VI. DISCUSSION

Nowadays, lactobacilli play an important role in food industry and may contribute to apply as probiotics in human and animal lives. They can generate the microbial balance condition in their habitat, the oral cavity, intestine and vagina (41). As the defenders in the oral cavity, Sookkhee *et al.* (15) reported that some oral lactobacilli which collected from healthy human volunteers possessed the antimicrobial activity against various oral pathogens. Likely to the present study, some intestinal lactobacilli which collected from healthy goats exhibited the inhibitory activity against *P.gingivalis* W50. It may be noted that goat's intestine is the source of potent lactobacilli which acted as probiotics.

In the aspect of the oral pathogens, they can be divided into 3 groups according to their oral manifestations namely, caries, periodontitis and candidiasis. The microorganisms in the red periodontal microbial complex including *P. gingivalis*, *P. intermedia* and *A. actinomycetemcomitans* play a significant role in the progression of periodontal diseases (150). Their virulence factors can contribute to adhere and colonize on the oral surface, escape themselves from innate immunity and invade tissue (165, 187, 197, 202). Chemotherapeutically, the regimen of metronidazole and either amoxicillin or ciprofloxacin was recommended for this prophylaxis (225). However, the incidences of antibiotic resistance of *P. intermedia* and *A. actinomycetemcomitans* were reported (234-239). These pathogens could reside in the oral biofilms. At that condition, the antibiotic-resistant molecular encoded from the antibiotic resistant strains may induce or transfer to its colleagues, *P. gingivalis*, and may lead to develop as the antibiotic resistant strain. From this hypothesis, the novel alternative concept for periodontal treatment has been established. One of all was the bacteriocin treatment. The several studies of bacteriocins produced from lactobacilli against food-borne and urogenital pathogens were reported (16, 113, 114, 233) while there was a few study of bacteriocin activity towards oral pathogens (4, 15). In the present study, the bacteriocin produced by *L. paracasei* subsp. *paracasei* was characterized, partially purified and determined the antimicrobial activity against *P. gingivalis*.

In our previous study, 1,000 isolates of lactic acid bacteria were primarily screened the antimicrobial activity with the basic bacterial indicators including *S. aureus* ATCC 25923, *S. lutea* ATCC 9341, *E. coli* ATCC 25922 and *B. subtilis* ATCC 6633 (226). These tested bacteria were established as the primary indicators of antimicrobial testing because of their high sensitivity (226). Afterwards, the oral pathogens namely *S. mitis*, *S. mutans* and *P. gingivalis* W50 were also performed as the indicators. They have played a role as the representatives of major oral pathogens. Agar-cup diffusion method which modified by Gordon *et al.* (227) was performed as a gold standard method for determining the antimicrobial activity. The broad spectrum lactic acid bacteria were recruited as the potent isolates. Among the potent isolates, the isolates which also exhibited the strong activity towards *P. gingivalis* W50 were selected. It was found that 10 isolates of lactic acid bacteria were demonstrated. Three of them namely B85/4, B282 and B63/8 possessed the strongest activity against *P. gingivalis* and were then carried out to perform in the present study. For the identification of these isolates, PCR technique was recommended to identify lactic acid bacteria (240). Due to the lack of primers, the biochemical technique was also recommended as the gold method (15, 241). This technique is rapid, color-detected, and comfort. On the biochemical basis, 2 commercial kits which commercially distributed in Thailand, namely API®-50 CHL (API®50 CHL; BioMérieux) and BBL crystal anaerobe identification (BD BBL CRYSTAL™; Becton Dickinson) were performed for these identifications. Between these commercial kits, the identifications were monitored according to their different methabolisms, sugar fermentation and anaerobic reactions. Also the different techniques, the color apparent and fluorescent techniques were performed for determining the reactions. According to API-50 CHL, it could not identify the lactobacillus isolates which belonged to *L. casei*. On the contrary, it could not recognize to *L. paracasei* after tested with BBL crystal anaerobe identification kit. It may suggest that the differentiate assay is required for these genera of lactobacilli. The caseinase activity or called as casein utilization was recommended (15). Following these examinations, the results showed that these three isolates were significant identified as *L. paracasei* subsp. *paracasei* from API®-50 CHL while they were identified as *L. casei* after testing with BBL™ anaerobic test kit. Therefore, the

