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Bacteriocin-like substances produced by *Lactococcus lactis* subsp. *lactis* CF4MRS isolated from fish intestine: Antimicrobial activities and inhibitory properties

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Abstract

In the present study, evaluation of antimicrobial activities of *Lactococcus lactis* subsp. *lactis* CF4MRS bacteriocin-like substances (BLIS) against various fish pathogens was performed using an agar well diffusion assay. The cell-free supernatant (CFS) was first pre-treated using four different bioassays. In the first treatment T1, CFS was treated with catalase, and the pH was adjusted to 6.5 with NaOH to eliminate the inhibitory effect of H_2O_2 and/or lactic acid. In T2, CFS was treated with only 1 mg/mL catalase. In T3, only the pH was modified and adjusted (6.5). For T4, no pretreatment was done on the CFS. Our results showed all tested pathogens: *Pseudomonas fluorescens* ATCC 13525, *P. aeruginosa* ATCC 10145, *Klebsiella pneumonia* ATCC 10031, *Escherichia coli* ATCC 25922, *Aeromonas hydrophila* ATCC 49140, *Edwardsiella tarda* BCRC 16703 and *Serratia marcescens* (Monash culture collection), were susceptible to *L. lactis* CFS (T4). This bacterial inhibition activity was presumably due to BLIS present in CFS. However, the CFS lost its antimicrobial activity when pH was adjusted and treated with enzyme catalase (T1 and T3). This inhibitory effect would be attributed to either organic acid or H_2O_2 produced by the bacterium. On the other hand, CFS treated with only catalase (T2) exerted similar inhibitory effect against the pathogens as showed by the untreated CFS (T4). BLIS in CFS were subsequently determined using HPLC method. Our results revealed that lactic acid in BLIS indeed plays the important role in bacterial inhibition, suggesting the bacteria could be potentially used in managing and controlling fish diseases.

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Introduction

Chemotherapy using antibiotics and other chemical antimicrobial agents in aquaculture has generated a growing concern among consumers. As a result, there has been a great interest in naturally produced antimicrobial agents for more natural fish farm production. In recent years, probiotics have emerged as a crucial feed formulation to develop sustainable aquaculture practices. The most commonly used probiotics in animal nutrition are single or mixed strains of lactic acid bacteria (LAB) such as *Lactobacillus acidophillus, L. plantarum* and *L. casei* (Jacobsen *et al*., 1999).

LAB have been known to produce a wide range of antimicrobial compounds with the ability to inhibit the growth or to kill a broad range of bacteria (Kumari and Garg, 2007; Kumari *et al*., 2008). They are generally recognized as safe microorganisms used in many industries such as food, fermentation and bio-preservation (Marrug, 1991; Kojić *et al.*, 2007; Yang *et al*., 2012). The bio-preservation effect exerted by LAB has recently became the main interest of food producers due to their advantages such as non-chemical properties, high heat stability, low susceptibility to degradation by proteolytic enzymes present in the digestive system, and strong ability to inhibit the growth of various primary pathogenic and spoilage microorganisms (Cleveland *et al.* 2001; Abriouel *et al*., 2003; Deegan *et al.*, 2006; Kojić *et al*., 2007). These antimicrobial compounds produced by LAB mainly comprised of organic acids (such as lactic acid) in which their secretions could lower pH to achieve anti-bacterial purposes (Daeschel, 1989). The other antimicrobial compounds produced by these bacteria groups include hydrogen peroxide (H_2O_2), carbon dioxide (CO_2), diacetyl (2,3-butanedione), acetaldehydrate, D-isomers of amino acids, reuterin, and bacteriocins (Cintas *et al*., 2001).

Bacteriocins have been extensively studied in the food industry due to their potential for food preservation and natural antimicrobial activity (Khay

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et al., 2011). Bacteriocins are peptide antibiotics synthesized ribosomally by bacteria in nature (Drider *et al*., 2006). In most cases, the bacteriocin-producing cells exhibit specific immunity action against their own bacteriocins (Montville *et al*., 1995). The bacteriocins produced by LAB exert their antibacterial effect by damaging the target cell membranes (Bruno and Montville, 1993; Abee, 1995; Moll *et al*., 1998). Autolysin is an enzyme in bacteriocins that is capable of causing bacterial autolysis (Bierbaum and Sahl, 1987; Shockman and Höltje, 1994). Over the years, a large number of bacteriocins produced by LAB have been identified and characterized. Nisin, a bacteriocin produced by *Lactococcus lactis* subsp. *lactis* have been extensively studied in food sectors. The use of nisin in bio-preservation has long been established due to its wide spectrum of antimicrobial activity in food preservation, and it does not contribute to the formation of resistance to pathogens (Hurst, 1981; Delves-Broughton *et al.*, 1996; Alla *et al.*, 2003).

