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Collagen Binding Activity of Bacteria Isolated from Pig and Cow Small Intestine

(Aktiviti Pengikatan Kolagen Bakteria Dipencilkan daripada Usus Kecil Babi dan Lembu)

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ABSTRACT

Bacterial adherence to connective tissue, especially to collagen has been vastly known for their invasive and infectious activities. However, the ability to exploit the unique and specific interactions between bacteria and collagen as a novel approach in detection of placental collagen has never been explored. This study aimed to determine bacteria with binding specificity to placental collagen (Type IV) derived from human and sheep. In order to do this, total bacteria from small intestines of pig and cow were isolated and their ability to bind to Type IV placental collagen (human and sheep) was determined. Interestingly, three bacterial samples; P5, P9 (pig small intestine origin) and B7 (cow small intestine origin) were found to be able to bind strongly to the placental collagen. The bacterial binding to human placental collagen was however, diminished after the bacteria were treated with trypsin, proteinase K (for removal of surface protein) and guanidine hydrochloride (for S-layer removal), suggesting that the interaction of these bacteria to placental collagen was promoted by protein(s) present at the bacterial surface. In addition, significant reduction of placental collagen-binding ability of the bacteria pre-incubated with soluble human placental collagen showed that there is a specific interaction between the bacteria and collagen. P5, P9 and B7 bacteria were found to share 95-97% 16S rRNA sequence similarity to Enterococcus faecalis *ZL,* Enterococcus hirae *ss33b and* Enterococcus faecium *M3-1, respectively. The results presented here may facilitate future studies in identifying bacterial surface protein(s) responsible for the specific binding of bacteria to collagen and opens new opportunity to utilize the protein(s) for the detection of placental collagen in nutraceutical and food supplements.*

Keywords: Bacterial binding; placental collagen; surface protein

ABSTRAK

Pelekatan bakteria kepada tisu penghubung, terutamanya kolagen telah diketahui dengan meluas untuk aktiviti invasif mereka. Walau bagaimanapun, dalam kajian ini, keupayaan untuk mengeksploitasi interaksi yang unik dan khusus antara bakteria dan kolagen sebagai pendekatan baru dalam pengesanan kolagen plasenta telah dikaji. Bakteria daripada usus kecil khinzir dan lembu telah diasingkan dan keupayaan mereka untuk mengikat kepada kolagen plasenta 'Type' IV (manusia dan biri-biri) telah dikenal pasti. Menariknya, tiga sampel bakteria; P5, P9 (daripada usus kecil babi) dan B7 (daripada usus kecil lembu) didapati mampu mengikat teguh kepada kolagen plasenta. Bagaimanapun, keupayaan bakteria untuk mengikat plasenta kolagen manusia berkurangan selepas bakteria dirawat dengan tripsin, proteinase K (untuk pembuangan protein permukaan) dan guanidin hidroklorida (untuk pembuangan lapisan S). Ini menunjukkan bahawa interaksi bakteria untuk kolagen plasenta dipromosikan oleh protein yang wujud pada permukaan bakteria. Selain itu, pengurangan yang ketara daripada keupayaan bakteria untuk mengikat kolagen plasenta selepas dieram dengan kolagen larut plasenta menunjukkan bahawa terdapat interaksi tertentu antara bakteria dan kolagen. Bakteria P5, P9 dan B7 didapati mempunyai 95-97% persamaan daripada segi jujukan 16S rRNA masing-masing dengan Enterococcus faecalis *ZL,* Enterococcus hirae *ss33b dan* Enterococcus faecium *M3-1. Hasil daripada kajian ini boleh digunakan sebagai asas kajian lanjut untuk mengenal pasti protein permukaan bakteria tertentu yang bertanggungjawab mengikat kolagen dan membuka peluang baru untuk menggunakan protein tersebut bagi pengesanan kolagen plasenta dalam makanan tambahan dan nutraseutikal.*