casein utilization was carried out to differentiate the *casei* species. All *L. casei* strain can utilize the casein by using their extracellular caseinase. The negative results of casein utilization were demonstrated after tested these isolates. It may be concluded that all of isolates were *L. paracasei* subsp. *paracasei*. As the previous study of Ward *et al.* (240), *L. casei*, *L. paracasei* and *L. rhamnosus* exhibited a closely related taxonomic group within the heterofermentative manner. They proposed that it was difficult to differentiate them according to traditional fermentation profiles. They also suggested the polymerase chain reaction method by using a randomly amplified polymorphic DNA (RAPD) as a standard method. The primer was specifically based on differences in the V1 region of the 16s rRNA gene primer. As the present results, it may suggest to carry out the above technique for the further identification of three lactobacillus isolates.

According to the agar-disc diffusion methd that modified by Kirby *et al.* (242) Mueller-Hinton agar have been recommended as the standard agar plate. Because of the fastidious property (228), blood agar was replaced to Mueller-Hinton agar for determining the antimicrobial susceptibility of three antimicrobial producing lactobacilli. Similarly to lactobacilli, *S. aureus* ATCC 25923 that is the member of Gram's positive bacteria was performed as the indicator of the quality control and interpretive standard. All isolates of lactobacilli were sensitive to erythromycin but resistant to penicillin G, ampicillin, cephalothin, ceptazidime and gentamicin and intermediate resistant to norfloxacin and chloramphenicol. The different clindamycin susceptibility patterns of B85/4, B282 and B63/8 isolates were shown to be intermediate resistant, resistant and susceptible, respectively. As the representatives of the sensitive and resistant results, erythromycin and clindamycin were selected for determining the MIC towards the above lactobacilli. A number of methods for example E-test (228), agar dilution (243) and broth dilution (244) have been established for study the MIC of bactericides. In the present study, E-test was performed because of its quantitatively, simply to use, comfortably and rapidly (228). Results of these isolates demonstrated the same manner of erythromycin susceptibility according to the interpretation of agar-disc method and E-test. But clindamycin showed the different results between these methods. Because of MIC determination, the results obtained from E-test seem to be more acceptable than the results of an

agar-disc diffusion. It may suggest that the sensitivity of method is important for determining the antimicrobial susceptibility of each isolate. MIC obtained from the present study was important for the selective growth of such isolate in the further study.

In the analysis of growth curve and generation time, each freshly culture in the amount of 10^4 CFU/ml as the starting inoculum was performed (229). The early-log, mid-log, late-log, stationary and declined phases were obtained from the OD curve plotting (85). The generation time of each isolate was determined from a time in the period of early- and mid-log phases. The time which was appropriated to harvest the bacteriocin containing in the culture supernatant could be assessed from the analysis of growth curve and generation time. The harvesting times of lactobacillus culture were reported in some studies (12, 85, 109, 245). Zamfir *et al.* (12) and van Reenen *et al.* (85) supposed that the mid-log phase is the appropriate incubation time for harvesting the bacteriocin produced by *L. acidophilus* and *L. plantarum*. Unlikely to the present study, the growth of isolates in the early-stationary phase were enriched for harvesting their bacteriocins. Theoretically, a bacterial cell may be growing in volume or mass, synthesizing enzymes, proteins, RNA and increasing in metabolic activity in the lag phase. The log phase of growth is a pattern of balanced growth where in all the cells are dividing regularly by binary fission, and are growing by geometric progression. The cells divide at a constant rate depending upon the composition of the growth medium and the conditions of incubation. Exponential growth cannot be continued forever in a batch culture. Population growth is limited by one of three factors; exhaustion of available nutrients, accumulation of inhibitory metabolites or end products, and the exhaustion of biological space. The stationary phase, like the lag phase, is not necessarily a period of quiescence. Bacteria that produce secondary metabolites, such as bacteriocin, do so during this phase. At the later time, the culture was also rapidly acidified by the effect of large amounts lactic acid or acetic acid after further incubation. For the decline phase, any waste toxic products from their metabolism were detected and the strong acidic condition and coaggregation-associated proteins may able to coaggregate other extracellular proteins and then form to bacterial pellets so the bacteriocin was lost from the culture supernatant (246).

