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Bacteriocins from Wild Elephant Faeces

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ABSTRACT: Twenty strains of lactic acid bacteria (LAB) were isolated from 20 samples of wild elephant faeces from the Kui Buri District, in Prachuap Khiri Khan Province. Five strains of lactic acid bacteria showed antibacterial activity as follows: strain P115 showed antibacterial activity on *Salmonella enteritidis* serovar Enteritidis, *Vibrio cholerae*, and *Escherichia coli*, strain P124 showed antibacterial activity on *Salmonella enteritidis* serovar Enteritidis, and *Vibrio cholerae*, strain P322 showed antibacterial activity on *Vibrio cholerae*, *Salmonella enteritidis* serovar Enteritidis and *Shigella dysenteriae,* strain P911 showed antibacterial activity on *Vibrio cholerae* and *Salmonella enteritidis* serovar Enteritidis, and strain P1112 showed antibacterial activity on *Staphylococcus aureus*.

Strain P322 had higher antibacterial activity than P115. Antibacterial activity on *Vibrio cholerae*, *Salmonella* Enteritidis and *Shigella dysenteriae* was about 400 AU/ml for all test strains. Strain P115 showed antibacterial activity on *Salmonella enteritidis* serovar Enteritidis, *Vibrio cholerae*, and *Escherichia coli* at 400, 400, and 200 AU/ml, respectively. Strain P115 and strain P322 were identified by Bergey's Manual of Systemic Bacteriology and Biochemical test kit API 20 Strep (BioMerieux) as *Lactobacillus casei* strain P115, and *Lactococcus lactis* subsp. *lactis* strain P322, respectively. Antibacterial substances, cell free culture neutralized supernatant (CFNS) of *Lactobacillus casei* strain P115 and *Lactococcus lactis* subsp. *lactis* strain P322 were digested by proteolytic enzyme with a consequent loss of antibacterial activity, thus the antibacterial substances are bacteriocins. The bacteriocins showed thermostable properties: 100% after treatment at 100°C for 10 minutes.

KEY WORDS: Lactic acid bacteria, *Lactobacillus casei, Lactococcus lactis,* Bacteriocin, Elephant Faeces.

INTRODUCTION

Bacteriocins are ribosomal antimicrobial peptides which are produced by bacteria. The bacteriocins produced by lactic acid bacteria (LAB) have received considerable attention during recent years for their possible use as food preservatives with a resultant reduction in the use of chemical preservatives. Many bacteriocins produced by lactic acid bacteria inhibit closely related species and a variety of species of gram-positive spoilage bacteria and food-borne pathogens such as pediocin PA1 of *Pedicococcus acidilatici* PAC 1.0 (Chikindas *et al*., 1993), Sakacin A of *Lactobacillus sake* LB706 (Axelsson & Holck, 1995) and lactocin 705 of *Lactobacillus casei* CRL705 (Vignolo *et al*., 1996) and leucocin OZ of *Leuconostoc* OZ (Osmana-gaoglu, 2007) that inhibited *Listeria monocytogenes* and OR-7 bacteriocin of *Lactobacillus salivarius* that inhibited *Campylobacter jejuni* (Stern *et al*., 2006).

The possible uses of bacteriocins are not only as preservatives for improving the microbial safety of food but also as probiotics

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in animals and humans to improve the balance of microflora and to inhibit pathogenic bacteria in intestinal tracts (Soomro *et al*., 2002). Furthermore, they are active in immune response stimulation and influence metabolic activity (Salminen *et al*., 1996).

At present there are only a few bacteriocins produced by lactic acid bacteria that are capable of inhibiting important food-borne pathogens such as *Salmonella sp., Shigella dysenteriae*, *Vibrio cholerae, Vibrio parahaemolyticus Staphylococcus aureus,* and *Escherichia coli* O157:H7.

Bacteriocin producing lactic acid bacteria are usually isolated from fermented food (Savadogo *et al.,* 2004) or fermented milk (Bagenda *et al.*, 2008; Bukola *et al.,* 2008) and the intestinal tracts of animals such as the gut of marine prawns (Karthikeyan & Santhose, 2009), Wista albino rat intestines (Patil *et al.,* 2007), and chicken intestines (Nithisinprasert *et al.*, 2000). The isolation of lactic acid bacteria from wild animals has not been reported.

