



Journal of
**Pharmacology and
Toxicology**

ISSN 1816-496X



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Inhibitory Effect of Metabolites from Probiotics *Lactobacillus acidophilus* Strains on Growth of Pathogenic Bacteria

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ABSTRACT

In the present study, the effects of combined metabolites produced by lactic acid bacteria were tested on food pathogenic and contaminant microorganisms. Totally, eight strains of *Lactobacillus acidophilus* (FTDC 2804, FTDC 0785, FTDC 8592, FTDC 1295, FTDC 4793, FTDC 4462, FTDC 0582 and FTDC 2916) were used which cultivated in four types of agro waste substrates including pineapple waste, soy whey, cabbage and molasses. The inhibitory effects of *L. acidophilus* metabolites (lactic acid, hydrogen peroxide, acetaldehyde, diacetyl and bacteriocin) were determined by agar well diffusion method on two pathogenic bacteria; *Staphylococcus aureus* and *Escherichia coli*. Metabolites of different *L. acidophilus* strains cultured in different agro waste substrates showed diverse inhibitory effects. Among all, the highest inhibition zone was obtained with the strains cultivated in pineapple waste, such as, *L. acidophilus* FTDC 4462 strain (10.57±0.10 mm for *S. aureus* and 11.13±0.45 mm for *E. coli*). It can be concluded that *L. acidophilus* species has the ability to grow in agro waste materials and produce beneficial metabolites with antibacterial activities.

Key words: *Lactobacillus acidophilus*, agro waste, antimicrobial activity, probiotics, metabolites

INTRODUCTION

Probiotics are living microorganisms that are similar to symbiotic microorganisms found in the human gut. They are also called "friendly bacteria" or "good bacteria" (Saavedra, 2001; Douglas and Sanders, 2008). According to a definition developed by the Food and Agriculture Organization of the United Nations (FAO) and World Health Organization (WHO), probiotics are the microorganisms and, when administered in adequate amounts, confer a health benefit on the host. (FAO/WHO, 2001). Probiotics are available to consumers mainly in the form of dietary supplements and foods. They can be used as Complementary and Alternative Medicine (CAM) (Day, 2002).

Most probiotics are bacteria similar to those naturally found in human guts, especially in those of breastfed infants who have natural protection against many diseases (NCCAM, 2008). Most often, the bacteria come from two groups, *Lactobacillus* or *Bifidobacterium*. Within each group, there are different species such as *Lactobacillus acidophilus* and *Bifidobacterium bifidus*, moreover, within each species, different strains or varieties (Heller, 2001; O'Mahony *et al.*, 2005).

The concept of probiotics progressed around 1900, when Elie-Metchnikoff hypothesized that the long and healthy lives of Bulgarian peasants were the outcome of their consumption of fermented milk and milk products (Kopp-Hoolihan, 2001).

Earlier studies on probiotics showed that the fermented products of probiotics possess strong anti-bactericidal effects against foodborne pathogens (Yesillik *et al.*, 2011). Similarly, in another study the isolated strain of *Lactobacillus paraplantarum* from Tea Leaves demonstrated potent inhibitory effect on the growth of *E. coli* and *S. aureus* (Gharaei-Fathabad and Eslamifar, 2011). Amin *et al.* (2009) reported the isolation of sixty lactobacilli strains, from fresh vegetables which were grown in Man-Rogosa-Sharpe medium (MRS) broth, exhibited remarkable antimicrobial activity against a panel of pathogenic bacteria such as *Escherichia coli*, *Salmonella typhi*, *Shigella dysenteriae*, *Bacillus anthracis* and *Staphylococcus aureus*.

Beneficial effects conferred by Lactobacilli, including inhibition of gram negative and positive pathogenic bacteria, were described by Maragkoudakis *et al.* (2006) and Charlier *et al.* (2008). Substantiating the antimicrobial activities of probiotics will affirm their use in the development of functional foods for the betterment of the health of the consuming public (Eduardo *et al.*, 2003).