Extracellular compounds contained in the cell-free supernatant of lactobacilli namely, lactic acid, acetic acid, ethanol, aldehydes, carbondioxide, hydrogen peroxide and bacteriocins were reported (20). Lactic acid, hydrogen peroxide and bacteriocins were acted as the major antimicrobial substances. For the characterization of these compounds contained in the cell-free supernatant, the supernatants were separately treated with 3 characterized methods. The original pH of the freshly prepared cell-free supernatant was 4.3. The residual antimicrobial activity against *P. gingivalis* W50 was still remained after adusted the cell-free supernatant to pH range 1-5. It may be suggested that the activity may cause by lactic acid and/or acidic proteins. There was no activity after adjusted the MRS broth to pH range 1-5. Therefore, the added acid were not affected the activity. After treated with NaOH, the activity was dramatically decreased in the alkaline pH range. Lactic acid contained in the cell-free supernatant was so neutralized. Acidic proteins were also inactivated. However, NaOH adjustment could not affect to the baseline activity of MRS. It seems to be that the acidic condition was the optimized condition for the antimicrobial activity of cell-free supernatant (109). For heat treatment, the activity caused from lactic acid could be exhibited but the antimicrobial proteinaceous cmpounds may be destroyed at the range of heat. If the activity is inactivated, the major activity will be caused from the antimicrobial proteins or called as bacteriocins. Results demonstrated the residual antimicrobial activity of supernatant after heated at 80°C. The residual activity was partially reduced after heated more than 100°C. At that temperature, the remained activity may be due to lactic acid. The reduced activity should be caused from the proteins. It was noted that some proteins contained in the cell-free supernatant was heat-resistant and potent as the bacteriocins. On the basis of hydrogen peroxide, it was spontaneously inactivated to be water and oxygen during the increase of heat. Therefore, it was not affected to the activity at the tested temperature. From these results, it may be supposed that the major antimicrobial compound contained in the cell-free supernatant was bacteriocin which potent in the acidic condition and stable at 80°C. The characteristics of bacteriocins in the cell-free supernatant were likely to the bacteriocins which found in the previous reported (108, 109). The antimicrobial activity which caused from the bacteriocins should be reduced after treated with the protease enzyme. Pepsin and trypsin which were the recommended enzymes (12, 15,

85, 109) were performed as the affecting enzyme in the present study. These enzymes were inactivated by heating at 75°C. Bacteriocins were not affected at this temperature. Results demonstrated that the activity was more sensitive to pepsin than trypsin. It may be suggested that the acidic condition of cell-free supernatant may influence to the proteolysis of these enzymes. Various reports described that the antimicrobial activity of bacteriocin was inactivated upon treatment by trypsin or pepsin (12, 13, 15, 85, 109, 241, 247). Zamfir *et al.* (12), Sookkhee *et al.* (15), van Reenen *et al.* (85) and Lash *et al.* (109) reported that the antimicrobial activities of their lactobacilli were significantly inactivated after treatment with pepsin, trypsin α-chymotrypsin, and proteinase K. The same manners of the inactivation were shown in the present study after treated with the proteolytic enzymes. These characterizations of the antimicrobial activity may suggest that the interesting antimicrobial compound was the bacteriocin. The present results corresponded with the study of Lash *et al.* (109) that demonstrated the high molecular weight bacteriocin contained in the cell-free supernatant of *L. plantarum*. It exerted the antimicrobial activity in acidic condition and was inactivated at high temperature and proteases.

The gold method for bacteriocin extraction in the present study was the precipitation or salting-out of ammonium sulphate (13). After the removal of water surrounded protein molecule, proteins with larger or more hydrophobic patches will aggregate and then precipitate before those with smaller or fewer patches, resulting in fractionation. In the present study, the proteins were precipitated in the acidic condition of lactobacillus culture. Some bacteriocins were reported that they were inactivated at alkaline pH such as nisin (248) and pediocin (249). Total bacteriocins were precipitated with the various saturations of ammonium sulphate, 20%, 40%, 60% and 80%. This study was indicated the optimal saturation of ammonium sulphate to precipitate the bacteriocins. The optimal saturation was differently indicated in any reports such as 40% saturated precipitation for acidophilin 801 with molecular weight < 6.5 kDa produced by *L. acidophilus* (12), 35% saturated precipitation for two bactericidal peptides with molecular weight 4.5 and 6.0 kDa produced by *L. amylovorus* (250) and 60% saturated precipitation for active bacteriocin with molecular weight 122 kDa produced by *L. plantarum* (109). The present results exhibited that crude bacteriocins were significantly precipitated in a

