Annals of Agri-Bio Research **27** (2) : 211-216, 2022 **Impact Score : 0.28 (Scopus)**

# *Lactobacillus gasseri* with Probiotic Properties Isolated from Thai Traditional Fermented Foods

PARICHAT PHUMKHACHORN AND PONGSAK RATTANACHAIKUNSOPON*

*Department of Biological Science, Faculty of Science, Ubon Ratchathani University, Warin Chamrap, Ubon Ratchathani-34190, Thailand*
**(e-mail : rattanachaikunsopon@yahoo.com; Contact : +66- 0867792834)*

(Received : February 20, 2022; Accepted : March 18, 2022)

**ABSTRACT**

Probiotic microorganisms are live microorganisms, when administered in adequate amounts, confer a health benefit. Most probiotic microorganisms generally belong to lactic acid bacteria. Therefore, this study was aimed at finding lactic acid bacteria (LAB) having probiotic properties. Twelve isolates of LAB isolated from four different Thai traditional fermented foods including Nham (fermented pork rind), PlaSom (fermented fish), Kung Jom (fermented shrimp paste) and Mum (fermented ground beef) were tested for their tolerance to acid and to bile salt, considering with important probiotic properties. It was found that LAB isolate M2, isolated from Mum, was the most tolerant isolate to acid at pH 2 and to bile salt at the concentration of 2%. Furthermore, the study showed that LAB isolate M2 had antimicrobial ability against *Escherichia coli* O157 : H7 ATCC 43895 and *Staphylococcus aureus* ATCC 25923. Based on 16S rDNA sequence analysis and phylogenetic tree analysis, the LAB isolate M2 was found to be closely related to *Lactobacillus gasseri* strain ATCC 33323 (accession number NR_075051.2). The LAB isolate with probiotic properties obtained from this study can be further characterized in order to utilize it in the future.

**Key words :** Fermented foods, lactic acid bacteria, *Lactobacillus gasseri*, probiotics

## INTRODUCTION

Microorganisms have been unintentionally used as part of our diets, especially fermented foods, for centuries. In 1907, Elie Metchnikoff, a Russian Noble Prize Winner, discovered that Bulgarian people, in the Caucasus Mountains, who were over 100 years old had a fermented yoghurt drink, containing *Lactobacillus bulgaricus*, on a daily basis (Velikova *et al.*, 2018). In 1935, Yakult, the first commercial drink containing a beneficial microorganism, *Lactobacillus casei* Shirota, was introduced (Ou *et al.*, 2019). The term "probiotics", meaning "for life", was first coined by Lilly and Stillwell in 1965 to describe substances secreted by one microorganisms stimulating growth of other microorganisms (Kerry *et al.*, 2018). Although many definitions of probiotics have been proposed, the internationally endorsed definition of probiotics is proposed by FAO/WHO in 2011 as "live microorganisms that, when administered in adequate amounts, confer a health benefit on the host" (Binda *et al.,* 2020). Most probiotic microorganisms belong to lactic acid bacteria, especially *Lactobacillus* spp. and *Bifidobacterium* spp. However, *Enterococcus* spp. has not been considered as probiotics in some countries because of their tendency to transfer antibiotic resistance genes (Hanchi *et al.*, 2018). Besides lactic acid bacteria, other microorganisms have been reported to have probiotic properties including *Bacillus* sp. (Mingmongkolchai and Panbangred, 2018), *Clostridium butyricum* (Guo *et al.*, 2020) and yeast *Saccharomyces boulardii* (Sen and Mansell, 2020). To be able to exert their health benefit in host bodies, probiotic microorganisms have to be resistant to the gastric acid environment and to bile salt in the small intestine (Li *et al.*, 2020). Therefore, these two properties are commonly used as major criteria for selection of probiotic microorganisms. Other criteria for probiotics selection, such as having beneficial immunological effect (Vitetta *et al.,* 2018), antimicrobial activity against pathogenic microorganisms (Igbafe *et al.,* 2020) antioxidant activity (Wang *et al.,* 2017) and antimutagenic activity (Lee *et al.,* 2020) are applied only when certain probiotic properties are required.

In this study, probiotic lactic acid bacteria were

isolated from various traditionally Thai fermented foods. They were also examined for their acid and bile salt tolerance. The selected one with the most acid and bile salt tolerance was tested for its antimicrobial activity against pathogenic bacteria and identified using the 16S rDNA sequence analysis and phylogenetic tree analysis.