Application of bacteriocin to control unwanted bacteria in animal feed stuff and fish microflora is a new area in aquaculture. Studies have shown that supplementation of LAB *(L. acidophilus)* to the diet of Nile tilapia could significantly increase fish survival during a challenge with *Aeromonas hydrophila*, and further improved the immune response of the fish. The bacteriocins produced by *L. acidophilus* also showed antibacterial activity against *A. hydrophila* and *Streptococcus agalactiae* (Villamil *et al.*, 2014). Nisin-producing LAB such as *L. lactis* subsp. *lactis* had recently suggested by Touraki *et al.* (2013) as probiotic to control *Vibrio* infection in European sea bass, *Dicentrarchus labrax.* The ability to inhibit other fish pathogens by bacteriocin-like inhibitory substances (BLIS) produced by the bacteria, however, is still limited. Hence, this present study aimed to investigate the bacteriocin activity on the antimicrobial spectrum produced by *L. lactis* subsp. *lactis i*solated from fish intestines. Bio-active compounds produced by the bacterium are further identified to elucidate the compounds that play the important role in the antimicrobial activity.

Materials and Methods

Bacterial strains and preparation of cell-free supernatants

The potential probiotic, *Lactococcus lactis* subsp. *lactis* CF4MRS (GenBank accession number: KM488626) was isolated from gastrointestinal (GIT) samples of the farmed fish, *Clarias batrachus* (Loh *et al*., 2014). The probiotic was cultured in MRS (Man-Rogosa-Sharpe, Oxoid, UK) culture broth at 26 ± 2°C for 24 h. Serial dilution was performed to obtain a cell concentration of 10^6 cfu/mL at $OD_{540.}$ Cell-free supernatant (CFS) was collected by centrifugation at 7,500 rpm for 15 min at 4°C with a high-speed refrigerated centrifuge (Universal 320R, Hettich, Germany). The CFS was then filtered through a sterile 0.22 µm pore size filter (Sortunos, USA). Filtrate of CFS was stored in vials at - 20°C until use.

Determination of antimicrobial spectrum and bacteriocin activity assay

Inhibitory activity against the indicator bacteria was determined by agar well diffusion assay as described by Cosentino *et al.* (2012). Seven pathogenic strains, commonly known as human-fish pathogens, were selected and tested. They are *Pseudomonas fluorescens* ATCC 13525, *P. aeruginosa* ATCC 10145, *Klebsiella pneumonia* ATCC 10031, *Escherichia coli* ATCC 25922, *Aeromonas hydrophila* ATCC 49140, *Edwardsiella tarda* BCRC 16703 and *Serratia marcescens* (Monash bacterial culture collection). Briefly, 1% (v/v) aliquot of overnight culture of indicator strains (at 10^6 cfu/mL of each strain) was mixed into 20 mL of soft molten MHA agar (37 ± 1°C), and poured into the Petri dishes. Four pre-treatments of CFS were prepared for the study. Firstly, the CFS was treated with catalase (1 mg/mL, Sigma Aldrich, USA) and pH was adjusted to 6.5 with 5M NaOH to eliminate the inhibitory effect of H_2O_2 and/or lactic acid (T1). In the second pre-treatment (T2), CFS was treated only with 1 mg/mL catalase. The third pre-treatment (T3), pH of the CFS was adjusted to 6.5 with 5M NaOH, while no pre-treatment was performed at the last treatment (T4), i.e. either catalase or NaOH on the CFS (untreated CFS). Lastly, a blank control (Con.) consisting of MRS broth without *L. lactis* subsp. *lactis* was also prepared. All pre-treated CFS were subsequently filtered through a 0.22 µm pore size syringe filter (Sortunos, USA) prior to the assay (Cosentino *et al.*, 2012). To each plate, five wells (6 mm diameter) per Petri dish were prepared and each well filled with 100 µL aliquot of the respective pre-treated and untreated *L. lactis* subsp. *lactis* CFS. The plates were subsequently kept at 4°C for 4 h to allow radial diffusion of the compounds in the supernatant, and incubated at 37°C for 24 h. The experiment was performed in triplicates. If inhibition zones were found around the wells, the CFS was considered capable to produce BLIS. The antimicrobial activity was expressed as the diameter of the inhibition zones (annular radius) formed around the wells.