range of 40-80% saturated ammonium sulphate. However, the protein increasing folds of crude bacteriocins at 40% and 60% were not significantly different. Forty percent of ammonium sulphate, a minimal concentration to precipitate them, was carried out as the protocol for bacteriocin extraction. There was a report proposed that high concentration of ammonium sulphate may interfere the purification procedure (246). The precipitated protein complex between anionic and cationic proteins could be entrapped with the ionic parts of ammonium sulphate. After redissolved with PBS pH 7.2, the bond between ionic parts of ammonium sulphate and proteins were broken. The proteins complexes could be removed the ions of salt by the dialysis step. The crude proteins were harvested after concentrated and removed the excess of PBS. In the present study, the membrane with cut off $\geq$ 10 kDa were performed to separate the large ($\geq$ 10 kDa) and small (< 10 kDa) protein molecules. The large proteins as the harvested proteins could be detected the protein amount by BCA assay. Bicincroninic acid (BCA) assay was performed as the protocol for determining the protein in the lactobacillus culture in the sensitivity range of 20-20,000 µg/ml (13, 241). Because of Tween 80 interference, Bradford's and Lowry's methods were not recommended to determine the amounts of proteins contained in the lactobacillus culture, supernatant and MRS broth. Moreover, the lower sensitivity to determine the proteins of these methods (20-200 µg and 2-100 µg, respectively) was inadequate for the small amounts of protein. Tween 80 is a nonionic detergent consisted in the formula of MRS broth. Tween 80 may form micelles with the proteins in the medium and lead to the more stability of bacteriocin activity (246). Crude proteins were analyzed by SDS-PAGE on 12% SDS-polyacrylamide gel and visualized by staining with Coomassie Brilliant Blue R250 (13).

The gold standard method for analyze charge and molecular size of protein is 2D-PAGE. Their charges were separated by pH gradient immobilization which able to immobilize any charge of protein at that pH value on the immobilized pH gradient strips (230). Isoelectric point which protein exhibited the neutral charge was called as isoelectric point or pI. The pI value of crude bacteriocins was important to the protein purification. In the first dimensional step of 2D-PAGE, IPG pH 3-10 gradient strips were used to analyze any protein charge. All acidic ($3 \leq pI \leq 6$), neutral (pI = 7) and

basidic ($8 \leq pI \leq 10$) proteins were immobilized according to their pI on any pH gradient strips. In the second dimensional step, molecular sizes of crude bacteriocins immobilized on the strip were separated in the 12.5% SDS-PAGE and visualized the protein spots by staining with Coomassie Brilliant Blue G250 with colloidal particle to avoid the excessive background staining on the gel (230). In the present study, the charges of crude bacteriocins of all isolated were detected in both anionic and cationic zones. In the anionic zone, the charges could be detected in the range of pI 3-6. The major clusters of anionic proteins were located at pI 4-6 and minor islets were located at pI 3-4. In the cationic zone, the charges of proteins were could be detected at pI 8-10, and some neutral proteins were found at pI 7. The molecular sizes of crude proteins were found in the range of 30-120 kDa. Interestingly, the major protein spots were detected at low molecular weight of 40-50 kDa and high molecular weight of 80-100 kDa. These results were demonstrated after compared with the pattern of SDS-PAGE. The antimicrobial activity of bacteriocin in the supernatant was stronger in the acidic condition. It may be suggested that the interested active bacteriocins produced by these isolates of *L. paracasei* subsp. *paracasei* was the acidic proteins. Likely to the present study, Atanassova *et al.* (108) reported that the anti-bacteria and anti-yeast activities of a proteinaceous compound produced by *L. paracasei* subsp. *paracasei* were maximally detected at pH 6 and the charge of this protein was shown at pI 5.6, respectively.