## MATERIALS AND METHODS

Thai fermented foods used as sources of lactic acid bacteria were Nham (fermented pork rind), PlaSom (fermented fish), Kung Jom (fermented shrimp paste) and Mum (fermented ground beef). Twenty-five g of each food sample were thoroughly mixed with 225 g of 0.85% (w/v) of NaCl by a stomacher. The liquid part of the mixture was 10-fold serially diluted in 0.85% NaCl to obtain dilutions ranging from $10^{-1}$ to $10^{-6}$. One hundred ml of three different dilutions ($10^{-4}$, $10^{-5}$and $10^{-6}$) were spread on MRS (de Man, Rogosa and Sharpe) agar supplement with 1% (w/v) $CaCO_3$. After incubation at 30°C for 18 h, bacterial single colonies surrounding with a clear zone were randomly selected. The isolated lactic acid bacteria were subjected to re-streaking on MRS agar to obtain pure bacterial isolates which were further examined by Gram staining and catalase test.

Twenty µl of 18 h culture of each LAB isolate were inoculated in 2 ml of fresh MRS broth adjusted pH to 2 and 7 (control). The LAB cultures are incubated at 30°C for 6 h before measuring their absorbance at the wavelength of 600 nm. The results were shown as per cent survival calculated from (absorbance of LAB culture with pH 2/absorbance of LAB culture with pH 7) x 100. The experiment was performed in triplicate.

Twenty µl of 18 h culture of each LAB isolate were inoculated in 2 ml of fresh MRS broth supplemented with bile salt (2% w/v) and with no bile salt. The LAB cultures were incubated at 30°C for 6 h before measuring their absorbance at the wavelength of 600 nm. The results are shown as per cent survival calculated from (absorbance of LAB culture with bile salt/absorbance of LAB culture with no bile salt) x 100. The experiment was performed in triplicate.

Study of antimicrobial activity of LAB against pathogenic bacteria consisted of 2 steps including the preparation of cell free culture supernatant of LAB (LAB-CFCS) and the detection of LAB-CFCS against pathogenic bacteria, *Escherichia coli* O157 : H7 ATCC 43895 and *Staphylococcus aureus* ATCC 25923. To prepare LAB-CFCS, each LAB was cultured in MRS broth at 30°C for 18 h. The culture was then centrifuged at 5,000 xg for 10 min. The supernatant was collected and passed through a 0.22-µm-pore-size sterile filter. The resulting LAB-CFCS was kept at 4°C before use.

Antimicrobial activity of LAB-CFCS was determined by swab paper disc assay. Each indicator microorganism (*E. coli* O157 : H7 ATCC 43895 or *S. aureus* ATCC 25923) was grown in BHI (Brain Heart Infusion) broth at 37°C for 18 h and then smeared onto BHI agar by a sterile swab. A sterile paper disc (6 mm in diameter) was placed onto the inoculated BHI agar. Ten µl of the previously prepared LAB-CFCS were dropped onto the paper disc. After incubation at 37°C for 18 h, an inhibition zone around the paper disc was observed. The experiment was performed in triplicate.

The LAB isolate with the most acid and bile salt tolerance was subjected to the bacterial identification using 16S rDNA sequence analysis. Genomic DNA of the LAB isolate was extracted by using Genomic DNA Extraction Kit Mini (RBC Bioscience, Taipei, Taiwan) and used as template DNA for PCR amplification of 16S rDNA. Two primers used in this experiment were FD1 (5'AGAGTTTGATCC TGGCTCAG3') and RP2 (5'ACGGCTACCTTGT TACGACTT3'). The PCR condition consisted of 1 cycle of 94°C for 5 min, followed by 30 cycles of 94°C for 1 min, 55°C for 1 min and 72°C for 1 min, and 1 cycle of 72°C for 7 min. The nucleotide sequence of PCR product was determined by BioDesign Co., Ltd. (Pathumthani, Thailand) and compared with the 16S rDNA sequences in the GenBank database using the NCBI Blast program. It was also subjected to multiple alignments using Clustal W and phylogenetic analysis using Neigbor-joining method. Evolutionary analyses were conducted in MEGA 7.

The data (means±SD) were analyzed by one way analysis of variance (ANOVA) followed by

Tukey's test to compare the means of all treatments to the mean of every other treatment in SPSS at P<0.05 levels.