Because wild elephants eat many different plants their intestinal tract may be a good source of bacteriocins. The purpose of this study is the isolation of bacteriocin producing lactic acid bacteria from wild elephant faeces from the Kui Buri District, Prachuap Khiri Khan Province, Thailand, 2007-2008.

MATERIALS AND METHODS

Elephant faeces collection

About 50 g. of each of 20 samples of fresh wild elephant faeces were collected in an area of pineapple plantations around the Kui Buri National Park. The samples were kept at 10ºC for 3–4 days for the isolation for lactic acid bacteria.

Isolation of lactic acid bacteria

Lactic acid bacteria were isolated from 20 samples of fresh wild elephant faeces. Ten-fold serial dilution of the samples was performed in 0.85% NaCl and the diluted samples were spread on to de Man Rogasa and Sharpe (MRS) agar (Difco, USA) which contained 0.8% $CaCO_3$. The plates were incubated at 30ºC for 24-48 hrs. Each colony on the MRS agar that produced a clear zone was selected for determination of bacteriocin production and propagated in MRS broth (Difco, USA). The bacterial strains were kept in MRS broth, containing 15% glycerol at -20°C.

Screening of antimicrobial substance producing lactic acid bacteria and determination of CFS antibacterial activity

Each colony in the MRS agar that produced a clear zone was selected for determination of bacteriocin production. The lactic acid bacteria were cultured for production of antibacterial substances in MRS broth at 30°C for 24 hrs. A cell free culture supernatant (CFS) of each strain was obtained by centrifugation of the culture at 10,000 rpm for 15 min. The cell-free supernatant was adjusted to a pH of 6 with 5 Molar NaOH solution. The bacterial cells were destroyed by heating the supernatant to 70° C for about 30 min. The CFS of each lactic acid bacterial strain was investigated for antibacterial action on several important pathogenic bacteria, such as *Salmonella enteritidis* serovar Enteritidis, *Staphylococcus aureus*, *Shigella dysenteriae, Vibrio cholerae,* and *Vibrio parahaemolyticus.*

The production of antibacterial substances was examined by the spot-on-lawn

method (Fujita *et al.*, 1999). Briefly, a nutrient agar (NA) plate was layered with 10 ml of nutrient soft agar (0.7% agar) which was then inoculated with 10 µl of 10^7 cfu/ml indicator strain culture. Then the double layer medium was spotted with 10 µl of each CFS of lactic acid bacteria or 2-fold serial diluted CFS. After an overnight incubation at 37°C, the bacterial lawns were checked for inhibition zones. The activity was defined as the reciprocal of the highest dilution causing a clear zone of growth inhibition in the indicator lawn and expressed in activity units (AU) per millilitre of a bacteriocin preparation. The arbitrary unit (AU) was defined as the reciprocal of the highest dilution producing a clear zone of growth inhibition of the indicator strain. The AU was calculated as (1000/10)D where D was the dilution factor (Parente *et al*., 1995). Lactic acid bacteria which displayed the highest inhibition activity against each indicator strain was selected and identified for further study.

Identification of antimicrobial substance producing lactic acid bacterial strains

The lactic acid bacterial strains showing the highest inhibition activity were identified according to the methods described in Bergey's Manual of Systematic Bacteriology (2004). Initially, the identification of the strains to genus was based on morphological and some biochemical characteristics determined by gram staining, catalase testing, gas production from glucose and xylitol, and growth characteristics.

The identification of lactic acid bacterial strains at species level was performed by using an API 20 Strep (BioMerieux, Frence) kit. The principle of this identification kit is based on different biochemical characteristics that are unique for each bacterium species.

Effect of enzymes on antibacterial activity

In order to investigate the effect of enzymes on the antimicrobial activity of the antimicrobial substances, the CFS's of lactic acid bacterial strains were adjusted to optimum pH activities for each enzyme such as proteinase K (Sigma; pH 7.0) trypsin (Sigma; pH 7.0), pepsin (Sigma; pH 3.0), α-amylase, lipase and lysozyme. The prepared CFS's were treated with the desired enzyme at a final concentration of 1 mg/ml at 37ºC for 4 hrs. The enzyme reaction was stopped by heating to 100ºC for 5 min. The retained antimicrobial activity of the CFS was determined by the spot-on-lawn method as previously described. *Salmonella enteritidis* serovar Enteritidis was used as the indicator strain.