Kata kunci: Kolagen plasenta; pengikatan bakteria; protein permukaan

INTRODUCTION

Bacteria have long been known for the capability to bind to connective tissue particularly for their infection and invasive activities. Probiotic bacteria (such as *Lactobacillus plantarum*) also have the same binding capability which determines the probiotic potential of the bacteria (Salzillo et al. 2015). Collagen is one of the major glycoproteins found in connective tissue which serves as a major target for bacterial attachment. Bacterial binding to collagen is usually catalyzed by a specific protein present on the bacterial surface. For instance, Ace from *Enterococcus faecalis* (Rich et al. 1999), CNE from *Streptococcus equi* (Lannergård et al., 2003) and cna from *Staphylococcus aureus* (Patti et al. 1992) are some of the surface proteins

that have been extensively studied. On the other hand, enolase from probiotic *Lactobacillus plantarum* has been shown to exhibit binding activity towards Type I collagen (Salzillo et al. 2015). These collagen binding proteins have been exploited for various usages. For instance, Ace and CNE have been used in vaccine development against diseases such as endocarditis (Rich et al. 1999) and strangles (Waller et al. 2007). Collagen binding protein from *Lactobacillus plantarum* 91 displayed anti-adhesion activity against *Escherichia coli* 0157:H7 (Yadav et al. 2013) which contributed to the probiotic potential of the bacteria.

Recent trends on consumption and use of placenta-containing products for health and cosmetics have increased among consumers. Placenta is predominantly composed of Type I, IV, V and VII collagen (Friess 1998). Albeit various collagen detection techniques using mass spectrometry (Zhang et al. 2009) and enzyme-linked immunosorbent assay (Venien & Levieux 2005) that have been developed, we propose here a novel approach for the detection of collagen from placenta using bacteria which exhibit specific binding to collagen derived from placenta.

In this work, bacteria isolated from pig and cow small intestines were examined for collagen-binding activity against collagen derived from human and sheep placenta. Bacteria that were able to bind strongly to human and sheep placental collagen were identified through solid-phase collagen binding assay. Probable specific components that are responsible for the adherence of the bacteria to human and sheep placental collagen were determined by applying several treatments on the bacteria such as removal of surface proteins and removal of S-layer components. The effects of these treatments on the bacterial capability to bind to placental collagen were studied. The species of the bacteria with strong binding capability to placental collagen were determined using partial sequencing of amplified 16S rRNA sequences. This finding will be beneficial for successive effort in developing techniques for detection of placental collagen in food supplements as well as in nutraceutical and cosmeceutical products.

Materials and Methods

Isolation and Enrichment and Identification of Bacteria Isolated from the Small Intestine of Pig and Cow

The sampling of pig small intestine was carried out at TipTop Meat Slaughter House, Rawang-Batang Berjuntai, Selangor, Malaysia. The sampling of cow small intestine was carried out at several abattoirs in Selangor and Perak, Malaysia. Approximately 5-8 cm of small intestine from randomly selected 10 healthy and freshly slaughtered pigs and cows was obtained using sterile utensils and placed into sterile plastic containers. The samples were transported to laboratory on ice and immediately processed. The small intestines were dissected using a sterile knife and collected into sterile tubes before being rinsed several times in TBS-Tween solution. Approximately, 10 g of the epithelial lining of the rinsed small intestines were scrapped using a sterile knife and put into sterile tubes.

A total of 90 mL maximum recovery diluent (Sigma Aldrich, USA) was added into each tube to ensure full recovery of bacteria from the samples. The sample mixtures were mixed homogeneously before being subjected to serial dilution using Phosphate-buffered saline (1×). 100 uL of the 10^4 and 10^6 dilutions were separately inoculated on MRS agar (Sigma Aldrich, USA). The inoculated MRS agar was then incubated at 37°C for 24 to 48 h.

Five bacterial colonies were randomly selected from each agar plate for enrichment. In order to enrich a purified bacterial species, single bacterial colony was cultured in MRS broth, followed by incubation at 37°C for 18 to 24 h. The bacterial cultures were then separately streaked onto MRS agar plates, followed by incubation at 37°C for 18 to 24 h. The enrichment step was repeated at least eight to ten times.