## RESULTS AND DISCUSSION

A total of 12 LAB isolates were obtained from the isolation of LAB from Thai fermented food on $CaCO_3$ supplemented MRS agar. Among them, three isolates were derived from each food sample including Nham (N1-N3), PlaSom (PS1-PS3), Kung Jom (KJ1-KJ3) and Mum (M1-M3). Preliminary microbiological examination revealed that all of them were Gram positive and did not produce catalase. LAB isolates from Nham and Mum were bacilli, whereas those isolated from PlaSom and Kung Jom were cocci.

LAB having a human origin or human strains generally possessed essential characteristics of probiotics which were acid and bile salt tolerance because they could survive in low pH condition of stomach and high concentration of bile salt in small intestine. However, this was not the case for those derived from foods. Therefore, tests for these characteristics are of necessity for the selection of probiotics derived from foods. Besides the essential characteristics of probiotics, some probiotics may have one or more of other characteristics, most of them related to health promotion, such as enhancing growth (Park *et al.*, 2017), preventing pathogenic bacteria to adhere to intestinal epithelium (Kerry *et al.*, 2018), improving immune system (Vitetta *et al.*, 2018), producing antioxidants (Wang *et al.*, 2017) and preventing diseases of gastrointestinal tract (Park *et al.*, 2017).

The pH of human stomach varies from 2-4 depending on its function. When proteins get to the stomach, it releases proteolytic enzyme, called pepsin, and hydrochloric acid (HCl) to aid in digestion (Martinsen *et al.*, 2019). By itself, HCl does not do the digestion work, but pepsin that cleaves proteins work best in an acidic environment or low pH. Therefore, LAB being used as probiotics must survive in the gastric acid environment. In this experiment, all 12 isolated LAB were tested for their tolerance at pH 2. The results are shown in Table 1. The acid tolerance of LAB was classified into three levels : high (% survival > 80%), moderate (% survival 50-80%) and low (% survival < 50%). From Table 1, LAB with high acid tolerance were N1 and M2, with moderate acid tolerance were N2, N3, PS1, PS3, KJ1, KJ2 and M1 and with low acid tolerance were PS2, KJ3 and M3.

**Table 1.** Acid tolerance of lactic acid bacteria isolated from fermented foods

| LAB isolate | Per cent survival of each experiment in triplicate experiments | | | Per cent survival (Mean±SD) |
|---|---|---|---|---|
| | 1 | 2 | 3 | |
| N1 | 89.47 | 87.64 | 85.33 | 87.48±2.08[a] |
| N2 | 52.53 | 55.27 | 50.04 | 52.61±2.62[d] |
| N3 | 66.23 | 67.45 | 69.38 | 67.69±1.59[c] |
| PS1 | 81.95 | 80.51 | 77.11 | 79.86±2.49[b] |
| PS2 | 44.37 | 43.33 | 40.27 | 42.66±2.13[e] |
| PS3 | 55.88 | 50.54 | 52.23 | 52.88±2.73[d] |
| KJ1 | 69.57 | 65.08 | 67.43 | 67.36±2.25[c] |
| KJ2 | 67.53 | 62.29 | 68.77 | 66.20±3.44[c] |
| KJ3 | 47.68 | 45.36 | 43.12 | 45.39±2.28[e] |
| M1 | 69.17 | 72.76 | 65.34 | 69.09±3.71[c] |
| M2 | 86.36 | 88.31 | 83.72 | 86.13±2.30[a] |
| M3 | 39.29 | 35.52 | 38.66 | 37.82±2.02[f] |

Mean values with different superscripts in a column are significantly different at P<0.05.

The human small intestine is the site where lipids are digested. Lipid digestion is required not only lipases but also bile as a lipid emulsifier. Therefore, bacteria being used as probiotics must be resistant to bile that is toxic to many bacterial strains. In general, human small intestine consists of 0.2-2% bile salt depending on the individual and the type and amount of food ingested (Hu *et al.*, 2018). When lipids are consumed, the concentration of bile salt in small intestine increases up to 2% to help lipid digestion. In this experiment, all 12 isolated LAB were tested for their tolerance to 2% bile salt. The results are shown in Table 2. The bile salt tolerance of LAB was classified into three levels : high (% survival > 80%), moderate (% survival 50-80%) and low (% survival < 50%). From Table 2, LAB with high bile salt tolerance was M2, with moderate acid tolerance were N1, N3, KJ1, KJ2 and KJ3 and with low acid tolerance were N2, PS1, PS2, PS3, M1 and M3.