Thermostability of antimicrobial substance

The CFS's of lactic acid bacterial strains were exposed to various heat treatments, 70º C for 30 min, 100ºC for 10, 20, and 30 min, and 121ºC for 15 min. The retention of antimicrobial activity of the CFS's was then determined.

RESULTS AND DISCUSSION

Screening of antimicrobial substance producing lactic acid bacteria

In all 98 acid-producing bacterial strains were obtained from 20 fresh wild elephant faecal samples that were collected from pineapple plantations around the Kui Buri National Park, Prachuap Khiri Khan Province, Thailand.

Only 20 stains of these acid-producing bacteria displayed the general characteristics of lactic acid bacteria: for instance, produced

acid and clear zones in the MRS agar which consisted of 0.8% $CaCO_3$, were gram positive, and were unable to produce catalase enzyme. These lactic acid bacteria comprised 12 strains of coccus shape and 8 strains of rod shape. The result differs from that reported by Gonzalez *et al*. (2000) who obtained rod shape lactic acid bacteria (237 strains) but only 12 strains of the coccus variety.

Five strains of the 20 lactic acid bacteria showed antimicrobial activity on some indicator bacteria. Strain P115 displayed antimicrobial activity on *Salmonella enteritidis* serovar Enteritidis, *Vibrio cholerae* (Fig. 1) and *Escherichia coli* O157:H7. Strain P124 shown antimicrobial activity on *Salmonella enteritidis* serovar Enteritidis, and *Vibrio cholerae.* Strain P322 showed antimicrobial activity on *Vibrio cholerae*, *Salmonella enteritidis* serovar Enteritidis and *Shigella dysenteriae*. Strain P911 displayed antimicrobial activity on *Salmonella enteritidis* serovar Enteritidis, and *Vibrio cholera*, while strain P112 displayed antimicrobial activity only on *Staphylococcus aureus* (Table 1).

Strain P322 was the most interesting antimicrobial substance producing lactic acid bacteria, because it was able to inhibit three important food-borne poisoning pathogens; *Salmonella enteritidis* serovar *Enteritidis, Shigella dysenteriae,* and *Vibrio cholerae*, that cause enteritis/food poisoning and typhoid fever, bacterial dysentery, and cholera, respectively. This strain displayed a high antimicrobial activity at 400 AU/ml on those three food-borne pathogens. Meanwhile strain P115 was also active, displaying antimicrobial activity on *Salmonella enteritidis* serovar Enteritidis, *Vibrio cholerae* and *Escherichia coli* O157:H7 (causing infant diarrhoea) at 400, 400, and 200 U/ml, respectively. This is the first report of lactic acid bacteria which show antimicrobial activity on *Vibrio cholerae*, an important food-borne pathogen in tropical countries. In the future, these lactic acid bacteria might be used as effective probiotics applied to livestock, and have industrial and medical uses. Therefore, P322 and P115 were selected for further investigation of bacteriocin production.

Identification of strain P322 and P115

According to the method described in Bergey's Manual of Systematic Bacteriology (2004), the lactic acid bacterial strain P115 was identified by morphological and biochemical characteristics (Table 2) as *Lactobacillus casei*. This lactic acid bacteria is a gram positive rod, non-spore forming bacteria, unable to produce catalase, producing acid, but not producing gas in glucose fermentation, and able to ferment mannitol. Strain P322 was identified as belonging to the genus *Lactococcus* but the specific species of this strain could not be determined by the general characteristics.

Lactic acid bacteria strain P322 was positively identified by using an API 20 Strep (BioMerieux, Frence) kit (Table 3), which is suitable for the identification of gram-positive coccus bacteria. The biochemical characteristics of this strain were computed by the API 20 Strep program. Finally, strain P322 was identified as *Lactococcus lactis* ssp. *lactis* with 90% certainty. *Lactococcus lactis* has been isolated from vegetables such as soybeans, cabbage, grass and potatoes (Teuber, 1995), which are probably the source of the *Lactococcus lactis* ssp. *lactis* strain P322 that is in the wild elephant faeces.

