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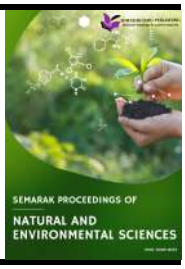

Bacteriocin-like inhibitory Substance (BLIS) Produced by *Lactococcus lactis* strain FA4 with Potential for Use in Aquaculture

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ABSTRACT

Lactococcus lactis is a lactic acid bacteria isolated from dairy environment which is considered as generally regarded as safe (GRAS). A *L. lactis* strain FA4 was previously isolated from Black tip shark have shown antagonistic properties against *Vibrio parahaemolyticus*, a shrimp and fish pathogen. In this study, cell free supernatant (CFS) from L. lactis FA4 has shown active antagonism against the growth of indicator strain *V. parahaemolyticus*, but not against *V. alginolyticus*. With *V. parahaemolyticus*, the minimum inhibitory concentration (MIC) recorded around 0.125AU/ml. The CFS component was found to be rather stable during brief heat treatment, retaining ~86% of residual activity, and alkaline treatment had retained ~60.8% of residual activity. SDS page analysis and zymogram studies revealed that the CFS contains proteinaceous substance that have molecular weight of approximately ~3-5kDa. Based on several previous studies, L. lactis strain FA4 produces a bacteriocin like substance (BLIS) that highly resembles to that of nisin. Therefore, the BLIS has future potential to be utilized as antimicrobial agent suitable to be used in aquaculture treatment.

Keywords: Lactococcus lactis, bacteriocin-like inhibitory substances, antagonistic activities, nisin

1. Introduction

Lactococcus lactis belongs a lactic acid bacterium (LAB) originally discovered and studied due to its deep involvement in making fermented dairy product. This strain is now considered to be categorized as Generally Recognized as Safe (GRAS) organism for human consumption [1]. Commonly found in human and animal microbiomes, *L. lactis* is a prevalent starter in fermented foods, plant materials, and silage. *L. lactis* has a long history of involvement in dairy fermentation, especially in cheese making [2]. *L. lactis* is a primary bacterium contributes to the development of desirable flavors and characteristics of cheese and buttermilk. Being common in food fermentation, this strain is also considered as human probiotics since many health benefits it brings such as reducing lactose intolerance and diarrhea, stimulating the immune system, and potential protection against colon cancer.

L. lactis produces bacteriocins or bacteriocin like substance BLIS, an antimicrobial peptide that can inhibit the growth of other related bacteria. Nisin, an example of BLIS produced by *L. lactis,* is a natural food preservative which has been commercialized [3]. It usually antagonizes Gram-positive bacteria and inhibiting bacterial spores by forming pores in their cell membranes. Due to its origin,

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nisin is considered safe for human consumption, reason for its widely use in dairy products, canned foods or oral health products. Recent study has explored its potential application beyond food preservation, including various therapeutic areas [4].

This work was carried out following a discovery of a *L. lactis* strain FA4 from the black tip shark *Carchahinus limbatus* [5]. This strain was found to inhibit the growth of a fish or shrimp pathogen *Vibrio parahaemolyticus*. In shrimp, this *V. parahaemolyticus* is notorious for Early Mortality Syndrome (EMS) outbreaks which has resulted in massive collapse in shrimp production sector. In light of these findings, a subsequent study evaluated the effectiveness of consuming *L. lactis* strain as probiotic by shrimp [6]. Despite of these promising results, it is still unclear on the nature and properties of inhibitory mechanism against *V. parahaemolyticus*. Previous studies have indicated that the cell free supernatant (CFS) from *L. lactis* FA4 isolates could have produced a BLIS that acts and kills Vibrio strain. Thus, in this work the present of nisin in the CFS of *L. lactis* FA4 was further characterized and verified. This work will open up future possibility of using nisin in aquaculture sector.

2. Methodology

2.1 Strains and medium

Lactococcus lactis strain FA4 [5] and the two indicator strains (*V. parahaemolyticus* and *V. alginolyticus*) were derived from existing glycerol stock collection available at Kulliyah of Science, International Islamic University Malaysia. Strains were revived on MRS agar (for *L. lactis* FA4) and NA agar (for *V. parahaemolyticus and V. alginolyticus*). The cells were sub-cultured on de Man, Rogosa, and Sharpe (MRS) agar or Nutrient agar (NA) until pure colonies have formed. All colonies of these strains were picked and used to inoculate either MRS or NA broth, respectively, followed by final incubation at 30°C for 12-18 hours until growth has reached to ~1.0 x 10^8 CFU (for Lactococcus strain), approximately 1.0 x 10^6 CFU (for Vibrio strains).