Based on high acid and bile salt tolerance, the LAB isolate M2 was selected for further

**Table 2.** Bile salt tolerance of lactic acid bacteria isolated from fermented foods

| LAB isolate | Per cent survival of each experiment in triplicate experiments | | | Per cent survival (Mean±SD) |
|---|---|---|---|---|
| | 1 | 2 | 3 | |
| N1 | 60.65 | 55.76 | 57.09 | 57.83±2.53$^{c}$ |
| N2 | 47.40 | 45.02 | 43.33 | 45.25±2.05$^{d}$ |
| N3 | 54.67 | 56.74 | 55.21 | 55.54±1.07$^{c}$ |
| PS1 | 48.55 | 42.79 | 45.36 | 45.57±2.89$^{d}$ |
| PS2 | 42.42 | 47.62 | 45.33 | 45.12±2.61$^{d}$ |
| PS3 | 45.86 | 46.64 | 43.90 | 45.46±1.41$^{d}$ |
| KJ1 | 73.89 | 70.55 | 68.32 | 70.92±2.80$^{b}$ |
| KJ2 | 58.28 | 55.54 | 54.95 | 56.26±1.78$^{c}$ |
| KJ3 | 70.27 | 68.66 | 71.22 | 70.05±1.29$^{b}$ |
| M1 | 33.58 | 35.18 | 30.02 | 32.93±2.64$^{e}$ |
| M2 | 82.96 | 80.51 | 84.29 | 82.59±1.91$^{a}$ |
| M3 | 23.91 | 25.82 | 20.07 | 23.27±2.93$^{f}$ |

Mean values with different superscripts in a column are significantly different at P<0.05.

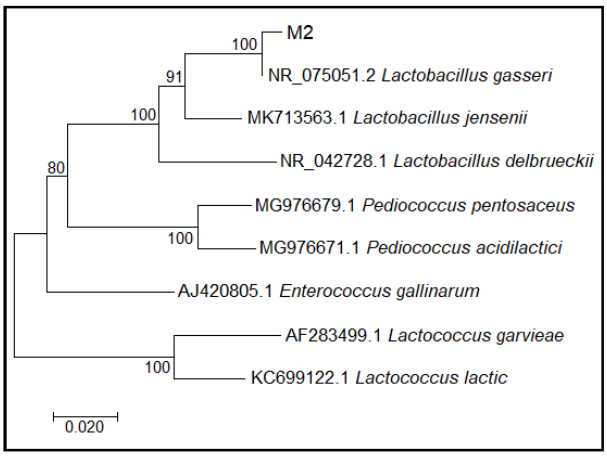


Fig. 1. Phylogenetic tree based on neighbour-joining method created with 16S rDNA sequences of LAB isolate M2 and other species of lactic acid bacteria. Values on nodes represent percentage bootstraps out of 1000 bootstrap samples; values less than 50% are not shown. Scale bar represents the number of mutations per sequence position.

experiments which were antimicrobial activity against pathogenic bacteria and bacterial identification by 16S rDNA sequence analysis.

When LAB isolate M1 was tested for its antimicrobial activity against *E. coli* O157 : H7 ATCC 43895 or *S. aureus* ATCC 25923, it was found that LAB isolate M2 was able to inhibit both pathogenic bacteria. Since LAB had ability to produce several antimicrobial substances such as organic acids, hydrogen peroxide and bacteriocins (Hernandez-Gonzalez *et al.*, 2021), further investigation is required to find out the actual substance responsible for antimicrobial activity of LAB isolate M2.

When genomic DNA of LAB isolate M2 was used as template DNA for PCR amplification of 16S rDNA, the obtained PCR product was about 1,496 bp in size. The nucleotide sequence of the PCR product was compared to the known bacterial 16S rDNA sequences in GenBankdatabase using Blast program, it showed 99% homology to 16S rDNA sequence of *Lactobacillus gasseri* strain ATCC 33323 (accession number NR_075051.2). Thus, the LAB isolate M2 was designated as *L. gasseri* M2. This result was confirmed by phylogenetic tree analysis showing that the LAB isolate M2 was closely related to *L. gasseri* (accession number NR_075051.2; Fig. 1). Many strains of *Lactobacillus* were previously reported to have probiotic properties such as *Lactobacillus plantarum* (Astuti *et al.*, 2018; Zheng *et al.*, 2020), *Lactobacillus johnsonii* (Delley *et al.*, 2015), *Lactobacillus acidophilus* (Delley *et al.*, 2015; Vemuri *et al.*, 2018), *Lactobacillus fermentum* (Naghmouchi *et al.*, 2020) and *Lactobacillus rhamnosus* (Martin *et al.*, 2019). Furthermore, some of them have already used in commercial products such as *L. casei* Shirota (Ou *et al.*, 2019), *L. johnsonii* (Delley *et al.*, 2015) and *L. acidophilus* (Vemuri *et al.*, 2018).