Collagen Binding Assay

The ability of the bacteria to bind to placental collagen was determined using a method described by Jakava-Viljanen and Palva (2007) with slight modification. Briefly, 250 mL of 50 ug/mL human and sheep placental collagen solution were separately coated on 8-well chamber slides (Permanox®, Nunc® Lab-Tek®, USA) method. The slides were then blocked with 1% (w/v) blocking reagent (Roche) in PBS. 100 uL of freshly overnight grown bacterial culture at a concentration of 5×10^7 to 5×10^9 cells per mL was used to inoculate the human and sheep placental collagen-coated glass slides. Incubation was carried out at 25°C for 2 h. Non-adherent bacteria were removed by washing three times with 1× phosphate buffered saline (PBS). Adherent bacteria were fixed with heat at 55°C for 20 min and stained with methylene blue. The number of bacteria adhering to human and sheep placental collagen was visualized and calculated using light microscopy. The assay was repeated with six replicates of each bacterial samples and placental collagen.

Percentage of bacterial binding to collagen was calculated using the formulae: (Number of adherent bacterial cells/number of total inoculated bacterial cells)×100 and subjected to statistical data analysis to evaluate the adherence capabilities of the bacteria.

Effect of Proteases Treatment on Bacterial Ability to Bind to Placental Collagen

Proteases were diluted to specific final concentrations in phosphate-buffered saline (25 ug/mL trypsin and 40 ug/mL proteinase K). 100 uL of each protease was added to 100 µL of the bacterial cell suspension and further incubated at 37°C for 60 min. Soybean trypsin inhibitor to a final concentration of 50 µg/mL and phenylmethanesulfonyl fluoride to a final concentration of 5 mM were separately added into trypsin and proteinase K-containing sample to stop the proteolytic activity. Bacterial cells were then

washed twice with phosphate-buffered saline (1×) before being subjected to collagen binding assay. The number of cells was adjusted to 5×10^7 to 5×10^9 cells per mL for use in collagen binding assay. Bacterial cells without protease treatment were served as controls. Binding percentages of the treated and control bacteria cells to the collagen will be subjected to statistical data analysis to determine the effect of the treatment to the collagen binding ability of the bacteria.

EFFECT OF S-LAYER REMOVAL ON BACTERIAL ABILITY TO BIND TO PLACENTAL COLLAGEN

Exponentially growing bacteria in 200 mL culture were harvested by centrifugation and then washed using ice-cold water. The cell pellet was re-suspended in 4 M guanidine hydrochloride and incubated for 1 h at room temperature. After that, the cells were pelleted by centrifugation and re-suspended in PBS and the numbers of cells were adjusted to be used in collagen binding assay. Bacterial cells that have not undergone S-layer removal were served as control. Binding percentages of the treated and control bacteria cells to collagen will be subjected to statistical data analysis to determine the effect of S-layer removal to the collagen binding ability of the bacteria.

COMPETITIVE INHIBITION OF BACTERIAL ADHERENCE BY THE SAME SOLUBLE PLACENTAL COLLAGEN

Bacterial suspensions were incubated with 10 μg of soluble human placental collagen at 25°C for 30 min. The bacteria were then washed with PBS and seeded on the glass slides for collagen binding assay using immobilized human placental collagen. Bacteria that have not undergone pre-incubation served as control. Binding percentages of the pre-incubated and control bacteria cells to collagen were subjected to statistical data analysis to determine the effect of S-layer removal to the collagen binding ability of the bacteria.

STATISTICAL DATA ANALYSIS

The statistical significance of the adherence capabilities of the bacterial cells to human and sheep placental collagen were evaluated by one-way analysis of variance (ANOVA) and Tukey HSD test for post-analysis of variance pairwise comparisons (Minitab 17 Software). The differences were considered significant when P values being obtained were less than 0.01. In order to evaluate the effect of treatment (protease treatment, S-layer removal and pre-incubation of bacterial cells with soluble collagen), the statistical significance of the binding percentages of the treated bacterial cells as compared to the untreated bacteria cells was evaluated by one-way analysis (ANOVA) and Dunnet Test for post-analysis of multiple pairwise comparisons between the mean of treated bacteria binding percentage and the mean of a control group. The differences were considered significant when the confidence intervals do not contain zero.

