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Inhibitory Property of Metabolite Combinations Produced from *Lactobacillus plantarum* Strains

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ABSTRACT

The inhibitory property of metabolite combinations produced from six different strains of *Lactobacillus plantarum* were evaluated in this study. The final pH of culture media and cell population of each strain were determined and the antimicrobial activity of the metabolite from individual strains and combinations were conducted against *Pediococcus acidilactici* as indicator microorganism. The lowest pH was observed in the media which were cultured with *L. plantarum* RG11 and RG14 ($P < 0.05$), while the highest cell population was found in *L. plantarum* RI11 and RG11. There was no significant difference in the inhibitory activity among the six individual strains. However, when the three strains of *L. plantarum* were combined, the combination of strains (TL1-RI11-RG11, strains RS5-RI11-RG11 and strains RI11-RG14-RG11) showed a higher level of inhibition. The metabolite combinations from *L. plantarum* strains could be potential pathogen inhibition compound in the food and feed industry.

Keywords: Lactobacillus plantarum, metabolite combination, inhibitory activity

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INTRODUCTION

Over the past fifty years, antibiotics have been extensively used in the industrial animal production. The widespread use of antibiotics to accelerate growth among livestock and to compensate for the unhealthy conditions found in

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confined animal feeding operations has led to an increase in virulent strains of pathogenic bacteria and occurrence of drug residues in animal products. Therefore, recommendations to restrict and limit the use of antibiotics in feed have been made gradually.

New approach, such as probiotics, has been suggested as an alternative to growth promoting antibiotics in animal feed. Beneficial or commensal bacteria, such as *Lactobacillus* spp., function as a Competitive Exclusion (CE) culture. When supplemented into the diet of animals, it could reside and colonize the intestinal tract, thereby reducing the colonization of pathogens in the gastrointestinal tract (Harvey *et al*., 2005). In addition, the metabolite produced by the probiotics contains inhibitory compounds such as bacteriocins and organic acids (Thanh *et al*., 2009; Thu *et al.*, 2011) which further restrict the survival of pathogens.

Various recent studies have shown the benefits of bacterial metabolite as a feed additive in farm animals. Reductions in faecal *Enterobacteriaceae* (ENT) and cholesterol level in the plasma were observed when the metabolite produced from *L. plantarum* was supplemented into the feed of broiler chickens (Thanh *et al*., 2009) and pigs (Thu *et al*., 2010). Similarly, the bacterial metabolite also exhibits a wide range of inhibitory activities against pathogens such as *Escherichia coli*, *Salmonella typhimurium*, *Listeria monocytogenes*, *vancomycin resistant enterococci* (VRE) due to the presence of bacteriocins and organic acids (Gaggia *et al*., 2010; Thanh *et al*., 2010). Therefore, the purpose of this study was to determine the inhibitory activity of the individual of cell free extracts and select the best metabolite combination produced by 6 strains of *L. plantarum* based on the inhibitory activity against an indicator bacterium. In this study, *Pediococcus acidilactici* was chosen as the indicator because it is a common food spoilage bacterium in both animal and human food products (Waite *et al*., 2009).

MATERIALS AND METHODS

Preparation of the Bacterial Cultures

Six different strains of bacteriocin producing *Lactobacillus plantarum* (UL4, TL1, RS5, RI11, RG11 and RG14) were used in this study. These *L. plantarum* were previously isolated from Malaysian fermented food by Dr. Foo Hooi Ling from the Department of Biotechnology, Universiti Putra Malaysia, Malaysia (Foo *et al*., 2003).

Revival of the Bacterial Cultures and Collection of Metabolite

The media used to revive and cultivate *L. plantarum* were MRS (Man ROGOSA and SHAPE) broth, MRS agar and soft agar (Merck, Darmstadt, Germany). The stock cultures of these strains were prepared by inoculating *L. plantarum* (2%, v/v) into MRS broth (Merck, Darmstadt, Germany) and incubating at 30°C anaerobically for 24 hours before glycerol was added to a final concentration of 20% (v/v). The stock cultures were kept at -20°C until required. Before each use, the stock cultures were

revived twice by transferring them into 10 mL MRS broth and were incubated overnight at 30°C anaerobically. Plate spreading was then conducted for the revived cultures, followed by 48 hours of incubation. A single colony of *L. plantarum* was picked and inoculated into 10 mL MRS broth and incubated anaerobically for 24 hours, followed by re-subculturing into 10 mL MRS broth and incubating anaerobically for another 24 hours. 2% (v/v) of overnight culture was inoculated into 1 L MRS broth and incubated overnight at 30°C. The metabolites were collected by separating the bacterial cells by centrifugation at 10,000 xg for 15 min. The crude cell-free extracts were then kept at 4°C until further usage, as described by Foo *et al*. (2003) and Loh *et al.* (2010). A total of 20 different metabolite combinations were established from these strains, with each combination (COM) containing 3 strains (Table 1).