2.2 Antagonistic Study, Temperature-alkaline Treatments and Minimum Inhibitory Concentration (MIC)

The cell free supernatant (CFS) of *L. lactis* FA4 was prepared by discarding the cell pellet after centrifuging resultant culture broth at 1000xg for 15 min, and filtered using 0.4µm Whatman cellulose acetate filter. Antagonistic study was carried out by transferring 100 µL CFS from *L. lactis* FA4 onto the well made on Nutrient Agar (NA) pre-streaked with the indicator strains. The size of zone of inhibition (ZOI) formed surrounding the well were recorded. The CFS was also subjected to heat and pH treatments. For heat treatment, CFS was subjected to 95°C for 5 min and applied to indicator organism *V. parahaemolyticus.* For pH treatment, 500 µl of CFS were adjusted to 10 with NaOH, and incubated for 4 hours at room temperature and the inhibitions were measured by agar well diffusion. The CFS of *L. lactis* FA4 was subjected to dilution series in 2 factor dilution (2N), i.e., dilution 2^1, 2^2, 2^3, and 2^4. Each 100µL CFS dilution was then used for antagonistic test as described above. The MIC (in arbitrary unit, AU) was defined as the reciprocal of the highest dilution showing visible zone of inhibition.

2.3 Zymogram and SDS poly-acrylamide gel electrophoresis

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was carried out using Tris-Buffer under denaturing condition [7]. Electrophoresis was run in duplicate gel in which one gel was

stained with Coomassie blue after separation completed. The other gel was spared for zymogram study with slight modification [8]. Separated gel was cut along the gel lane, and overlayed on NA agar pre-streaked with indicator strain *V. parahaemolyticus*. Following incubation at 30°C for 12 hours, any inhibition zones formed at the periphery surrounding the gel cut was recorded.

3. Results & discussions

The agar well diffusion method was performed to assess the antimicrobial activity of the BLIS present in the CFS of *Lactococcus lactis* against two indicator strains *Vibrio parahaemolyticus* and *Vibrio alginolyticus*. The zone of inhibition (ZOI) was observed with inhibition size of 0.80 ± 0.03cm (diameter) showing antimicrobial activity against the indicator strain *V. parahaemolyticus*. In contrast, ZOI was almost invisible when *Vibrio alginolyticus* was used as indicator strain (see Table 1). Thus, the CFS was more active against some Vibrio strains.

Minimum inhibitory concentration (MIC) was determined using indicator strain *V. parahaemolyticus*. As shown on Figure 1, *L. lactis* FA4 CFS of different dilution factors have showed varying inhibition zones that diminished as dilution factor increase (see Figure 1). The highest dilution factor showing visible ZOI was at 3 factors of two folds or at 2^3 dilutions. Dilution factor 24 onwards recorded no observable inhibition. Thus, estimated minimal inhibitory concentration (MIC) of the BLIS from CFS of *L. lactis* corresponded to $1/2^3$ dilution factor, or assigned as ±0.125 AU ml^{-1}. Nevertheless, the absolute MIC values cannot be directly comparable to other reported figure for nisin since different experimental condition or unit used resulted in different bias in MIC values [9].

Table 1
Result following antagonistic test using *V. parahaemolyticus* and *V. alginolyticus* used as indicator strain

Antagonistic test	Diameter of inhibition (cm) ± SD
Vibrio parahaemolyticus	0.800 ± 0.02
Vibrio alginolyticus	0.033 ± 0.03

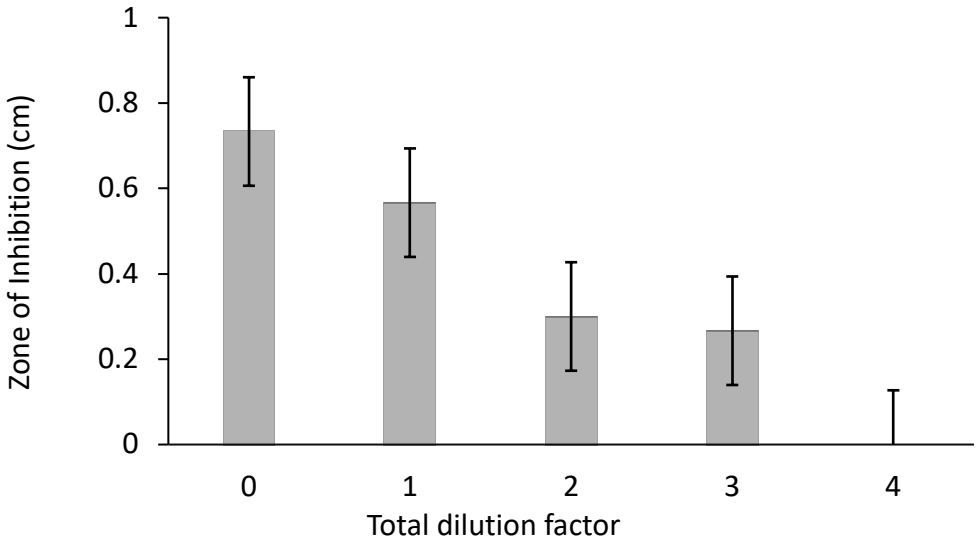

Fig. 1. Minimum inhibitory concentration (MIC) of CFS from *L. lactis FA4* against indicator strain *V. parahaemolyticus*