The aim of this study was to investigate the effect of *L. acidophilus* strains metabolites cultivated in four different types of agro waste substrates namely, pineapple, soy-whey, cabbage and molasses against *Staphylococcus aureus* (gram positive) and *Escherichia coli* (gram negative) pathogenic bacteria.

MATERIALS AND METHODS

Microorganisms and culture media reagents: Man-Rogosa-Sharpe (MRS) broth was obtained from (Hi-media, India). Nutrient agar and nutrient broth were purchased from Merck, Darmstadt, Germany. Waste substrates such as pineapple, soy-whey, cabbage and molasses were collected from the local market. The other reagents used in the experiment were analytical grade.

The pathogenic microorganisms, *E. coli* and *S. aureus* were obtained from General Hospital. Penang, Malaysia. The bacterial species were cultivated and maintained in nutrient agar slants in screw-capped tubes. The agar slants were preserved in a refrigerator at 4°C until use.

Strains of *L. acidophilus* (FTDC 2804, FTDC 0785, FTDC 8592, FTDC 1295, FTDC 4793, FTDC 4462, FTDC 0582 and FTDC 2916) were obtained from the Culture Collection Center of the School of Industrial Technology, Universiti of Sains Malaysia (Penang Malaysia). The strains were inoculated in sterile MRS broth (dextrose 20.0 g L⁻¹; meat peptone 10.0 g L⁻¹; beef extract 10.0 g L⁻¹; yeast extract 5.0 g L⁻¹; sodium acetate 5.0 g L⁻¹; disodium phosphate 2.0 g L⁻¹; ammonium citrate 2.0 g L⁻¹; tween 80 1.0 g L⁻¹; magnesium sulfate 0.1 g L⁻¹, manganese sulfate 0.05 g L⁻¹) at 37°C for 36 h. The organisms were activated for three successive times prior to use. The culture centrifuged at 5000 rpm for 15 min and then washed two times by normal saline. The cells re-suspended in four different waste substrates mentioned above (1% v/v inoculum size). All the substrates were incubated at 37°C for 36 h. After incubation the bacterial suspension was centrifuged at 4°C for 15 min at 5000 rpm (Beckman, USA). The metabolites were next evaluated for antimicrobial activity. The entire work was conducted at Department of Pharmaceutical Technology, School of Pharmaceutical Sciences, Univeristi Sains Malaysia, Penang, Malaysia in year of 2010.

Evaluation of antimicrobial activity: This assay was performed using the agar well diffusion method (Fooks and Gibson, 2002; Toure *et al.*, 2003) and is widely used to determine the antimicrobial activity against different types of pathogenic microorganisms.

The antimicrobial activities were determined by measuring the diameter of zone of growth inhibition formed around the wells. To further check whether the pathogens were inhibited or killed, a swab was taken from the growth inhibition zone and then inoculated into nutrient broths

and incubated at 37°C for 24 h. The broth tubes were then checked for growth. Presence of growth in the broth was interpreted as an inhibitory activity, while no growth was interpreted as microbicidal activity. Each experiment was conducted in two individual trials, each in triplicate.

Statistical analysis: The results were analyzed statistically using one-way analysis of variance (version 13.0, SPSS, USA). When there was a statistically significant difference, post-hoc Tukey Honestly Significant Difference test was applied. ANOVA data with $p < 0.001$, $p < 0.01$ and $p < 0.05$ were classified as statistically significant.

RESULTS AND DISCUSSION

The selection of suitable probiotics starter culture among the one naturally present in fermented products should be emphasized. Earlier studies showed that the probiotics grown in modified MRS media exerted considerable antagonistic effect against the human pathogens *Bacillus cereus*, *Listeria monocytogenes*, MRSA (Methicillin Resistance *Staphylococcus aureus*) and *Pseudomonas aeruginosa* the causative agents of food borne disease, Listeriosis, skin infections and lung disease, respectively (Bhilabutra *et al.*, 2007). Yesillik *et al.* (2011) showed that the antibacterial effects of probiotic yoghurt on viability of *Salmonella typhimurium*, *Escherichia coli*, *Pseudomonas aeruginosa* and *Staphylococcus aureus* was investigated by using disc diffusion method. In their study it was determined that the most sensitive pathogenic bacteria to the metabolites of probiotics were *Salmonella typhimurium* whereas the least sensitive pathogen was *Pseudomonas aeruginosa*.