SPECIES IDENTIFICATION OF BACTERIA

The bacteria species designated as P5, P9 and B7 were determined through partial 16S rRNA sequencing. Firstly, genomic DNA of the bacteria was extracted using Wizard Genomic DNA Purification Kit (Promega, USA) following the manufacturer's protocol. 50-100 mg of bacterial genomic DNA was used as a template in PCR amplification of 16S rRNA fragment utilizing 0.5 uM forward 27F: 5'AGTTTGATCCTGGCTCAG3' and reverse primer 1429R:5'GTTACCTTGTTACGACTTC3'. The PCR was conducted on Mastercycler Gradient PCR machine (Eppendorf) using 1X TopTaq MasterMix (Qiagen) according to the following PCR program: Initial denaturation at 94°C for 3 min, 35 cycles of denaturation at 94°C for 3 min, annealing at 55°C for 30 s and extension at 72°C for 1 min followed by final extension at 72°C for 10 min. The PCR product, which corresponds to the amplified 16S rRNA fragment were visualized on 1% agarose gel and purified using Wizard SV Gel and PCR Clean Up Kit (Promega, USA) before being sent for sequencing (FirstBase Laboratories, Singapore). The obtained 16S nucleotide sequences were then searched against non-redundant nucleotide database in NCBI through BLASTn software.

RESULTS

Forty bacteria samples isolated from small intestine and gastrointestinal tract of pig and cow were assessed for binding to human and sheep placental collagen. Three bacterial samples designated as P5, P9 and B7 were found to exhibit strong binding to human and sheep placental collagen. 16 samples were found to exhibit binding percentage between 30-70% and 21 others exhibited less than 30% binding to human and sheep placental collagen (Figure 1). 16S rRNA sequencing showed that these three strong binding bacteria are of Enterococcus species. P5 shared 96% homology to *Enterococcus faecalis* ZL, P9 shared 97% homology to *Enterococcus hirae* ss33b and B7 shared 96% homology to *Enterococcus faecium* M3-1 (Table 1).

In order to further examine the component responsible for the binding of bacteria to placental collagen, P5, P9 and B7 bacteria treated with trypsin, proteinase K and guanidine hydrochloride were subjected to solid phase collagen binding assay. The ability of the treated bacteria to bind to placental collagen was compared to the untreated bacteria. Trypsin and Proteinase K were used to remove proteins at the bacterial surface, while guanidine hydrochloride was employed for removal of S-layer protein on the bacterial surface. Binding percentages of treated bacteria to human placental collagen were compared to the untreated bacteria using Dunnet Simulataneous Statistical Test with 95% confidence intervals. As expected, it was found that the treatment of bacteria with trypsin, proteinase K (for removal of surface protein) and guanidine hydrochloride (for removal of S-layer protein) imposed a significant effect on the bacterial ability to bind to the

