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Characteristics and Antibacterial Activity of Metabolites from *Lactobacillus acidophilus* Strains Produced from Novel Culture Media

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Abstract: The search for new culture media that will encourage the growth and production of active antimicrobial metabolites by probiotics has become of utmost importance in the light of increasing bacterial resistance and the high costs of commercial media to cultivate the probiotics. Hence, the study was aimed at characterizing and determining the activity of metabolites obtained from *Lactobacillus acidophilus* strains cultivated in two novel media against pathogenic bacteria. Eight strains of *L. acidophilus* were cultivated in *Morinda citrifolia* juice and *Glycine max* extract. Bacterial metabolites were harvested and evaluated for antibacterial activity against two human pathogens, *Staphylococcus aureus* and *Escherichia coli*. All tested metabolites of *L. acidophilus* strains showed significant bactericidal activity, as indicated by zone of inhibition in the culture plates compared with control. There was a statistically significant difference in the activity of the metabolites against *S. aureus* and *E. coli*. Metabolites of *L. acidophilus* FTDC-4462 strain exhibited the highest zone of inhibition against *S. aureus* and *E. coli* in both growth media. Metabolites of *L. acidophilus* strains were more effective against *S. aureus* than *E. coli*. There were no significant differences in the growth media on antimicrobial effect of the metabolites of *L. acidophilus* against *E. coli* and *S. aureus*. Metabolites of *L. acidophilus* significantly inhibited the growth of both pathogenic bacteria used and can be used as potential antibiotic or probiotic agents. *M. citrifolia* juice and *G. max* extract could be used as novel and cheap culture media for *L. acidophilus* to produce antibacterial metabolites.

Key words: *Lactobacillus acidophilus*, *Morinda citrifolia* (noni), *Glycine max* (soya bean), antibacterial activity, probiotics, metabolites

INTRODUCTION

Probiotics are live microorganisms, similar to symbiotic microorganisms which are found in the human gut (Saavedra, 2001; Douglas and Sanders, 2008) and act as beneficial microorganisms to humans when administered in adequate quantities. Probiotics serve as dietary supplements to enhance the growth and health of the host animals. Therefore, they have received increased attention as a means of disease control, digestion aids, immune booster and supplementing or replacing the use of antimicrobial compounds in the field of health and medicine (Chantharasophon *et al.*, 2011). In addition, probiotic microorganisms have been used as a food preservative and antimicrobial agent more than other chemical agents due to the probiotic effects on human and other animal foods.

There is a growing interest among researchers to study the therapeutic effects of probiotics against various diseases such as chronic intestinal inflammatory disease

(Mach, 2006), pathogen-induced diarrhea (Yan and Polk, 2006), urogenital infections (Reid, 2008) and atopic diseases (Vanderhoof, 2008). Probiotics in alimentary duct increases the immunological response (Hung *et al.*, 2008) and reduces host cholesterol levels (Usman and Hosono, 2000). It has also showed significant beneficial effects compared with antibiotics or other medicines with no side effects, allergy, or toxicity. The best example of probiotics is breast milk, which develops the innate immunity of infants (NCCAM, 2008).

Metabolites derived from probiotics have strong bactericidal effects against food-borne pathogens (Yesillik *et al.*, 2011), broad bacteriostatic effects on *E. coli* and *S. aureus* (Gharaei-Fathabad and Eslamifar, 2011) and antimicrobial agents for pathogenic bacteria such as *E. coli*, *S. typhi*, *S. dysenteriae*, *B. anthracis* and *S. aureus* (Amin *et al.*, 2009).

Various types of microorganism strains have been employed for probiotic production (Heller, 2001; O'Mahony *et al.*, 2005). Among these are *Lactobacillus*

and *Bifidobacterium* (Critchfield *et al.*, 2011), which have not shown any risk to humans (Saxelin *et al.*, 1996; Naidu *et al.*, 1999). A metabolite such as bacteriocin, isolated from lactic acid bacteria is one of the antimicrobial substances which is proteinaceous in nature and mostly acts against closely related species (Bali *et al.*, 2011). However, the feed back regulation of bacteriocin is not clear. On the other hand, the bactericidal and inhibition effects depend on factors such as low pH, other organic acids, hydrogen peroxide (H₂O₂), ethanol and low oxidation-reduction (Pitt *et al.*, 2000).