In the present study, the results of the antibacterial activities of probiotic *L. acidophilus* strains cultivated in pineapple agro waste juice against *S. aureus* and *E. coli* is depicted in Fig. 1. The inhibition zones obtained in *S. aureus* cultures were in the range of 10.08-10.57 mm, whereas, the inhibition zones observed in *E. coli* cultures were in the range of 9.77-11.13 mm. The highest inhibition was obtained with *L. acidophilus* FTDC 4462 against *S. aureus* (10.57 mm) and *E. coli* (11.13 mm). While, the lowest inhibition effect was obtained with *L. acidophilus* FTDC 0582 against *S. aureus* (10.08 mm) and *L. acidophilus* FTDC 8592 against *E. coli* (9.77 mm).

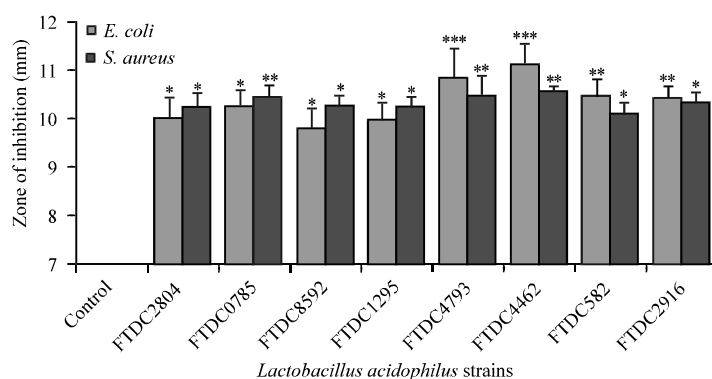


Fig. 1: The antibacterial activities of probiotic *Lactobacillus acidophilus* strains cultivated in pineapple agro waste juice against *Staphylococcus aureus* and *Escherichia coli*. Results are expressed as Mean±SD; each data point is the average of measurement from six independent replicates (n = 6). ***: Significant when compared to the control ($p < 0.001$), **: Significant when compared to the control ($p < 0.01$), *: Significant when compared to the control ($p < 0.5$)

Figure 2 illustrates the antibacterial activities of probiotic *L. acidophilus* strains cultivated in soy-whey waste juice against *S. aureus* and *E. coli*. The inhibition zones measured in *S. aureus* cultures were in the range of 9.38-10.83 mm, whereas, the inhibition zones found in *E. coli* cultures were the range 9.67-10.50 mm. The highest inhibition was obtained with *L. acidophilus* FTDC 0785 towards *S. aureus* (10.83 mm) and *E. coli* (10.50 mm), while, the lowest inhibition effect was obtained with *L. acidophilus* 8592 against *S. aureus* (9.38 mm) and *L. acidophilus* FTDC 2804 in *E. coli* (10.50 mm).

Figure 3 illustrates the antibacterial activities of probiotic *L. acidophilus* strains cultivated in cabbage waste against *S. aureus* and *E. coli*. The inhibition zones measured in *S. aureus* cultures were in the range of 9.27-11.12 mm, whereas, the inhibition zones found in *E. coli* cultures were the range 9.25-10.67 mm. The highest inhibition was obtained with *L. acidophilus* FTDC 4462

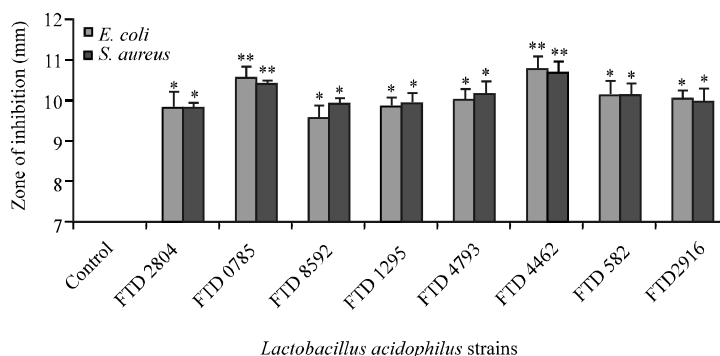


Fig. 2: The antibacterial activities of probiotic *Lactobacillus acidophilus* strains cultivated in soy-whey waste against *Staphylococcus aureus* and *Escherichia coli*. Results are expressed as Mean±SD; each data point is the average of measurement from six independent replicates (n = 6). Comparison between the different strain grown in the same media (p<0.05). **: significant when compared to the control (p<0.001), *: Significant when compared to the control (p<0.01)