BLIS analysis using high performance liquid chromatography (HPLC)

Bacteriocin detection was performed using Agilent 1200 Infinity HPLC system (Agilent Technologies, Palo Alto, CA, USA) equipped with 1260 Infinity Refractive Index Detector (RID). Chromatographic separation was performed on Agilent Hi-Plex H column, 7.7 × 300 mm, 8 µm (Agilent Technologies, Santa Clara, CA, USA) at 65°C. MilliQ water containing 0.005 M H_2SO_4 was used as mobile phase, and acts as isocratic gradient at a flow rate of 0.6 mL/min. The RID detector was set at 35°C. Twenty µL of 0.22 µm-filtered samples (CFS and MRS broth - control) were injected into the system via a HPLC syringe (Thermo Scientific, UK) at a 20 µL injection loop connected with the HPLC system. Each run lasted at 25 min. To detect and identify inhibitory compounds, lactic acid was used as standard (0.0156, 0.0625, 0.25, 1.0 and 4.0 g/L) to compare chromatographs taken from the CFS and control MRS broth. Each sample was analyzed in triplicates.

Results

Antimicrobial spectrum and bacteriocin activity assay

The antimicrobial activities of *L. lactis* subsp. *lactis* CF4MRS CFS against seven human-fish pathogens, namely *P. fluorescens, P. aeruginosa, K. pneumonia, S. marcescens, E. coli, A. hydrophila* and *E. tarda* were determined in the study. The effects of pre-treatments on CFS, such as neutralization of the pH, elimination of H_2O_2 from the CFS, combination of both chemical treatments and solely untreated CFS, reveals the role of BLIS as the antimicrobial compound (Figure 1). It is interesting to note that, all pathogenic strains were suppressed when untreated CFS was added into the culture media. The CFS lost its antimicrobial activity following by the treatment with pH adjustment (T3), and during the combined treatment of NaOH and with catalase (T1). However, CFS treated with catalase (T2) only exerted similar inhibitory effect against the pathogens as demonstrated by the untreated CFS. The CFS produced by *L. lactis* subsp. *lactis* without pH adjustment but with H_2O_2 (T2) showed stronger inhibitory strength towards *P. fluorescens*, *S. marcescens* and *E. tarda*, where the clearing zones were recorded at the range of 2.2 to 2.3 mm (Table 1). On the contrary, the inhibitory effect of *L. lactis* subsp. *lactis* CFS (T2) was slightly lower against *P. aeruginosa, K. pneumonia, A. hydrophila* and *E. coli* (ranged from 1.3 to 1.5 mm). Generally, CFS without any chemical treatment (T4) revealed