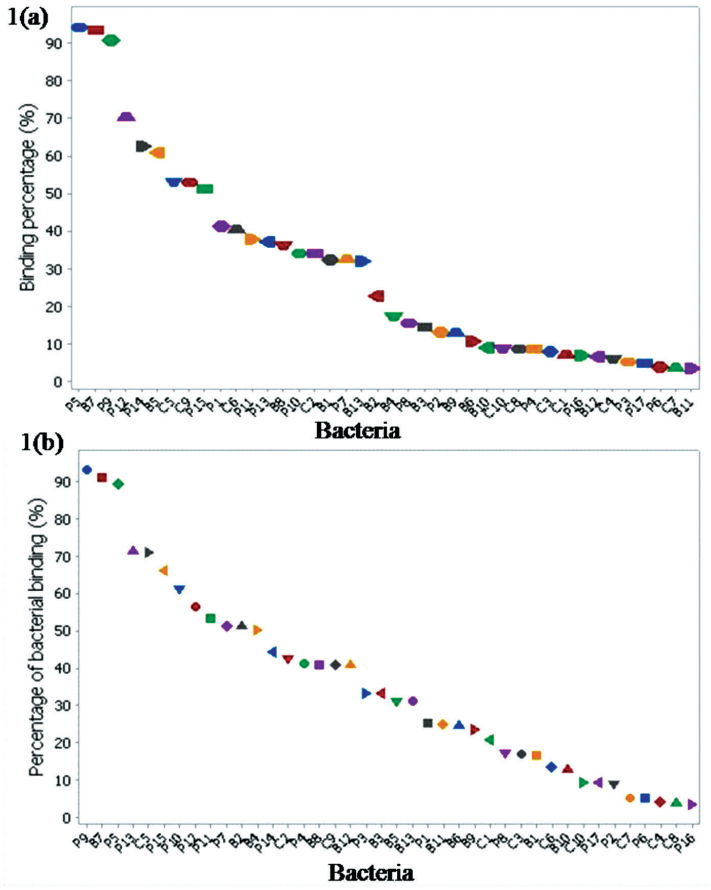

FIGURE 1. Binding percentage of bacteria isolated from pig and cow small intestine to sheep (1a) and human (1b) Type IV placental collagen

TABLE 1. 16S rRNA sequence analysis of P5, P9 and B7 bacteria

No	Bacteria	Species	Strain	Similarity
1	P5	*Enterococcus faecalis*	*ZL*	96%
2	P9	*Enterococcus hirae*	*ss33b*	97%
3	B7	*Enterococcus faecium*	*M3-1*	96%

placental collagen in which, the binding of treated bacteria to placental collagen was almost diminished to less than 8% (Figure 2). Further confirmation of bacterial binding to placental collagen was carried out by solid phase collagen binding assay using P5, P7 and B9 bacteria pre-incubated with soluble human placental collagen. In comparison to the bacteria that was not incubated with soluble human placental collagen, the ability of the pre-incubated bacteria were significantly reduced (less than 10%) thus further strengthening the evidence that the bacterial binding to placental collagen was promoted by specific proteins on the bacterial surface (Figure 3).

DISCUSSION

Collagen has been categorized to thirteen types according to the length of the helix and the nature and the size of the non-helical portions. Type IV collagen has been exclusively found in basement membranes and contains up to six genetically distinct alpha-chains. Glanville et al. (1979) successfully developed a procedure for the isolation of type IV collagen from the basement membranes of human placenta. In this study, type IV collagen from sheep and human placenta were used (Sigma Aldrich, USA) to determine binding capability of bacteria isolated from porcine and bovine intestine.

Three bacterial samples with strong binding capability to type IV human and sheep placental collagen, designated as P5, P9 and B7, shared 96 to 97% homology to *Enterococcus faecalis* ZL, *Enterococcus hirae* ss33b and *Enterococcus faecium* M3-1, respectively. The sensitivity of the bacterial binding to protease treatment showed that the binding relies on specific determinants present on the bacterial surface. The capability of most bacteria to bind to extracellular matrix protein (ECM) lies on the bacterial ability to produce microbial surface components