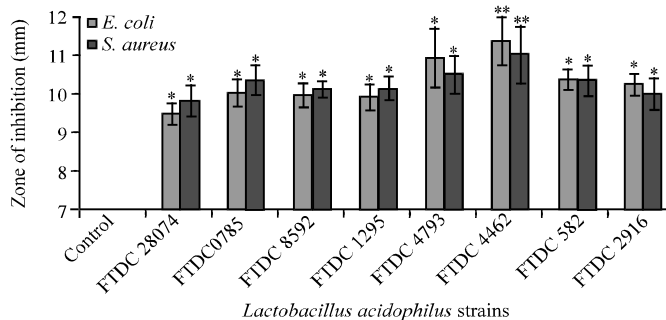


Fig. 3: The antibacterial activities of probiotic *Lactobacillus acidophilus* strains cultivated in cabbage waste against *Staphylococcus aureus* and *Escherichia coli*. Results are expressed as Mean±SD; each data point is the average of measurement from six independent replicates (n = 6). Comparison between the different strain grown in the same media (p<0.05). **: Significant when compared to the control (p<0.01), *: Significant when compared to the control (p<0.5).

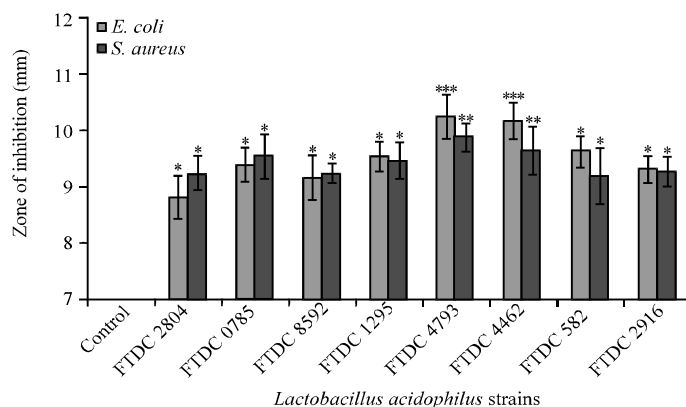


Fig. 4: The antibacterial activities of probiotic *Lactobacillus acidophilus* strains cultivated in molasses waste against *Staphylococcus aureus* and *Escherichia coli*, Results are expressed as Mean±SD; each data point is the average of measurement from six independent replicates (n = 6). Comparison between the different strain grown in the same media (p<0.05). ***: Significant when compared to the control (p<0.001)

towards *S. aureus* (11.28 mm) and *E. coli* (10.67 mm), while, the lowest inhibition effect was obtained with *L. acidophilus* 2804 against *S. aureus* (9.27 mm) and *L. acidophilus* FTDC 2916 in *E. coli* (9.25 mm).

Figure 4 showed the antibacterial activities of probiotic *L. acidophilus* strains cultivated in molasses waste against *S. aureus* and *E. coli*. The inhibition zones calculated in *S. aureus* cultures were in the range of 8.48-10.48 mm, whereas, the inhibition zones observed in *E. coli* cultures were the range 9.18-10.08 mm. The highest inhibition was obtained with *L. acidophilus* FTDC 2916 towards *S. aureus* (10.48 mm) and *L. acidophilus* FTDC 1295 towards *E.coli* (10.08 mm), while, the lowest inhibition effect was obtained with *L. acidophilus* 2804 against *S. aureus* (8.48 mm) and *L. acidophilus* FTDC 0582 in *E. coli* (9.18 mm).

All results were expressed as Mean±SD; each data point is the average of measurement from six independent replicates (n = 6).