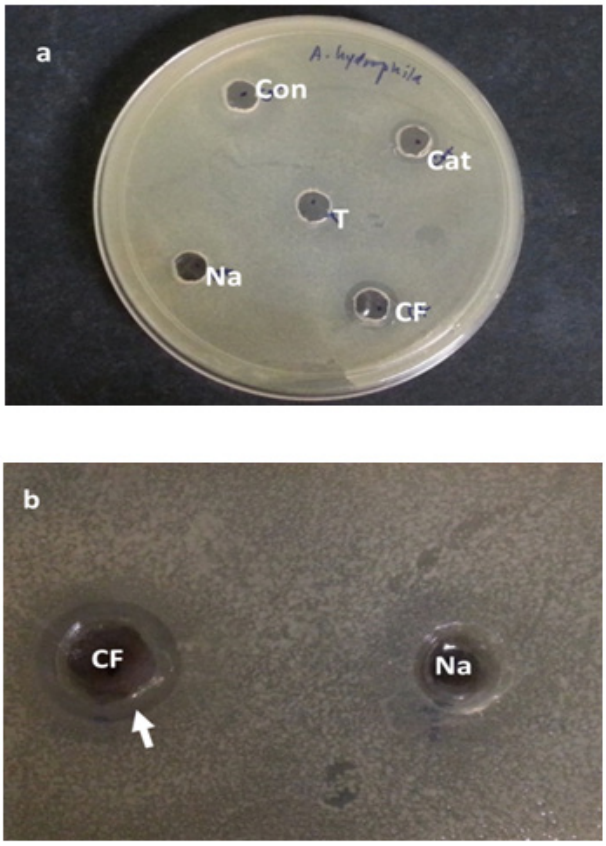

Figure 1. Antimicrobial activities of cell-free supernatant (CFS) against (a) *A. hydrophila*. Wells labeled as follows: T - CFS pre-treated with combined treatment of catalase and NaOH (T1); Cat - CFS pre-treated with catalase (T2); Na - CFS pre-treated with NaOH (T3); CF - CFS only without no pre-treatment (T4) and Con - blank MRS broth. (b) The well on the left showed a clearing zone (arrow) – labeled with CF (CFS only), and no inhibition was found on the right well – labeled with Na (CFS pre-treated with NaOH)

similar inhibitory effect as H_2O_2-treated CFS (T2) in the inhibition of *P. aeruginosa* and *E. coli*. The *L. lactis* subsp. *lactis* CFS pre-treated with catalase (T2) showed higher suppression effect against *P. fluorescens* and *A. hydrophila*. Table 1 indicated similar inhibitory effect was also observed in T4 for all pathogenic strains.

Lactic acid detection in bacteriocin-like substance (BLIS)

The HPLC chromatograph (Figure 2) showed lactic acid was detected in the BLIS of *L. lactis* subsp. *lactis* CF4MRS CFS. The retention time at 11.455 min was detected as lactic acid when compared to pure lactic acid as the external standards. Reference to mobile phase revealed no lactic acid was detected in the blank MRS broth (Figure 2). Based on lactic acid standard curve ($y = 183080x$; $R^2 = 0.9996$), the peak at 11.455 min was calculated as 1.145 g/L.

Table 1. Antimicrobial spectrum and bacteriocin activity of cell-free supernatant (CFS) produced by *L. lactis* subsp. *lactis* following various chemical pre-treatments

Inhibition zone (annular radius, mm) inhibited by CFS of *L. lactis subsp. lactis*					
Pathogenic strains	**(Con) Control***	**(T1) Combined treatment (NaOH + Catalase)****	**(T2) H_2O_2 eliminated (Catalase)****	**(T3) pH adjusted (NaOH)****	**(T4) CFS only*****
P. fluorescens	-	-	2.3 ± 0.3	-	2.8 ± 0.8
P. aeruginosa	-	-	1.5 ± 0.9	-	1.5 ± 0.5
K. pneumoniae	-	-	1.5 ± 0.5	-	1.3 ± 0.3
S. marcescens	-	-	2.3 ± 0.6	-	2.0 ± 0.0
A. hydrophila	-	-	1.3 ± 0.3	-	1.7 ± 0.3
E. tarda	-	-	2.2 ± 0.3	-	2.0 ± 0.0
E. coli	-	-	1.5 ± 0.5	-	1.5 ± 0.0

Legend: * Sterile MRS broth as a control; ** Bacterial cell-free supernatant treated with respective chemicals; *** Bacterial cell-free supernatant only (untreated); "±" Mean ± standard deviation of three replicate experiments; "-" No inhibition detected. All treatment plates were incubated at 37°C for 24 h

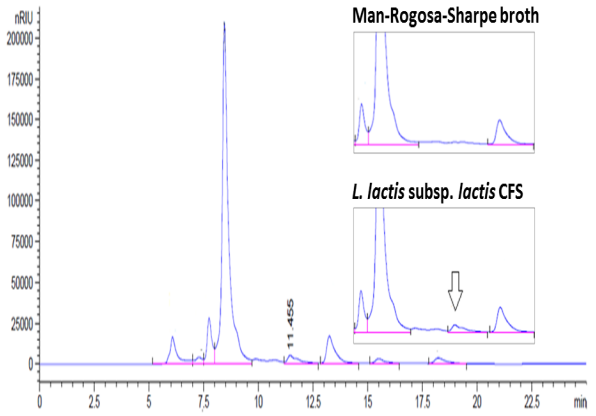

Figure 2. Detection of lactic acid in the cell-free supernatant (CFS) of *Lactococcus lactis* subsp. *lactis* CF4MRS. Lactic acid was not detected in MRS broth. In the CFS (inoculated with *L. lactis* subsp. *lactis*), peak (arrow) at the retention time of 11.455 min was identified as lactic acid