Total Viable Plate Count

The population of LAB was determined by carrying out a 10-fold serial dilution of bacterial culture using sterile distilled water. A total of 0.1 mL aliquot of diluted bacterial suspension was spread on MRS agar using spread plate method. The number of colony forming unit (CFU) was enumerated and recorded after 48 h of incubation at 37°C. The CFU per millilitre of the sample was calculated as follows (Thu *et al.,* 2010):

$$\text{Total viable plate count (CFU/mL)} = \frac{\text{Number of colonies X Dilution factor}}{\text{Volume of Sample (mL)}}$$

Optical Density and pH Determination

Optical density is a means of numeric expression of the turbidity of a suspension, such as a bacterial culture. A total of 100 µL metabolite was mixed with 900 µL of MRS broth. The optical density for each strain was adjusted and measured

TABLE 1
Twenty different metabolite combinations established from 6 strains of *L. plantarum*, each combination (COM) containing 3 strains

COM	strains	COM	strains
COM123	UL4, TL1 and RS5	COM234	TL1, RS5 and RI11
COM124	UL4, TL1 and RI11	COM235	TL1,RS5 and RG14
COM125	UL4, TL1 and RG14	COM236	TL1,RS5 and RG11
COM126	UL4, TL1 and RG11	COM245	TL1, RI11 and RG14
COM134	UL4, RS5 and RI11	COM246	TL1, RI11 and RG11
COM135	UL4, RS5 and RG14	COM256	TL1, RG14 and RG11
COM136	UL4, RS5 and RG11	COM345	RS5, RI11 and RG14
COM145	UL4, RI11 and RG14	COM346	RS5, RI11 and RG11
COM146	UL4, RI11 and RG11	COM356	RS5, RG14 and RG11
COM156	UL4,RG14 and RG11	COM456	RI11, RG14 and RG11

using a spectrophotometer (Novaspec III, Biochrom, Cambridge, UK) at 600 nm wavelength. The pH of each strains of *L. plantarum* was determined using Mettler-Toledo pH meter with a glass electrode (Mettle-Toledo., England).

Inhibitory Assay

The inhibitory assay against an indicator bacterial (*Pseudococcus acidilactici*) was determined by using the Agar-well diffusion method (Tagg & McGiven, 1971). The cell-free supernatant (CFS) containing bacteriocins was collected and diluted 2-fold until level 5 with normal saline (0.85% w/v), followed by dispensing 20 µL of each diluted CFS into MRS agar wells (5.5 mm diameter). The diluted CFS was allowed to diffuse for about 2 hours at room temperature before overlaid with 3 mL of MRS soft agar containing 1% (v/v) indicator strain of *P. acidilactici*. The plates were then sealed with parafilm and incubated at 30°C for 24 hours under anaerobic condition. The inhibitory activity was examined for a clear inhibition zone surrounding each agar well. The diameter of the inhibition zone was measured to indicate the magnitude of the inhibitory activity.

The antimicrobial activity of the cell-free extract was calculated from the reciprocal of the highest dilution producing a clear zone of growth inhibition on the indicator media under standardized condition, and it is expressed as Arbitrary Unit (AU) as described by Thanh *et al.* (2010).

$$\text{Inhibitory activity unit} = \frac{\text{Highest dilution with clear zone AU/mL}}{\text{Volume of CFS in each well}}$$

Statistical Analysis

The data were analyzed using one-way analysis of variance (ANOVA) and the General Linear Model procedure by SAS 1998 (SAS Inst., Inc., Cary, NC). Meanwhile, the Duncan's Multiple Range Test System was used to compare the significant differences of the treatments at $P < 0.05$. The data were presented as the mean ± standard error of the mean (SEM).