To date there are no reports of the isolation of bacteriocins or antimicrobial substance producing lactic acid bacteria from wild elephant faeces or other wild animal faeces. Most of the reports related to the isolation of lactic acid bacteria are from domestic animals, such chicken intestines (Nitisinprasert *et al.,* 2000; Stern *et al.,* 2006) and fermented foods, such as Nigerian fermented food (Bukola *et al.,* 2008), Boza (Todorov and Dicks, 2000).

There are many reports of bacteriocin production but bacteriocins that inhibit gram-negative food-borne pathogens are less common, especially those inhibiting *Salmonella sp.* and *Vibrio cholerae*. Therefore, *Lactobacillus casei* strain P115 and *Lactococcus lactis* ssp. *lactis* strain P322, might be a useful source of bacteriocins, since they displayed antibacterial activity on important gram-negative pathogens.

Table 1. Antimicrobial spectrum of lactic acid bacterial strains.

indicator / strain	Antimicrobial activity on					
	*S.*Enteritidis	*V. cholerae*	*V. parahaemolyticus*	*E. coli*	*S. dysenteriae*	*S. aureus*
P115	-	+	-	+	-	-
P118	-	-	-	-	-	-
P121	-	-	-	-	-	-
P122	-	-	-	-	-	-
P124	+	+	-	-	-	-
P125	-	-	-	-	-	-
P322	+	+	-	-	+	-
P411	-	+	-	-	-	-
P511	-	-	-	-	-	-
P512	-	-	-	-	-	-
P521	-	-	-	-	-	-
P611	-	-	-	-	-	-
P612	-	-	-	-	-	-
P811	-	-	-	-	-	-
P911	+	+	-	-	-	-
P1011	-	-	-	-	-	-
P1112						+
P1211	-	-	-	-	-	-
P1212	-	-	-	-	-	-

Remark ; + : show inhibition zone on the indicator bacteria
- : no inhibition zone on the indicator bacteria

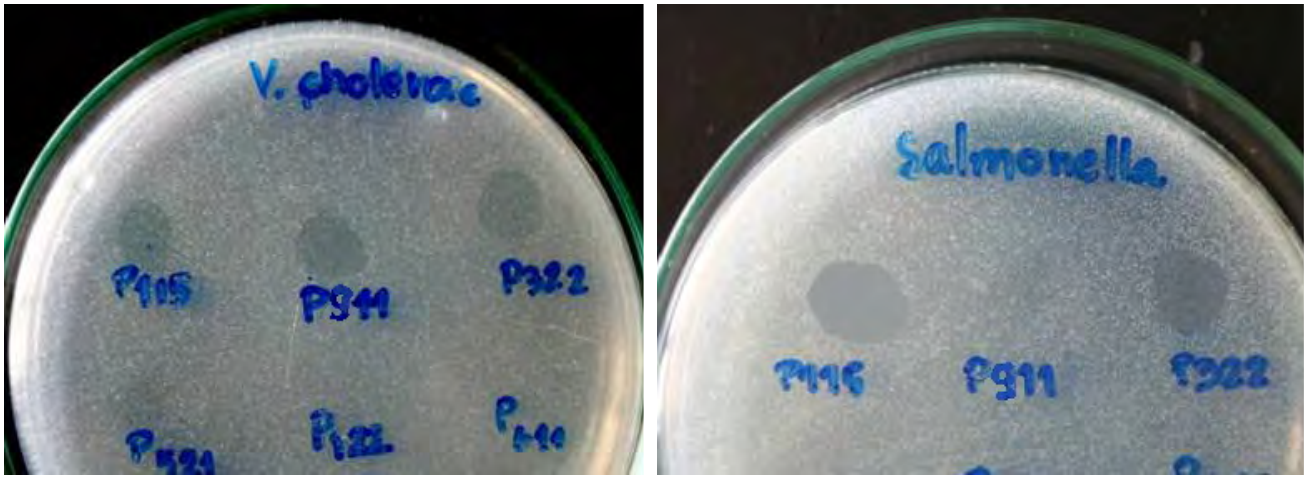

Figure 1. Antibacterial inhibition on *Vibrio cholerae* and *Salmonella enteritidis* serovar Enteritidis of the CFS produced by some lactic acid bacteria isolated from wild elephant feces.

Table 2. General characteristics of lactic acid bacteria strain P115 and P322.