At the pI value, a protein can bind to a positively charged medium or called as anion exchanger. In the procedure of protein purification, anion exchange column chromatography was performed as the protocol for capturing the interested anionic proteins. This process was done by using fast protein liquid chromatography (FPLC) apparatus as the recommended performance (231). FPLC is the high performance liquid chromatography system for fast and easy purification of protein. The sepharose anion exchange column was carried out to purify the anionic protein in the present study (250). It is based on a matrix of 90 μm particles made from 6% agarose and highly cross-linked for chemical and physical stability and contained the quarternary ammonium ($-O-CH_2N^+(CH_3)_3$, Q) group as a strong ion exchange groups. It can maintain their charge over a broad pH range, allowing selection of the most suitable pH for each application. In the elution steps, Tris-HCl pH 8.0 (buffer A) and Tris-

HCl pH 8.0 contained 1M NaCl (buffer B) was also used as the appropriate eluting buffers of these proteins. The anionic proteins with pI 4-6 were interesting. In the elution mode of purification, 3 segmented gradient steps of NaCl concentration in buffer B was the optimal eluting condition in the protocol. This step gradients elution could give the high resolution of each peak separation. The volumes of eluting buffer called as column volume (CV) was set up for optimal eluting of unbound cationic substance and anionic proteins. The column volumes of buffer A for unbound substance eluting were set in 10 column volumes which could completely remove the unbound substance from the column. The column volumes of buffer B for anionic proteins eluting in the first, second and third steps were 0, 25 and 10 column volumes and the target concentration of NaCl in the first, second and third segments were 35%, 55% and 100%, respectively. The unbound fractions which contained with salts, impurities and cationic proteins were completely eluted out the column in the length of eluting column volume of buffer A as demonstrated in the fraction 3-5 (A2-A4). Two peaks of anionic fractions were shown in the step of NaCl gradient elutions. The first anionic peak of each isolate was completely eluted out the column in the length of segmented eluting column volume of buffer B with NaCl gradient concentration and then the second peak was completely eluted out. For the first anionic peak, the asymmetry peak with rapidly increased absorbance in the front ascending slope and decreased absorbance in the back descending slope was observed. It was noted that it tended to have a sharp front and pronounced tailing since they contained more than one component. In the elution step, any weak acidic proteins were more easily eluted out the column than the strong acidic proteins by low ionic strength of buffer B. The sharp front in fraction 23-27 (C1-C5) may be suggested as the weak acidic proteins in pI value 5-6. Tailing back in fraction 28-32 (C6-C10) may be the acidic proteins in pI value 4-5 after compared with the proteins detected in 2D-PAGE gel. The groups of proteins in the tailing back were not completely separated as any single peaks because their charges were nearly located as shown in the 2D-PAGE. They were also closely eluted together in this ionic strength (251). The second anionic peak in fraction 47-52 (E2-E7) eluted out in the third segment elution was a single peak. It may be suggested that it was the strong acidic protein in pI value 3-4 compared with 2D-PAGE. The total protein concentrations of unbound and front peak pooled fractions

were shown in a large amount while other pooled fractions were shown in a small amount or not detectable in some lactobacilli. These results were corresponded to the eluted protein peak of each pooled fraction in any absorbance values. The molecular weights of the partial purified bacteriocin of all isolates were found that their sizes were > 30 kDa. For the anionic pooled fractions, the proteins in the front slope of the first anionic peak were visualized into 3 bands for B282 isolate and 2 bands for B85/4 and B63/8 isolate. For B85/4 proteins, their molecular sizes were 38 and 87 kDa, the sizes; 43, 46 and 87 kDa for B282 and size 39 and 91 kDa for B63/8. The proteins in the back slope of the first anionic peak were found in the SDS-PAGE gel. The protein in the second anionic peak was not detected in the gel. These protein bands related to the total protein concentration as shown in Table 14 and 2D-PAGE with showed the major clusters of acidic proteins at pI 5-6 as the weak acidic proteins. The protein band of MRS's crude proteins was not detected in both total protein concentration and SDS-PAGE which related to the purification chromatogram. For the limitation of this purification method, the solubility and/or viscosity of the sample may affect to the detected quantity (251). High sample viscosity can cause instability of the separation and an irregular flow pattern resulting in broad, distorted peaks and problems with back pressure. The amount of sample which can be applied to a column depends on the dynamic binding capacity of the ion exchange column chromatography medium and the degree of resolution required. Consequently, in order to achieve satisfactory resolution, the total amounts of proteins applied and bound to the medium should not exceed the total binding capacity of the packed column.

