR513, AR237, AR171, AR495 and AR326.

Although their biomass did not change in the presence of cholesterol, their cholesterol-removal rates changed, indicating that the cholesterol-removal rate of these strains was not dependent on their biomass. Another study indicated that the proportions of UFAs and saturated fatty acids (SFAs) in cells cultured in MRS with cholesterol were higher than in the control without cholesterol.[13] Analysis of variance showed that the UFA content of *L. rhamnosus* ASCC 1521, *L. acidophilus* CSCC 2400 and *B. bifidum* CSCC 5286 significantly increased in the presence of cholesterol.[30] Eight of the strains studied herein were consistent with the above findings, but the UFA proportion of *L. plantarum* AR300 remained almost unchanged.

The relationship between cholesterol removal and BSH in *L. plantarum* has been widely studied. In acidic environments, cholesterol removal is the result of co-precipitation of both cholesterol and deconjugated bile salts.[14] Our results showed that the nine strains had different deconjugation abilities with the same bile salt, and adding a BSH inhibitor can decrease the cholesterol removal rate. For *L. plantarum* AR113, AR511 and AR513, there was a positive correlation between the cholesterol lowering ability and the concentration of free bile acid released, and the cholesterol-removal rate was also significantly reduced after a BSH inhibitor was added. This was in accordance with previous research.[23] However, the cholesterol removal capability of *L. plantarum* AR495, AR171 and AR237 did not correlate with the concentration of cholic acid released. These results suggested that the effects of BSH on the cholesterol-removal rates were strain-specific. Previous research has shown that the co-precipitation effects of cholesterol are related to the pH of the medium.[31] Under *in vitro* conditions, cholesterol was removed by co-precipitation at low pH.[22] However, another study has shown that the co-precipitation of cholesterol was not completely dependent on pH.[23] Whether the cholesterol-removal rate of these strains is affected by pH is controversial. Our results indicated that for some of the studied strains pH was the key factor for improving the cholesterol-removal rate, and thus the effect of pH was strain-specific.

5. Conclusions

Some *Lactobacillus* strains have demonstrated the ability to remove cholesterol from their medium. All of the strains investigated in this study clearly indicated their ability to remove cholesterol, but to greatly varying extents. The factors that affected their ability to lower cholesterol were also different. *L. plantarum* AR113 and AR326 were influenced by growth status and BSH activity and pH. *L. plantarum* AR511 was affected by ox bile and growth status. *L. plantarum* AR495 was affected by growth status and pH. *L. plantarum* AR300 was affected by pH and BSH activity. *L. plantarum* AR513 and AR501 were affected by BSH activity and ox bile. Finally, *L. plantarum* AR171 and AR237 were affected only by pH and ox bile, respectively. This work indicated that these *L. plantarum* strains had different cholesterol-lowering abilities and were affected by different factors, suggesting that there are different mechanisms for lowering cholesterol, which need to be further explored.

Conflicts of interest

There are no conflicts to declare.

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