RESULTS

Characteristics of the Metabolites Produced by L. plantarum Strains

The optical density, final pH and cell population are shown in Table 2. Nonetheless, there is no significant difference ($P > 0.05$)

TABLE 2
Optical density (OD), pH and LAB counts of *L. plantarum* strains

	UL4	TL1	RS5	RI11	RG14	RG11
OD_{600nm}	9.64±0.12^{c}	9.85±0.08^{ab}	9.92±0.11^{a}	9.91±0.18^{a}	9.89±0.14^{ab}	9.91±0.15^{a}
pH	4.34±0.22^{a}	4.16±0.18^{ab}	4.27±0.12^{a}	4.12±0.16^{b}	3.83±0.16^{c}	3.85±0.12^{c}
LAB counts	8.14±0.05^{d}	8.79±0.03^{c}	8.85±0.03^{b}	8.95±0.02^{a}	8.82±0.04^{b}	8.92±0.03^{a}

[1] The results are presented as mean values ± SEM.
[2] [a-d] Means in the same row not sharing a common superscript are significantly different ($P < 0.05$)

in OD_{600nm} between TL1, RS5, RI11, RG14 and RG11. The mean optical density ranges from 9.64 to 9.91, with the highest optical density observed in RS5, RI11 and RG11 as compared with the other strains. In contrast, the lowest OD was observed in UL4 compared with the other strains. Meanwhile, the pH value was significantly lower ($P < 0.05$) in the culture media of RG14 and RG11 compared to the other strains. The highest pH value was observed in the medium which had been cultured with UL4. In general, the mean pH value ranges from 3.83 to 4.34. Among the six strains, RI11 and RG11 had the highest LAB counts ($P < 0.05$), and these were followed by TL1, RG14 and RS5. UL4 produced the lowest cell count ($P < 0.05$) compared with other strains.

The Inhibitory Activity of Individual L. plantarum Strains

The inhibitory activity of the metabolite produced by *L. plantarum* strains against *P. acidilactici* is shown in Table 3. As shown, there is no significant difference ($P > 0.05$) in the bacteriocin inhibitory activity among the strains. However, the largest diameter of inhibitory zone was observed in RG11 and RG14, followed by RS5, TL1 and RI11. The smallest diameter of the inhibitory zone was found in UL4.

The Inhibitory Activity of Different Metabolite Combinations

The inhibitory activity of the metabolite combinations against *P. acidilactici* is shown in Table 4. Two levels of inhibitory activities were observed in this experiment. The highest inhibitory activity (1600 AU/mL; $P < 0.05$) was observed in COM246, COM345 and COM456. Among the 3 combinations, COM456 showed the largest diameter of inhibitory zone. The remaining 17 metabolite combinations produced a lower level of inhibitory activity (800 AU/mL).

DISCUSSION

Optical Density, pH and LAB Counts

The optical density is an expression of transmittance of an optical element, under a known wavelength. The turbidity or optical density of a suspension of cells is directly related to cell mass or cell number. In this experiment, the OD was adjusted prior to the production of the active cultures where metabolite is harvested. The observation is

TABLE 3
The inhibitory activity and diameter of inhibitory zone exhibited by different *L. plantarum* strains

	UL4	TL1	RS5	RI11	RG14	RG11
Inhibitory activity (AU/mL)	800	800	800	800	800	800
Inhibitory zone (mm)	8.52±0.14[d]	8.85.±0.21[c]	9.17±0.17[b]	8.85±0.18[c]	9.57±0.23[a]	9.61±0.28[a]

[1] The results are presented as mean values ± SEM.
[2] [a-d] Means in the same row not sharing a common superscript are significantly different ($P < 0.05$).

TABLE 4
The inhibitory activity and mean diameter of bacteriocin inhibitory zone exhibited by different metabolite combinations of *L. plantarum* strains

Combinations	Inhibitory activity (AU/mL)	Inhibitory zone	Combinations	Inhibitory activity (AU/mL)	Inhibitory zone
COM123	800[b]	9.23±0.18[ab]	COM246	1600[a]	8.26±0.23[b]
COM124	800[b]	9.34±0.10[a]	COM345	1600[a]	8.28±0.29[b]
COM125	800[b]	9.15±0.13[b]	COM456	1600[a]	9.35±0.32[a]
COM126	800[b]	9.27±0.11[ab]			
COM134	800[b]	8.86±0.13[bc]			
COM135	800[b]	8.70±0.14[bc]			
COM136	800[b]	9.28±0.15[ab]			
COM145	800[b]	9.17±0.13[b]			
COM146	800[b]	9.45±0.16[a]			
COM156	800[b]	8.83±0.15[bc]			
COM234	800[b]	9.49±0.21[a]			
COM235	800[b]	8.84±0.04[bc]			
COM236	800[b]	8.72±0.14[bc]			
COM245	800[b]	8.69±0.12[bc]			
COM256	800[b]	8.54±0.15[c]			
COM346	800[b]	9.14±0.11[b]			
COM356	800[b]	9.34±0.12[a]			

