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Full Length Research Paper

Inhibitory activity and organic acid concentrations of metabolite combinations produced by various strains of *Lactobacillus plantarum*

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Metabolite combinations produced by *Lacrtobacillus plantarum* strains were studied by inhibitory activity test using *Pediococcus acidilactici* as indicator microorganism. The pH and cell population of *L. plantarum* strains were also determined. *L. plantarum* RG14 strain has significant (P < 0.05) lower pH but higher cell count than the other four *L. plantarum* strains. Inhibitory activity of metabolite combination was stronger than single strain metabolite. However, there was significantly higher (P < 0.05) inhibitory activity in Com 2 (TL1, RG11 and RI11 strains), Com 5 (TL1, RG14 and RS5 strains) and Com 7 (RG11, RG14 and RI11 strains) than other 7 metabolite combinations, which was attributed to the present of bacteriocin inhibitory compound. Lactic and acetic acids were also present in high amount in metabolite combinations. Therefore, metabolites produced by *L. plantarum* strains containing mainly organic acids and bacteriocin possess vast potential as feed additives for animals.

Key words: *Lactobacillus plantarum*, metabolite combination, inhibitory activity, lactic acid, acetic acid.

INTRODUCTION

Lactobacillus is the main genus of lactic acid bacteria (LAB) and plays an important role in balancing microflora in the gut ecosystem of animals (Lee, 1997; Brashears et al., 2005; Canibe et al., 2007). Many reports have shown that the antimicrobial substances produced by *Lactobacillus* sp. could fight against *Salmonella* and other harmful pathogens affecting animal health and the agent of food poisoning (Adams and Hall, 1988; Savadogo et al., 2006; Thanh et al., 2010). Metabolites are intermediates and or final products in metabolism of *Lactobacillus* sp., which contain mainly lactic acid, acetic acid and bacteriocins (Foo et al., 2005; Thanh et al., 2009). Recently, these metabolites have been shown to have many beneficial probiotic effects on animal growth performances and particularly in the gut health when used as additive in animal diet (Thanh et al., 2009; Loh et al., 2010; Thu et al., 2010). Bacterial metabolites also display a wide inhibitory activity against various species of pathogens such as *Escherichia coli*, *Salmonella typhimurium* and *Clostridium perfringens* (Savadogo et al., 2006; Gaggìa et al., 2010; Thanh et al., 2010) and other gram-positive bacteria such as *Bacillus cereus, Staphylococcus aureus, Streptococcus pneumonia* and

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Abbreviations: LAB, Lactic acid bacteria; **CFS,** cell free supernatant; **VFA,** volatile fatty acids; **ENT,** entrobacteriaceae; **VRE,** vancomycin resistant enterococci.

Enterococcus faecium (Foo et al., 2003; Mollendorff et al., 2006). Thanh et al. (2010) reported that metabolites of *L. plantarum* had broad inhibitory spectrum against *E. coli, Listeria monocytogenes, S. typhimurium* and vancomycin resistant enterococci (VRE) due to the presence of 2 classes of bacteriocins, plantaricin EF and plantaricin W (Moghadam et al., 2010). On the other hand, the low pH of metabolites would provide suitable environment for LAB development (Cintas et al., 2001; Thu et al., 2010).

Many studies have demonstrated the probiotic effects of metabolite combinations produced by *L. plantarum* on animal growth performance, such as increasing volatile fatty acids (VFA) and LAB count, while reducing *Entrobacteriaceae* (ENT) count in rats (Foo et al., 2003; Loh et al., 2008), broiler chickens (Thanh et al., 2009; Loh et al., 2010) and piglets (Thu et al., 2010). Therefore, metabolites produced by *L. plantarum* strains could be a potential alternative to in-feed antibiotics. However, the information pertaining to the inhibitory activity of metabolite combinations produced by *L. plantarum* strains against pathogens is very limited. Thus, the objectives of this study were to identify the best metabolite combinations produced by 5 strains of *L. plantarum* based on bacteriocin inhibitory activity and organic acid concentrations.

MATERIALS AND METHODS

Media preparation

Medium used for reviving and cultivation of *L. plantarum* strains was de Man-Rogosa-Sharpe (MRS) medium (Merck, Darmstadt, Germany). The medium was prepared as described by Loh et al. (2008). Soft agar was prepared using 0.75% (w/v) bacteriological agar in MRS broth and then autoclaved at 121°C for 15 min.