The results showed that the metabolites of all eight strains exerted considerable antibacterial effect, as the zone of inhibition results were significant when compared to the control. Among the eight strains cultivated in four different growth medium, there was a statistically significant difference in the anti-bacterial activities of the metabolites of *S. aureus* and *E. coli*. Some of the strains of *L. acidophilus* were more effective against *S. aureus*, whereas, the other strains were more effective against *E. coli*.

The results indicated that the agar diffusion method was comparatively more superior in the study of antimicrobial activity of *L. acidophilus* strains. This could be explained by a good diffusion of metabolites from *L. acidophilus* strains in the well method and thus this may probably be resulted in the growth inhibition of the pathogenic microorganisms. The results revealed that the metabolites from almost all strains tested were microbicidal as there was no growth found in the cultures collected by swabbing from inhibition zones. It is evident from the results of the measurement of the diameters of zone of inhibition that the metabolites are significantly effective. This can be explained from the fact that the metabolites produced by the probiotics include bioactive products such as organic acid, hydrogen peroxide (H₂O₂) and bacteriocins. It was reported that the

cell-free supernatant solution from strains of lactic acid bacteria exhibited antimicrobial activity which prevented the growth of different strains of *S. aureus* and *E. coli* (Lavermicocca *et al.*, 2000). It is reported that the principal metabolites of probiotics bacteria are acetic acid and lactic acid in ratio 3:2 and these acids are responsible for the consequent drop in pH and may be sufficient to antagonize many pathogenic bacteria belonging to both Gram-positive and Gram-negative bacteria (Cheikhoussef *et al.*, 2007).

The results showed no significant difference in anti-bacterial activities of *L. acidophilus* cultivated in pineapple waste substrate, soy-whey substrate and cabbage waste substrate ($p > 0.05$) whereas, it showed significant differences with molasses waste substrate ($p < 0.05$). The results of the inhibition zone diameter of all substrates were considerable and suggested good antimicrobial activity in these waste substrates against pathogenic bacteria. The major advantages of these media are that all the tested media are not toxic, in addition they are suitable for human consumption if they prepared in a suitable formulation and in contrast, MRS medium was toxic to human. The genus *Lactobacillus* has a long history of safe use and it plays a major role in fermented milk and other food products (Karska-Wysocki *et al.*, 2010). Huttunen *et al.* (1995) reported that Lactic acid bacterial strains are potentially promising because they generate bactericidal bioactive peptides (bacteriocins) and enzymes that are able to control bio-film formation and the growth of the pathogens. Certain *Lactobacillus* strains have been reported to be highly antagonistic to *S. aureus* (Ammor *et al.*, 2006). The current study provides the evidence that the beneficial effects of probiotics are strain specific. According to the World Gastroenterology Organisation (WGO) Practice Guidelines on Probiotics and Prebiotics, "the potential probiotic health benefits can only be attributed to the strain or strains tested and not to the species or the whole group of lactic acid bacteria or other probiotics (WGO, 2008). Therefore, it is obvious that *L. acidophilus* is effective against *E. coli* and *S. aureus* and acted as a bactericidal agent against human pathogenic bacteria. These results were consistent with the findings of other research groups (Choi and Beuchat, 1994; Arihara *et al.*, 1996; Ryan *et al.*, 1996; Aktypis *et al.*, 1998; Jacobsen *et al.*, 1999; Parente and Ricciardi, 1999).

CONCLUSION

The growth of the pathogenic bacteria was successfully inhibited when metabolites of *L. acidophilus* from all waste substrates were tested. There was a significant inhibition of bacterial growth in well diffusion method as depicted by the zone of inhibition. The effect of metabolites was found to be bactericidal on both pathogenic bacteria, *E. coli* and *S. aureus*. It can be concluded that *L. acidophilus* cultivated in waste substrates produced metabolites with strong bactericidal property and could provide medicinally value added advantages to the human beings.

ACKNOWLEDGMENT

The present study was financially supported by the Universiti Sains Malaysia (USM) Grant No: (1001/PFARMASI/843084) and a USM Graduate Assistance Scheme.

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