[1 a-b] Means in the same column not sharing a common superscript are significantly different ($P < 0.05$)

coincided with that of Thu *et al.* (2011) who found a significant difference in the final OD among the different strains of *L. plantarum*. This is mainly because of the variation in the biochemical and physiological properties between the different strains of *L. plantarum.* Under a similar condition, different strains of bacteria tend to grow and produce varied levels of metabolite which may affect the reading of the OD.

Meanwhile, the differences in the LAB counts and pH among the strains of *L. plantarum* were due to the production of metabolic compounds such as organic acids. In general, the increase in the active cell population leads to a higher production of metabolite. The metabolite production rate is correlated with a lot of factors, including growth, pH of culturing media, and temperature of incubation (Foo *et al.*, 2005; Barugue-Ramos *et al.*, 2006).

Inhibitory Activity of Metabolite and Combinations

There was no significant difference in the inhibitory activity of the single strain *L. plantarum.* However, a higher inhibitory activity was observed in COM246, COM345 and COM456 when the metabolite combination from 3 strains of *L. plantarum*

was produced and used against the indicator (*P. acidilactici)*. This is in agreement with the finding of Thu *et al*. (2011), whereby a higher inhibitory activity was observed in some of the 3 strains metabolite combinations which derived from *L plantarum.* The ability of the metabolite in expressing antimicrobial activity is mainly due to the presence of the compounds such as bacteriocins, organic acids and hydrogen peroxide (Reid, 2001). Bacteriocin from *L. plantarum* is an antimicrobial peptide in nature, and it is capable of inhibiting the growth of pathogens at cellular and molecular levels (Drider *et al*., 2006).

In addition, organic acid acts as an acidifying agent, reducing the pH of surrounding and survivability of non-acid-tolerant pathogens. Besides, organic acid could retard the enzymatic activity of the pathogens and force the bacterial cell to use the remaining energy to expel the excess of protons H^+, which would ultimately result in death by starvation (Holyoak *et al.*, 1998).

According to Yeaman and Yount (2003), the combination of metabolites from the different strains of bacteria enhanced the inhibitory activity against the pathogens due to the synergistic effects of the different antimicrobial peptides. This coincides well with the study by Luders *et al*. (2003), where a stronger inhibitory effect was observed when mixed bacteriocin from LAB was used against *E. coli*. Another study showed the inhibitory potential of *L. plantarum* where pathogens, such as *E.coli*, *L. monocytogenes*, *S. typhimurium* and vancomycin resistant *Enterococcus,* could be effectively inhibited when metabolites from all the six strains used in the study were mixed (Thanh *et al.,* 2010). Meanwhile, Bouttefroy and Milliere (2000) induced greater inhibitory effects against *L. monocytogenes* when niacin and curvacin were used.

The presence of other beneficial compounds in the metabolites, such as organic acid and hydrogen peroxide, could further potentiate the inhibitory activity of bacteriocin (Jack *et al*., 1995; Stiles, 1996). The acidification of organic acid may increase the solubility of bacteriocins and facilitate the translocation of the molecule through the cell wall. Buncic *et al*. (1995) showed that the sensitivity of *L. monocytogenes* to niacin increased when lactic acid was added.

CONCLUSION

This experiment concluded that the variations in OD, pH and LAB counts were observed in single strains. However, they produced a similar level of inhibitory activity. On the contrary, when three different strains of *L. plantarum* strains were combined, some combinations produced higher inhibitory effects. Among the 20 combinations of metabolites, the combination of strains TL1-RI11-RG11, strains RS5-RI11-RG11 and strains RI11-RG14-RG11 showed a higher level of inhibitory activity. The full potential of the metabolites in the single strains or even in combinations was still undetermined. However, this experiment could serve as a basis to show the enormous potential of the metabolites produced by *L. plantarum* strains, which could then be applied to the animal food and feed industry.

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