Bacterial growth condition and metabolite preparation

Five *L. plantarum* strains (TL1, RG11, RS5, RG14 and RI11) employed in this study were previously isolated from Malaysian foods (Lee, 2002; Foo et al., 2003; Lim et al., 2006) and kept at -20°C in MRS broth containing 20% (v/v) glycerol. The stock culture was revived twice in MRS broth and incubated anaerobically at 30°C for 48 h. The active culture of *L. plantarum* strains were grown in 10 ml MRS broth prior to the production of metabolites according to the procedure described by Foo et al. (2003). The metabolites were kept at 4°C until further experiment. Ten combinations of metabolites were prepared by mixing equal volume of metabolite produced by each strain of *L. plantarum* as shown in Table 1.

Determination of pH, optical density and *Lactobacillus* population

Final pH value and cell population were determined for each active strain after 24 h of incubation. The final pH was determined by a pH meter. Spectrophotometry method and total plate count were used for the *Lactobacillus* cell determination. The spectrophotometry method expressed as optical density at 600 nm (OD_{600nm}) was determined by using spectrophotometer (Novaspec III, Biochrom Ltd. Cambridge CB4, England) on 10% (v/v) of active culture diluted with MRS broth. The total plate count expressed as colony forming unit per ml (cfu/ml) of active strain was carried out for ten-fold serial diluted active strain carried out by using sterile peptone water (Merck, KGaA, Darmstadt, Germany). An aliquot of 100-µl cell suspension was plated on MRS agar plate and incubated anaerobically at 30°C for 48 h.

Lactic and acetic acids determination

Lactic and acetic acids were determined by UV-spectrophotometry (Varian Technologies Pte. Ltd. Singapore) method at 340 nm wavelength using commercial kits (R-Biopharm, Landwehrstr, Darmstadt). The analysis of both lactic and acetic acids were performed according to the methods described by the manufacturer at temperature of 20 to 25°C.

Inhibitory activity

Metabolites produced by *L. plantarum* strains were separated by centrifugation at 10,000 xg for 10 min at 4°C. The cell free supernatant (CFS) containing metabolite was collected and diluted 2-fold with normal saline (0.85 % w/v), followed by dispensing 20 µl of diluted CFS into MRS agar wells (5.5 mm diameter). The diluted CFS was allowed to diffuse for 1 to 3 h at room temperature before overlaid with 3 ml of MRS soft agar containing 1% (v/v) indicator strain of *Pediococcus acidilactici*. The plates were then sealed with parafilm and incubated at 30°C for 24 h under anaerobic condition. The inhibitory activity was examined for the clear inhibition zone surrounding each agar well. The diameter of inhibition zone was measured to indicate the magnitude of inhibitory activity. Arbitrary unit (AU) defined as the lowest inhibitory concentration of CFS (Thanh et al., 2010) was used to quantify the inhibitory activity of the metabolite produced by *L. plantarum* strains in this study.

Data analysis

One-way analysis of variance was used to analyse the data using the General Linear Model procedure by SAS 1998 (SAS Inst., Inc., Cary, NC). Duncan's multiple range test system was used to compare the significant differences of treatments at $P < 0.05$. The data is presented as the mean ± standard error of the mean (SEM).

RESULTS

Cell population, final pH and inhibitory activity of *L. plantarum* strains

The OD_{600nm} of RG14 was significantly higher ($P < 0.05$) than RI11 and RS5 strains but no significant differences ($P > 0.05$) were found among TL1, RG11 and RG14 strains (Table 2). However, the OD_{600nm} values of TL1 and RG11 were significantly higher ($P < 0.05$) than RI11 strain determined at 24 h of incubation. As for the final pH, the pH value was significantly lower ($P < 0.05$) in metabolites produced by RG14 and RI11 strains as compared to the other 3 strains. The inhibitory activity of *L. plantarum* metabolites against *P. acidilactici* indicator is shown in Table 2. Among these strains, the strongest inhibitory activity was observed in RG14 strain ($P < 0.05$), but no significant differences ($P > 0.05$) were found

Table 1. Ten metabolite combinations prepared by mixing any three metabolites produced by 5 strains of *L. plantarum.*

Combination	*L. plantarum* strains
Com 1	TL1, RG11 and RG14
Com 2	TL1, RG11 and RI11
Com 3	TL1, RG11 and RS5
Com 4	TL1, RG14 and RI11
Com 5	TL1, RG14 and RS5
Com 6	TL1, RI11 and RS5
Com 7	RG11, RG14 and RI11
Com 8	RG11, RG14 and RS5
Com 9	RG11, RI11 and RS5
Com 10	RG14, RI11 and RS5

Table 2. Cell population and characteristics of metabolites produced by various strains of *L. plantarum.*