## CONCLUSION

*Lactobacillus gasseri* M2 was LAB strain isolated from Mum, Thai traditional fermented ground beef. It had high survive rates in both acidic (pH 2) condition and condition with high bile salt concentration (2%). It also inhibited *E. coli* O157 : H7 ATCC 43895 and *S. aureus* ATCC 25923. The LAB strain obtained from this study had potential to be developed for being used as a probiotic bacterium.

## ACKNOWLEDGEMENT

The authors appreciate the support of the Department of Biological Science, Faculty of Science, Ubon Ratchathani University, Thailand for providing assistance with tools, equipment and chemicals.

## REFERENCES


Astuti, W. D., Wiryawan, K. G., Wina, E., Widyastuti, Y., Suharti, S. and Ridwan, R. (2018). Effects of selected *Lactobacillus plantarum* as probiotic on *in vitro* ruminal fermentation and microbial population. *Pak. J. Nut.* **17** : 131-139.

Binda, S., Hill, C., Johansen, E., Obis, D., Pot, B., Sanders, M. E., Tremblay, A. and Ouwehand, A. C. (2020). Criteria to qualify microorganisms as "Probiotic" in foods and dietary supplements. *Front. Microbiol.* **11** : 1662. *https://doi.org/10.3389/fmicb.2020.01662.*

Delley, M., Bruttin, A., Richard, M., Affolter, M., Rezzonico, E. and Bruck, W. M. (2015). *In vitro* activity of commercial probiotic *Lactobacillus* strains against uropathogenic *Escherichia coli. FEMS Microbiol. Letters* **362** : fnv096. *https://doi.org/10.1093/femsle/fnv096.*

Guo, P., Zhang, K., Ma, X. and Pingli, H. (2020). *Clostridium* species as probiotics : Potentials and challenges. *J. Anim. Sci. Biotech.* **11** : 24. *https://doi.org/10.1186/s40104-019-0402-1.*

Hanchi, H., Mottawea, W., Sebei, K. and Hammami, R. (2018). The genus *Enterococcus* : Between probiotic potential and safety concerns–An update. *Front. Microbiol.* **9** : 1791. *https://doi.org/10.3389/fmicb.2018.01791.*

Hernandez-Gonzalez, J. C., Martinez-Tapia, A., Lazcano-Hernandez, G., Garcia-Perez, B. E. and Castrejon-Jimenez, N. S. (2021). Bacteriocins from lactic acid bacteria. A powerful alternative as antimicrobials, probiotics and immunomodulators in veterinary medicine. *Animals* **11** : 979. *https://doi.org/10.3390/ani11040979.*

Hu, P. L.,Yuan, Y. H., Yue, T. L. and Guo, C. F. (2018). Bile acid patterns in commercially available oxgall powders used for the evaluation of the bile tolerance ability of potential probiotics. *PLoS One* **13** : e0192964. *https://doi.org/10.1371/journal.pone.0192964.*

Igbafe, J., Kilonzo-Nthenge, A., Nahashon, S. N., Mafiz, A. I. and Nzomo, M. (2020). Probiotics and antimicrobial effect of *Lactiplantibacillus plantarum*, *Saccharomyces cerevisiae* and *Bifidobacterium longum* against common food-borne pathogens in poultry. *Agric.* **10** : 368. *https://doi.org/10.3390/agriculture10090368.*

Kerry, R. G., Patra, J. K., Gouda, S., Park, Y., Shin, H. S. and Das, G. (2018). Benefaction of probiotics for human health : A review. *J. Food Drug Anal.* **26** : 927-939.