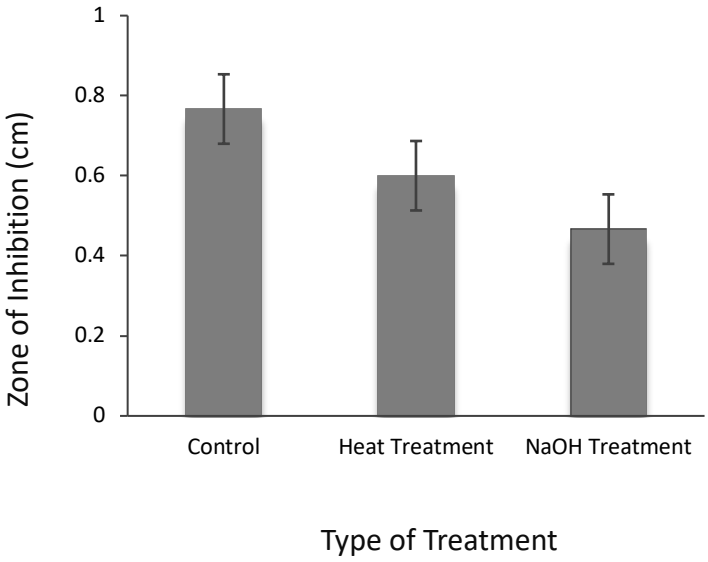

Fig. 2. Heat and alkaline treatments on CFS from *L. lactis FA4*

The CFS from *L. lactis* FA4 was subjected to different treatments. Based on Figure 2, the control antimicrobial activity recorded the largest ZOI (0.767 cm ± 0.033) and CFS was found to be thermally stable with inhibitory effects was depleted only ~14% upon treated with brief heat at 90°C for 10 mins. NaOH treatment has retained 60.8% of the inhibitory activity. This observation is therefore consistent with previous work in which the antimicrobial in CFS has properties similar to Nisin which was partially inactivated with alkaline treatment and was rather stable after a brief heat treatment [5]. Nisin a rather heat stable molecule shows general stability in acidic pH, but decreases in stability as pH increases above 6.0 [10].

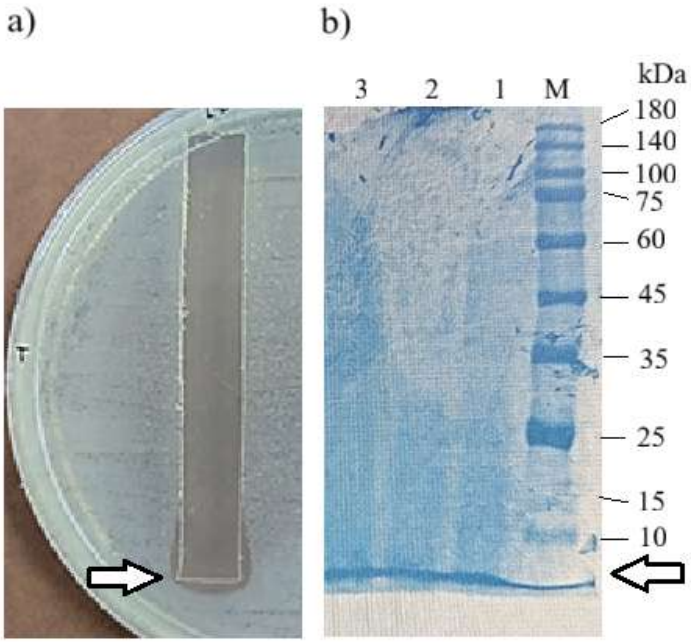

Fig. 3: (a) Zymogram studies showing electrophoresed gel cut in lane-wise, overlayed on the lawn of indicator strain *V. parahaemolyticus*. (b) Coomassie blue stained of SDS electrophoresed samples from cell free supernatant (CFS) from *L. lactis* FA1. White arrows indicate the zone of inhibition (a); and the putative protein bands for the BLIS (b)

As shown in Figure 3 above, results from protein electrophoresis indicated a protein band corresponded to region lower than 10kDa molecular weight. This band reflected the molecular weight of nisin which has corresponding molecular weight of ~3 to 5kDa. The duplicate gel run without protein staining was also overlayed on the lawn of indicator strain *V. parahemolyticus*. Following incubation, the region of ZOI could also be observed at the bottom end of the gel corresponding to 3-5 kDa molecular weight region comparable to the similar region in the stained gel. This indicated that the CFS contained proteinaceous compound similar to nisin. BLIS in this case it is active against *V. parahaemolytics*, but not with *V. alginolyticus*.

4. Conclusions

Our work indicated that the cell free supernatant (CFS) from L. lactis strain FA4 contains a bacteriocin like substance (BLIS) highly similar to nisin. The BLIS however showed potent antimicrobial activity toward the growth *of V. parahaemolytics*, a shrimp pathogen. Future studies on the molecular characteristic of this BLIS will unravel if the actual properties are similar or it is just a new varian of nisin. Thus, we can harness this nisin for aquaculture use, beyond food preservation.

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