Characteristic	*L. plantarum strains*				
	TL1	RG11	RG14	RI11	RS5
OD_{600nm}	10.20 ± 0.16^{ab}	10.27 ± 0.06^{ab}	10.69 ± 0.30^{a}	9.42 ± 0.22^{c}	9.85 ± 0.12^{bc}
Final pH	4.10 ± 0.03^{a}	4.02 ± 0.02^{b}	3.94 ± 0.02^{c}	3.93 ± 0.01^{c}	4.08 ± 0.02^{ab}
Total plate count (log_{10} CFU/ml)	8.98 ± 0.03^{b}	8.73 ± 0.03^{d}	9.66 ± 0.04^{a}	8.83 ± 0.04^{c}	8.67 ± 0.02^{d}
Bacteriocin activity (AU/ml)	800^{b}	800^{b}	1600^{a}	800^{b}	800^{b}
Diameter of inhibition zone (mm)	12.23±0.14	11.90±0.21	10.33±0.17	12.73±0.23	13.27±0.23

The results were presented as mean values ± SEM.
^{abcd} Values expressed with different superscripts within the same row are significantly different at $P < 0.05$.

Table 3. Inhibitory activity and organic acid concentration of metabolite combinations.

Metabolite combination	Inhibitory activity (AU/ml)	Diameter of inhibition zone (mm)	Lactic acid (g/l)	Acetic acid (g/l)
Com 1	800^{b}	14.07±0.29	3.28 ± 0.06^{de}	1.07 ± 0.08^{abc}
Com 2	1600^{a}	12.50±0.00	3.64 ± 0.06^{ab}	1.13 ± 0.05^{ab}
Com 3	800^{b}	13.67±0.17	3.52 ± 0.11^{bcd}	1.18 ± 0.04^{a}
Com 4	800^{b}	15.00±0.12	3.60 ± 0.04^{bcd}	0.93 ± 0.01^{c}
Com 5	1600^{a}	12.76±0.17	3.81 ± 0.13^{a}	1.18 ± 0.04^{a}
Com 6	800^{b}	14.10±0.10	3.35 ± 0.08^{cde}	1.17 ± 0.04^{a}
Com 7	1600^{a}	12.93±0.29	3.43 ± 0.05^{bcde}	1.18 ± 0.07^{a}
Com 8	800^{b}	14.23±0.43	3.20 ± 0.08^{e}	1.15 ± 0.03^{ab}
Com 9	800^{b}	12.30±0.15	3.17 ± 0.11^{e}	0.99 ± 0.06^{bc}
Com 10	800^{b}	13.23±0.15	3.34 ± 0.06^{cde}	1.06 ± 0.06^{abc}

The results are presented as mean values ± SEM.
^{abcde} Values expressed with different superscripts within the same column are significantly different at $P < 0.05$.
Com indicates the combination of metabolites produced by any three out of five strains of *L. plantarum* used in this study as shown in Table 1.

in the other 4 strains. The highest number of cell count was also observed for RG14 strain. In comparison, TL1 had a significant higher count ($P < 0.05$) than RI11 strain, and RI11 strain count was significantly higher ($P < 0.05$) than RG11 and RS5 strains, respectively.

Inhibitory activity and organic acids of metabolite combinations

Two levels of inhibitory activities (AU/ml) of metabolites were observed in this study (Table 3). The metabolite

combination groups of Com 2 (TL1, RG11 and RI11 strains), Com 5 (TL1, RG14 and RS5 strains) and Com 7 (RG11, RG14 and RI11 strains) showed significant stronger ($P < 0.05$) inhibitory activity than the other 7 combinations used in this study. However, no significant differences ($P > 0.05$) were found among 7 metabolite combinations. Table 3 shows the diameter of inhibition zone of metabolite combinations and no significantly different results ($P > 0.05$) were observed for the diameter of inhibition zone produced by metabolite combinations.

Lactic acid was found to be a major organic acid present in the metabolites produced by *L. plantarum* strains (Table 3). The concentration of lactic acid was significantly higher ($P < 0.05$) in Com 5 as compared to other metabolite combinations, except for Com 2. Moreover, Com 3 and Com 4 were significantly higher ($P < 0.05$) than Com 8 and Com 9 but no significant differences ($P > 0.05$) were found in Com 1, Com 6, Com 7 and Com 10. Acetic acid concentration for Com 3, Com 5, Com 6 and Com 7 was significantly higher ($P < 0.05$) than Com 4 and Com 9. In contrast, no significant differences ($P > 0.05$) were found among Com 1, Com 2, Com 8 and Com 10, respectively.

DISCUSSION

According to Jack et al. (1995), the cell population and bacteriocin productivity reflected by inhibitory activity (AU/ml) is closely related to cell growth. Therefore, an increase of cell population would lead to higher bacteriocin productivity and lower final pH value (Casadei et al., 2009). As in this study, the highest OD_{600nm} value and cell count were observed in RG14 strain. The value of OD_{600nm} relates well with the final pH value, peptides density and enzymatic activity (Cintas et al., 2001). Furthermore, bacteriocin production is affected by final pH and incubation temperature (Jack et al., 1995).