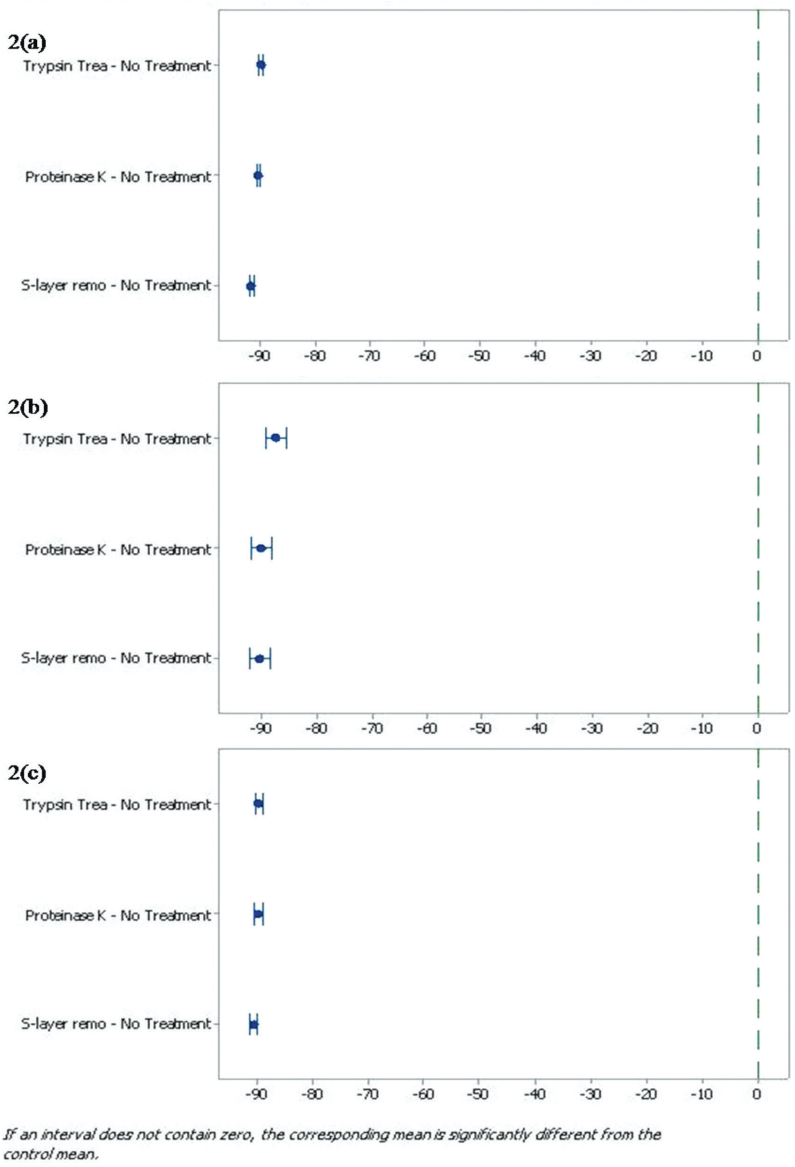

FIGURE 2. Effect of trypsin treatment, proteinase K treatment and S-layer removal on P5 (2a), B7 (2b) and P9 (2c) bacterial binding to human placental collagen

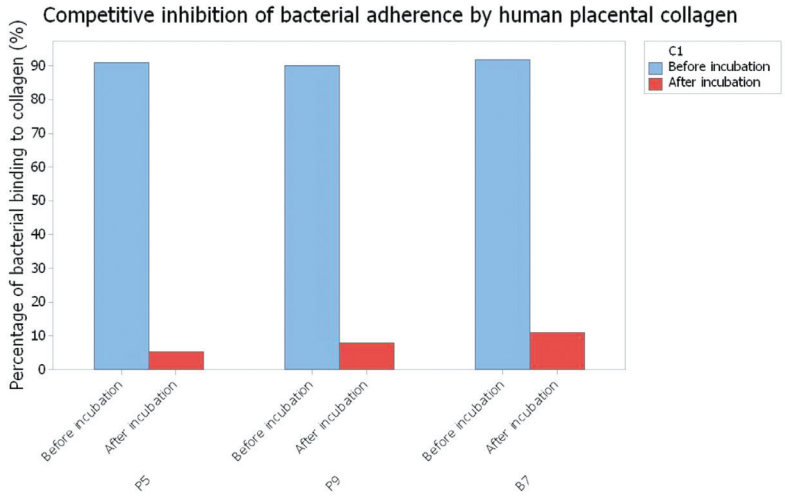

FIGURE 3. Binding percentage comparison of non-incubated bacteria to bacteria pre-incubated with human placental collagen

recognizing adhesive matrix molecule (MSCRAMM) on their surfaces. For instance, Ace, a collagen-binding MSCRAMM from *Enterococcus faecalis* has been reported to be responsible for the bacterial capability to bind to Type I and Type IV collagen and laminin (Nallapreddy et al. 2000). In order to utilize the binding characteristics of bacteria in placental collagen detection, identification of the specific proteins may be carried out by analyzing trypsin-digested peptides of the bacterial surface protein against type IV placental collagen. This method has been widely utilized in profiling the surfacome of *Staphylococcus aureus* (Dreisbach et al. 2010). Despite the inherent effect observed on bacterial binding capability after S-layer removal, to the best of our knowledge, S-layer protein expression in *Enterococcus* species has not been previously reported. Thus, further test to investigate the presence of S-layer protein in P5, P9 and B7 should be carried out through PCR amplification targeting S-layer protein genes.

CONCLUSION

The capability of P5, P9 and B7 bacteria to bind to Type IV placental collagen should be further explored through surface-shaving method to identify surface protein(s) that are responsible for their binding to collagen. The findings result will serve as basis for further development of placental collagen detection based on specific interactions of the surface protein to collagen.

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