Generally, *L. acidophilus* has been grown in various media such as Man-Rogosa Sharpe (MRS) medium (De Man *et al.*, 1960), Rogosa medium (Rogosa *et al.*, 1951), Lactobacillus Selective (LBS) medium (Mitsuoka, 1978; Atlas, 1997) and Lactobacillus Anaerobic MRS with Vancomycin and Bromocresol green (LAMVAB) medium (Hartemink *et al.*, 1997) for various fermentation processes and probiotic production. However, those commercially available media are expensive and this implies increased production cost as well as probiotic cost. To overcome the high production cost, there is need to find alternative natural sources of growth media with same probiotic nature as *L. acidophilus* (Naveena *et al.*, 2005; Shahravy *et al.*, 2012).

Morinda citrifolia (noni) and *Glycine max* (soya bean) are traditional medicinal plants that have been extensively used in folk medicine by Polynesians for over 2000 years (Hirazumi *et al.*, 1996). The plants have been reported to have a broad range of therapeutic and nutritional properties (Singh *et al.*, 1984) including antibacterial, antiviral, antifungal, anticancer activities, analgesic, hypotensive, anti-inflammatory and immune enhancing effects (Selvam *et al.*, 2009) in addition to being useful in treatment of liver and heart conditions (Wang *et al.*, 2008).

So far, the utilization of noni juice and soya bean extract as alternative natural sources of growth media with the probiotics *L. acidophilus* has not been documented. Therefore, the main objective of the present study is to determine the bactericidal activity of metabolites isolated from *L. acidophilus* strains cultivated in *M. citrifolia* (noni) juice and *G. max* (soya bean) against two human pathogens, *S. aureus* (Gram positive) and *E. coli* (Gram negative).

MATERIALS AND METHODS

Microorganisms and culture media reagents: Man Rogosa Sharpe (MRS) broth was purchased from Himedia, India. Nutrient agar and nutrient broth were

purchased from Merck, Darmstadt, Germany. *M. citrifolia* (noni) and *G. max* (soya bean) were purchased from a local supplier in Penang, Malaysia.

Preparation of fermentation substrates

Preparation of *M. citrifolia* substrate: Freshly harvested ripe *M. citrifolia* fruits were collected from a local supplier in Penang, Malaysia in 2010. The fruits were thoroughly washed in lukewarm water to remove extraneous materials. The seeds were separated by manual splitting.

The *M. citrifolia* fruit juice was prepared with fruits and water (1:1). The juice was extracted using a juice squeezer and filtered through cheese cotton cloth, then stored at -20°C until use. The fresh juice of *M. citrifolia* was used for inoculation. Before inoculation, the pH of *M. citrifolia* juice was fixed at 6.5 using 1 M NaOH followed by sterilization for 15 min at 121°C.

Preparation of soy milk substrate: Dried soybeans (*Glycine max*) were purchased from a local supplier in Penang, Malaysia. They were soaked overnight to promote swelling and then blended with distilled water at a ratio of 1:6 (w/v). The blended mixture was filtered with muslin cloth and the resultant soy milk was pasteurized at 95°C for 15 min. The resultant milk was directly used for inoculation.

Test strains: The pathogenic microorganisms *Staphylococcus aureus* (Clinical isolate) and *Escherichia coli* (Clinical isolate) were collected from the Pathology Laboratory, General Hospital, Penang, Malaysia. The bacterial strains were cultivated in nutrient agar slants and preserved at 4°C.

Probiotics culture and growth conditions: Strains of *L. acidophilus* FTDC 2804, *L. acidophilus* FTDC 0785, *L. acidophilus* FTDC 8592, *L. acidophilus* FTDC 1295, *L. acidophilus* FTDC 4793, *L. acidophilus* FTDC 4462, *L. acidophilus* FTDC 0582 and *L. acidophilus* FTDC 2916 were obtained from culture Center of School of Industrial Technology, Universiti Sains Malaysia, Penang, Malaysia. The strains were cultivated 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 broth was collected, centrifuged (5000 rpm) and washed three times to remove other substrates. The cells were re-suspended in *M. citrifolia* (noni) and *G. max*

(soya bean) substrate (1% v/v inoculum size) and 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 evaluated for antimicrobial activity.

Evaluation of antibacterial activity: The antibacterial assay was quantified by modifying the well diffusion method assay procedure of Fooks and Gibson (2002) and Savadogo *et al.* (2004). The well diffusion method assay has been widely used to determine the bactericidal activity against different types of pathogenic microorganisms (Yesillik *et al.*, 2011). The antimicrobial activities were determined by measuring the diameter of zone of growth inhibition formed around the wells. To confirm the bactericidal and bacteriostatic effects, swab was taken from the growth inhibition zone and then inoculated into nutrient broths and incubated at 37°C for 24 h and evaluated for growth.