The final pH was mainly attributed to the production of organic acids by the *L. plantarum* strains. Our finding is in agreement with the result reported by Foo et al. (2005) who suggested that the increase of biomass cells and organic acids production are the main reasons for pH reduction in fermented food. During the production of metabolite by *L. plantarum* strains, lactic and acetic acids are produced to promote the growth of producer cells (Foo et al., 2003; Savadogo et al., 2006). Both lactic and acetic acids are the major contributors to the acidic environment and they maintain the acidic pH of metabolites (Cintas et al., 2001; Brashears et al., 2005; Thanh et al., 2010). Lactic acid is the major final product of carbohydrate fermentation of homofermentative *Lactobacillus* sp. (Ross et al., 2002). The proliferation of spoilage organisms and food-borne pathogens can be prevented by low pH and high concentrations of organic acids (Adam and Hall, 1988; Cintas et al., 2001).

The bacteriocin inhibitory compound present in metabolites could also fight against harmful pathogens. The protective ability of bacteriocin as food bio-preservative and gut health have been demonstrated extensively (Lee, 1997; Brashears et al., 2005). In this study, the bacteriocin activity of *L. plantarum* metabolites was determined as the inhibitory activity against *P. acidilactici*. The current results show that among the individual strains of *L. plantarum*, the inhibitory ability of RG14 was the strongest (1600 AU/mL). This result is in accordance with the inhibition of *L. plantarum* metabolites against *E. coli*, *L. monocytogenes, S. typhimurium,* vancomycin resistant enterococci (VRE) and *P. acidilactici* (Thanh et al., 2010). The strongest inhibitory activity of RG14 strain correlated well with the higher number of producer cell and bacteriocin production during fermentation.

Additionally, the inhibition ability of metabolite combinations was due to the presence of various types and concentration of antimicrobial substances, such as bacteriocins and organic acids (Savadogo et al., 2006). Antimicrobial activity of bacteriocin produced by *L. plantarum* in Boza could inhibit a broad range of pathogens such as *S. aureus, L. monocytogenes, B. cereus, C. perfringens* and *E. coli* (Ross et al., 2002; Mollendorff et al., 2006). Bacteriocin is defined traditionally as proteinaceous compounds produced by bacteria, which inhibit bacteriostatically or killed bacteria (Jack et al., 1995; Savadogo et al., 2006). This function of bacteriocin could improve quality and safety of food, especially the fermented products (Lee, 1997; Ross et al., 2002). Bacteriocin produced by *Lactobacillus* has been reported to be able to permeate the outer membrane of bacteria, and hence it could inactivate gram-negative bacteria together with other antimicrobial environment factors such as temperature, acidic condition and nutrient availability (Brashears et al., 2005; Savadogo et al., 2006; Casadei et al., 2009; Thanh et al., 2010). Furthermore, Gaggìa et al. (2010) demonstrated that the application of antimicrobial substances produced by *Lactobacillus* could improve the criteria of biochemical quality, microbial aspects and safety of food.

The results of this study demonstrated that the inhibition abilities of metabolite combinations were higher than that of single strains. The magnitude of inhibitory activity of metabolite combinations is reflected by the diameter of inhibition zone. Thanh et al. (2010) reported that the inhibitory activity of the metabolite combination of 3 or 4 strains is stronger than that of the combination of 1 or 2 strains of *L. plantarum.* This was also in agreement with Cintas et al. (2001) who reported that the application of multiple *Lactobacillus* strains would be more useful than single strain due to the presence of various types of bacteriocin secreted by multiple *Lactobacillus* strains. These results also supported a previous study conducted by Thu et al. (2010) in which feeding of metabolite combinations produced by *L. plantarum* reduced *Entero-bacteriaceae* and increased volatile fatty acids in the gut of piglets. In addition, a high number of *Lactobacillus* in

in gut was reported due to the feeding of metabolite combinations, which would compete with pathogens in the gut (Loh et al., 2008).

Conclusion

The metabolites from different individuals or combinations of *L. plantarum* strains contained different amount of organic acids and bacteriocin. RG14 is the best candidate among the strains since it produced the highest cell population and inhibitory activity. High amount of lactic and acetic acids present in metabolites produced by *L. plantarum* strains, could lower the pH to inhibit pathogens. In comparison, the metabolite combinations have a stronger inhibitory activity than single strains of *L. plantarum*, particularly Com 2 (TL1, RG11 and RI11 strains), Com 5 (TL1, RG14 and RS5 strains) and Com 7 (RG11, RG14 and RI11 strains). The metabolite combinations possess vast potential to be used as feed additive of animal diets.

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