Among these fractions, unbound, pooled fraction 23-27 (front slope), pooled fraction 28-32 (back slope) and pooled fraction 47-52 (second anionic peak), they were carried out to determined the percentage of residual antimicrobial activity by using agar-cup diffusion method (227). The experiment was assessed the fraction which possessed the strongest antimicrobial activity against *P. gingivalis* W50 after compared with the antimicrobial activity of cell-free supernatant. From Figure 20-22, the strongest of residual activity of pooled fraction 23-27 of all isolates was shown. These results supported our hypothesis that bacteriocins which possessed the high antimicrobial activity may be the anionic proteins. This pooled fraction was shown in

the SDS-PAGE with molecular weight about 87-90 and 38-46 kDa and gave the high antimicrobial activity against *P. gingivalis*. It may be suggested that the bacteriocin was contained in the pooled anionic fraction 23-27 (front slope) and called as the partially purified bacteriocin. Likely to the study of Atanassova *et al.* (108), they reported that the antimicrobial proteinaceous compound produced by *L. paracasei* subsp. *paracasei* was purified by anion exchange, reversed phase chromatography and HPLC and detected the molecular weight by SDS-PAGE was 45 kDa. This partially purified bacteriocin of all isolates was carried to determine bacteriocin activity. Four diluents included Tris-HCl pH 8.0, acetate buffer pH 5.0, MRS broth and mMRS broth, were used for redissolving the protein and determining the activity. Bacteriocin of B282 and B63/8 isolate which dissolved in MRS broth and bacteriocin of B85/4 isolate which dissolved in mMRS broth demonstrated the strongest activity which represented as the highest percentage of residual antimicrobial activity compared with the residual activity of cell-free supernatant and unbound fraction which exhibited lower activity in each solvent. For the unbound fractions, they were tested the activity with each diluent. Their lower antimicrobial activity compared with cell-free supernatant and anionic fractions confirmed that the bacteriocin was not exhibited in the cationic protein. The antimicrobial activity of bacteriocin B85/4 was exhibited in mMRS broth which depleted peptone and beef extract as N-source and bacteriocin B282 and B63/8 was exhibited in MRS broth. Various supplements in MRS broth were responsible to support bacterial growth, production, bacteriocin secretion and enhancement of antimicrobial activity. Some reports have been demonstrated that the addition of peptone or yeast extract to the MRS resulted in the notable increase of the antimicrobial activity of some bacteriocins produced by lactobacilli (252, 253). So, each appropriate diluent of bacteriocin was carried out to determine their bacteriocin activity against *P. gingivalis* W50.

The bacteriocin activity of cell-free supernatant, crude bacteriocins and partially purified bacteriocin represented in the bacteriocin unit or BU was determined by the serial dilution microtiter plate assay (231) with the appropriate diluent. The activity in the crude bacteriocins of all isolates was exhibited the low activity compared with cell-free supernatant and partially purified bacteriocin. It may be noted that proteins contained in crude bacteriocins may exhibit as the neutral manner

and their activity was not enhanced with any diluents (231). On the other hand, the partially purified bacteriocin presented with the negative charge of acidic protein was exhibited the enhanced activity with MRS diluent. The purification folds and activities recovered of all isolates were exhibited. Among these lactobacilli bacteriocins in the present study, the partially purified bacteriocin produced by B85/4 isolate showed the potent bacteriocin activity and gave the activity recovered of 306.73 %. This result was likely to the study of Batdorj *et al.* (241) that proposed the purified bacteriocin produced by *Enterococcus durans* which exhibited the high activity recovered after the first purification step. As the results, the partially purified bacteriocin of B85/4 isolate was carried to study the time killing assay toward *P. gingivalis* W50.

The time killing assay was performed to assess the minimal exposure time between the active compound and the tested bacteria (232). MIC of cell-free supernatant, partially purified bacteriocin and ampicillin were separately carried out to determine the time. Their MICs were carried out to determine the killing time toward *P. gingivalis* W50. The results were found that the time killing profiles of ampicillin at MIC with 99.9% growth inhibition was 3 hrs after compared with bacterial control. The killing time of partial purified bacteriocin at MIC and 2-fold MIC level was the same manner with the significant inhibition at 6 hr as shown in Table 16.

In the present study, the crude bacteriocins of B85/4 isolate were partially purified by anion exchange chromatography. The partially purified bacteriocin was anionic, 38 and 87 kDa with pI 5-6. It may be suggested that such bacteriocin was belong to class III bacteriocins according to its molecular weight. These proteins should be further purified with gel filtration for separating these proteins by size exclusion. Among these proteins, the bacteriocin with exhibited the strongest antimicrobial activity toward *P. gingivalis* W50 may be analyzed in the level of proteome (254) to characterize and identify the bacteriocin.