Morphological / biochemical test	P115	P322
shape	rod	coccus
Gram's stain	Gram positive	Gram positive
Catalase test	-ve	-ve
Growth at		
pH 4.4	-ve	+ve
pH9.6	+ve	+ve
10ºC	-ve	-ve
45ºC	+ve	+ve
Growth in		
6.5% NaCl	+ve	+ve
18% NaCl	-ve	-ve
Mannitol fermentation	+ve	+ve

Remark ; +ve : positive reaction
-ve : negative reaction

Table 3. Biochemical characterization of lactic acid bacteria strain P322 by API20 Strep kit.

Reaction	Strain P322
Voges-Proskauer	+ve
Hippurate hydrolysis	+ve
Esculin hydrolysis	-ve
Pyrrolidonyl aminbopeptidase	-ve
α-galactosidase	-ve
β-galactosidase	-ve
γ-galactosidase	-ve
Alkaline phosphatase	+ve
Leucine arylamidase	+ve
Arginine dihydrolase	-ve
Ribose	+ve
Arabinose	+ve
Mannitol	+ve
Sorbitol	+ve
Lactose	+ve
Trehalose	+ve
Inulin	+ve
D-raffinose	+ve
Hydrolyse amindon	+ve
glycogen	-ve

Remark ; +ve : positive reaction
-ve : negative reaction

Table 4. Effect of enzymes on antimicrobial substances.

Enzyme	Antimicrobial activity of substances produced by:	
	Lactobacillus casei strain P115	*Lactococcus lactis* ssp. *lactis* strain P322
Control	400	400
Proteinase K	0	0
Trypsin	0	0
α-amylase	400	400
Lipase	400	400
Lysozyme	400	400

Note: the antimicrobial activity was determined on *Salmonella enteritidis* serovar Enteritidis by spot-on-lawn method.

Effect of enzymes on antimicrobial substances

The properties of the antimicrobial substances *of Lactobacillus casei* strain P115 and *Lactococcus lactis* ssp. *lactis* strain P322 were determined by the antimicrobial activity on *Salmonella enteritidis* serovar Enteritidis after treatment with various enzymes, such as proteinase K, trypsin, α-amylase, lipase and lysozyme, for 3 hrs at 37ºC in order to prove that they were bacteriocins. The antimicrobial activity on *Salmonella enteritidis* serovar Enteritidis of these substances were lost after treatment them with proteinase K and trypsin (Table 4). Meanwhile, they maintained their antimicrobial activity after treatment with others (Table 4). From these results, we suggest that these antimicrobial substances are bactericins, antimicrobial peptides produced by bacteria,

since they were sensitive to the proteolytic enzyme.

Thermostability of bacteriocins

The antimicrobial substances or bacteriocins of the *Lactobacillus casei* strain P115 and *Lactococcus lactis* ssp. *lactis* strain P322 were tested for thermostability by exposure at 70ºC for 30 min, 100ºC for 10, 20, and 30 min, and 121ºC for 15 min and subsequent determination of their retention of antimicrobial activity on *Salmonella enteritidis* serovar Enteritidis. The results showed that both bacteriocins maintained their activity 100% after exposure at 100ºC for 10 min. The bacteriocin of *Lactococcus lactis* ssp. *lactis* strain P322 was more stable than the bacteriocin of *Lactobacillus casei* strain P115, because only the bacteriocin of *Lactococcus lactis* ssp. *lactis* strain P322 maintained its antibacterial activity after exposure at 121ºC for 15 min., about 50% (200 AU/ml) of the control.

Since both bacteriocins displayed thermostable properties, we suggest that these bacteriocins might be classified as class II bacteriocins and be useful in industrial applications. However, it is necessary to confirm this suggestion with further study.

CONCLUSION

Both *Lactobacillus casei* strain P115 and *Lactococcus lactis* ssp. *lactis* strain P322: lactic acid bacteria isolated from fresh wild elephant faeces in the Kui Buri District, Prachuap Khiri Khan, Thailand produced interesting novel bacteriocins that showed antibacterial activity on important food-borne pathogens such as: *Salmonella enteritidis* serovar Enteritidis, *Vibrio cholerae, Shigella dysenteriae, and Escherichia coli* O157:H7. Both the P115 and P322 strains displayed some thermostable characteristics, a crucially important property of useful bacteriocins and an important reason for considering their possible eventual use in the future in the food industry as probiotics.

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