Lee, S. B., Cosmas, B. and Park, H. D. (2020). The antimutagenic and antioxidant activity of fermented milk supplemented with *Cudrania tricuspidata* powder. *Foods* **9** : 1762. *https://doi.org/10.3390/foods9121762.*

Li, M., Wang, Y., Cui, H., Li, Y., Sun, Y. and Qiu, H. J. (2020). Characterization of lactic acid bacteria isolated from the gastrointestinal tract of a wild boar as potential probiotics. *Front. V. Sci.* **7** : 49. *https://doi.org/10.3389/fvets.2020.00049.*

Martin, R., Chamignon, C., Mhedbi-Hajri, N., Chain, F., Derrien, M., Escribano-Vazquez, U., Garault, P., Cotillard, A., Pham, H. P., Chervaux, C., Bermudez-Humaran, L. G., Smokvina, T. and Langella P. (2019). The potential probiotic *Lactobacillus rhamnosus* CNCM I-3690 strain protects the intestinal barrier by stimulating both mucus production and cytoprotective response. *Scientific Rep.* **9** : 5398. *https://doi.org/10.1038/s41598-019-41738-5.*

Martinsen, T. C., Fossmark, R. and Waldum, H. L. (2019).The phylogeny and biological function of gastric juice-microbiological consequences of removing gastric acid. *Int. J. Mol. Sci.* **20** : 6031. *https://doi.org/10.3390/ijms20236031.*

Mingmongkolchai, S. and Panbangred, W. (2018). *Bacillus* probiotics : An alternative to antibiotics for livestock production. *J. Appl. Microbiol.* **124** : 1334-1346.

Naghmouchi, K., Belguesmia, Y., Bendali, F., Spano, G., Seal, B. S. and Drider, D. (2020). *Lactobacillus fermentum* : A bacterial species with potential for food preservation and biomedical applications. *Critical Rev. Food Sci. Nut.* **60** : 3387-3399.

Ou, Y., Chen, S., Ren, F., Zhang, M., Ge, S., Guo, H., Zhang, H. and Zhao, L. (2019). *Lactobacillus casei* strain Shirota alleviates constipation in adults by increasing the pipecolinic acid level in the gut. *Front. Microbiol.* **10** : 324. *https://doi.org/10.3389/fmicb.2019.00324.*

Park, Y., Lee, S., Hong, J., Kim, D., Moniruzzaman, M. and Sungchul, C. B. (2017). Use of probiotics to enhance growth, stimulate immunity and confer disease resistance

to *Aeromonas salmonicida* in rainbow trout (*Oncorhynchus mykiss*). *Aquaculture Res.* **48** : 2672-2682.

Sen, S. and Mansell, T. J. (2020). Yeasts as probiotics : Mechanisms, outcomes and future potential. *Fungal Gen. Biol.* **137** : 103333. *https://doi.org/10.1016/j.fgb.2020.103333.*

Velikova, P., Petrov, K., Lozanov, V., Tsvetanova, F., Stoyanov, A., Wu, Z., Liu, Z. and Petrova, P. (2018). Microbial diversity and health-promoting properties of the traditional Bulgarian yogurt. *Biotechnology and Biotechnological Equipment* **32** : 1205-1217.

Vemuri, R., Shinde, T., Shastri, M. D., Perera, A. P., Tristram, S., Martoni, C. J., Gundamaraju, R., Ahuja, K. D. K., Ball, M. and Eri, R. (2018). A human origin strain *Lactobacillus acidophilus* DDS-1 exhibits superior *in vitro* probiotic efficacy in comparison to plant or dairy probiotics. *Int. J. Med. Sci.* **15** : 840-848.

Vitetta, L., Vitetta, G. and Hall, S. (2018). Immunological tolerance and function : Associations between intestinal bacteria, probiotics, prebiotics and phages. *Front. Immunology* **9** : 2240. *https://doi.org/10.3389/fimmu.2018.02240.*

Wang, Y., Wu, Y., Wang, Y., Xu, H., Mei, X., Yu, D., Wang, Y. and Li, W. (2017). Antioxidant properties of probiotic bacteria. *Nutrients* **9** : 521. *https://doi.org/10.3390/nu9050521.*

Zheng, Z. Y., Cao, F. W., Wang, W. J., Yu, J., Chen, C., Chen, B., Liu, J. X., Firrman, J., Renye, J. and Ren, D. X. (2020). Probiotic characteristics of *Lactobacillus plantarum* E680 and its effect on hypercholesterolemic mice. *BMC Microbiology* **20** : 239. *https://doi.org/10.1186/s12866-020-01922-4.*