Statistical analysis: The results were analyzed statistically using one-way analysis of variance (version 17.0, SPSS, USA). When there was a statistically significant difference, *Post-hoc* Tukey's fair Significant Difference test was applied. ANOVA data with $p < 0.001$, $p < 0.01$ and $p < 0.05$ were classified as statistically significant.

RESULTS

The results of bacteriostatic and bactericidal properties of the metabolites of *L. acidophilus* strains cultivated from noni and soy-milk on the growth of *S. aureus* and *E. coli* are presented in Table 1. Results show that all of the probiotic strains grown in both culture media were able to inhibit the growth of *E. coli* and *S. aureus*. The results were expressed as Mean±SD, each data point is the average of measurement from six independent replicates, (n = 6). The results of the antibacterial activities of probiotic *L. acidophilus* strains cultivated in noni juice and soy-milk against *S. aureus* are given in Fig. 1. This clearly depicts the inhibition zones obtained in *S. aureus* culture in noni juice were in the range of 9.50-10.50 mm, while the inhibition zones observed in *S. aureus* cultures in soy-milk were 9.58- 10.43 mm. The highest inhibition was obtained with *L. acidophilus* FTDC 4462 against *S. aureus* cultivated in noni juice with the inhibition zone 10.50 mm ($p < 0.01$) and *S. aureus* cultivated in soy-milk with zone of inhibition 10.43 mm ($p < 0.01$), while, the lowest inhibition effect was obtained with *L. acidophilus* FTDC 2804 against

Table 1: Antibacterial effect of probiotics from noni and soy-milk on the growth of *S. aureus* and *E. coli*

<i>L. acidophilus</i> strains	Effect of growth media			
	Noni juice		Soy-milk	
	<i>E. coli</i>	<i>S. aureus</i>	<i>E. coli</i>	<i>S. aureus</i>
FTDC 2804	BS	BS	BS	BS
FTDC 0785	BC	BC	BC	BC
FTDC 8592	BC	BC	BC	BC
FTDC 1295	BC	BC	BC	BC
FTDC 4793	BC	BC	BC	BC
FTDC 4462	BC	BC	BC	BC
FTDC 0582	BC	BC	BC	BC
FTDC 2916	BC	BC	BC	BC

BS: Bacteriostatic, BC: Bactericidal

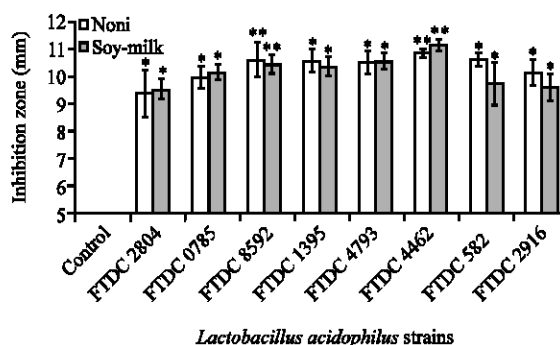


Fig. 1: The antibacterial activities of probiotic *L. acidophilus* strains cultivated in noni juice and soy-milk against *S. aureus*. Values are Mean±SD (n = 6), **Significant when compared with the control ($p < 0.01$), *Significant when compared to the control ($p < 0.01$)

S. aureus cultivated in noni juice (9.50 mm) and *L. acidophilus* FTDC 2804 against *S. aureus* cultivated in soy-milk (9.58 mm) (Fig. 1).

This study shows that *L. acidophilus* is effective against *E. coli* and *S. aureus* and it could act as bactericidal agent against human pathogenic bacteria as the diameters of the inhibition zones were considerably high and significant. This suggests the potent bactericidal activity of *M. citrifolia* and *G. max* as natural substrates for *L. acidophilus* against the tested pathogenic bacteria. Figure 2 shows the antibacterial activities of probiotic *L. acidophilus* strains cultivated in noni juice and soy-milk against *E. coli*. The inhibition zones measured in *E. coli* cultures in noni juice were in the range of 9.37-10.84 mm, whereas, the inhibition zones found in *E. coli* cultures in soy-milk were in the range of 9.52-11.12 mm. The highest inhibition was obtained with *L. acidophilus* FTDC 4462 towards *E. coli* (10.84 mm) cultivated in noni juice and *E. coli* cultivated in soy-milk

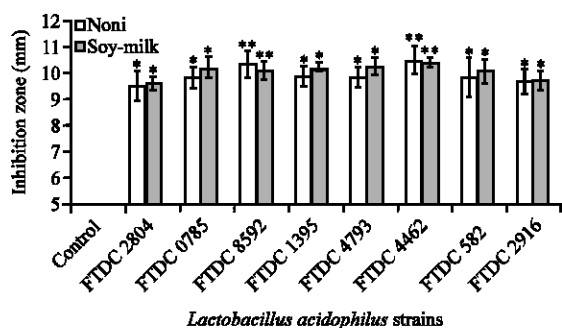


Fig. 2: The antibacterial activities of probiotic *L. acidophilus* strains cultivated in noni juice and soy-milk against *E. coli*. Values are Mean±SD (n = 6). **Significant when compared with the control (p<0.001), *Significant when compared to the control (p<0.01)

(11.12 mm). Lowest inhibition effect was noted with *L. acidophilus* 2804 against *E. coli* (9.37 mm) cultivated in noni juice and *E. coli* (9.52 mm) cultivated in soy-milk.

DISCUSSION

Selection of suitable probiotics starter from the natural source is a highly critical factor in the production of fermented products. Earlier studies (Eklund, 1989; Gan *et al.*, 2002; Schillinger and Lucke, 1989; Tharmaraj and Shah, 2009) showed that the probiotics grown in MRS media exerted considerable antagonistic effect against the human pathogens *B. cereus*, *L. monocytogenes*, Methicillin Resistance *S. aureus* (MRSA) and *P. aeruginosa*, the causative agents of food borne diseases, listeriosis, skin infections and lung disease, respectively (Bhilabutra *et al.*, 2007).

Overall results showed that the metabolites of all eight strains exerted considerable antibacterial effect, as the zone of inhibition results were significant when compared with the control. The growth media showed no significant influence on the antimicrobial effect of metabolites of strains *L. acidophilus* against *E. coli* and *S. aureus*, both growth media did not show any significant influence on the antimicrobial effect of metabolites of the strains. *Staphylococcus aureus* was more sensitive to the inhibitory substance produced by the *L. acidophilus* isolates than *E. coli*. Similar results have been reported by Toure *et al.* (2003) and Tadese *et al.* (2005). The resistance of Gram negative bacteria is attributed to the particular nature of their cellular envelopes. According to Bhunia *et al.* (1991) bacteriocin produced by *Lactobacillus* strains interact

with lipoteichoic acids that are absent in Gram-negative bacteria. On the other hand, this could be due to the fact that these isolates are acidocin producers and the primary target of acidocin is pore forming bacteriocin that creates cell membrane channels through the 'barrel-stave' mechanism (Ahmed *et al.*, 2010)

The results revealed that the agar diffusion method was suitable for the study of antimicrobial activity of *L. acidophilus* strains. This could be explained by the good diffusion of metabolites from *L. acidophilus* strains in the well method and this may have resulted in the growth inhibition of the pathogenic microorganisms.

It is evident from the results of the measurement of the diameters of zone of inhibition that the metabolites are significantly effective. This could be due to the fact that the metabolites produced by the probiotics include bioactive products such as organic acid, hydrogen peroxide (H₂O₂) and bacteriocins. Other researchers 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; Arokiyarny and Sivakumar, 2011). Further, Cheikhoussef *et al.* (2007) reported that the principal metabolites of probiotics bacteria are acetic acid and lactic acid in the ratio of 3:2 and these acids are responsible for the consequent drop in pH, which may be sufficient to antagonize many pathogenic bacteria belonging to Gram-positive and Gram-negative class.

In addition, compared with commercial media such as MRS and Rogosa medium, the noni and soya milk media were nontoxic and are suitable for human consumption. 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). Lactic acid bacteria strain produced the bacteriocin (bactericidal bioactive compounds) and that they are capable of controlling the growth and formation of biofilm of pathogen and they are highly antagonistic to pathogenic microorganism (Ammor *et al.*, 2006).

CONCLUSION

The growth of pathogenic bacteria, *Staphylococcus aureus* and *Escherichia coli* were significantly inhibited by metabolites of *L. acidophilus* cultured in *Morinda citrifolia* (noni) juice and *Glycine max* (soya bean) extract growth media. Metabolites isolated from *L. acidophilus* cultivated in the media derived from natural products exhibited strong

bactericidal property, which could provide novel value added medicinal benefits to human beings. The new media could serve as cheap alternative culture media compared to the presently available commercial ones.

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