Discussion

The results here showed that untreated CFS inhibited all test pathogens. However, pH-neutralized CFS (T3) did not produce antimicrobial activity towards any of the pathogenic bacteria. Similar results were also reported by Reinheimer *et al.* (1990) and Yang *et al.* (2012) in which strong antimicrobial effects of some LAB bacteria appeared to be a direct result of the organic acids and H_2O_2 present in the CFS. In our study, organic acids were found to be the main inhibitory substances rather than H_2O_2 and BLIS. For the untreated CFS (T4), pH was recorded at 1.5–2.0 which is highly sensitive to many bacteria. The inhibitory effect is presumably attributed to organic acid excreted by *L. lactis* subsp. *lactis*, this hypothesis was in the agreement with Dalié *et al.* (2010). The authors claimed the bacteriostasis and death of susceptible bacteria were indeed due to the neutralization of organic acids on the cyctoplasmic membrane, and thus increasing its permeability. This mechanism causes the cell ruptures and eventually kills the bacteria. In contrast, several studies demonstrated that treatment of the neutralized supernatant of *L. lactis* subsp. *lactis* with catalase and NaOH did not alter their inhibitory activity against foodborne pathogens e.g. *Enterococcus faecium, Listeria monocytogenes, L. innocua, L. ivanovii* and *Lactobacillus sakei* subsp. *sakei.* This inhibitory effect is presumed to be attributed to the bacteriocin-like inhibitory substances (BLIS) (Khay *et al.*, 2011; Cosentino *et al.*, 2012; Yang *et al.*, 2012). Nevertheless, bacterial inhibition was dependent on the concentration of the bacteriocin and the exposure time of the pathogens (Kumari *et al.*, 2009). Bacterial inhibition due to BLIS was not observed in the present study, the possible reason could be the occurrence of low concentration of BLIS in the CFS, which unlikely to demonstrate the capability to suppress the pathogenic bacteria.

The production of many bacteriocins such as nisin, lactococcin B and pediocin PA-1 production are closely rely on the growth medium (Venema *et al.*, 1997; Miladinov *et al.*, 2001; Zendo *et al.*, 2008). In many cases, LAB culture medium such as de Man-Rogosa-Sharpe (MRS) medium is used to produce BLIS in a small scale. However, no sign of nisin was detected when MRS was used to culture *L. lactis* subsp. *lactis* in the present study. This was perhaps due to the degradation of the bacteriocin by some impurities or peptones such as pepsin and trypsin contained in the agar medium (Khalil *et al.*, 2009). These impurities could be the key factors causing the loss of antimicrobial activity in bacteriocin (Yang *et al.*, 2012). To confirm the antimicrobial properties, the BLIS produced by the LAB was subjected to HPLC analysis. From the chromatographic result, we

demonstrated that lactic acid could be the perpetrator contributing to the microbial inhibition. Yang *et al.* (2012) also found that inhibitory effect against several foodborne pathogens e.g. *Listeria innocua, B. cereus, P. fluorescens, Erwinia carotovora* and *Leuconostoc mesenteroides* subsp. *mesenteroides* was indeed organic acids derived from LAB isolated from cheese and yogurt. These organic acids comprise mainly of acetic acid, citric acid and lactic acid. They are typically used to enhance the safety of food products in food industry (Yang *et al*., 2012). Among these, lactic acid was reported as the most effective antimicrobial agent against several pathogens such as *Shigella* species (In *et al*., 2013). This lactic acid production is the major end metabolite of the carbohydrate fermentation of most LAB, which is also the key bioactive compound inhibiting microorganisms (Françoise, 2010).

In the present study, *L. lactis* subsp. *lactis* (at 10^6 cfu/mL) demonstrated ability to inhibit not only *E. tarda* as shown in Loh *et al* (2014), but also against other fish pathogens, notably to *P. fluorescens* and *S. marcescens*. These two pathogens have been associated with septicemia and ulcerative conditions (etiological agent: *P. fluorescens*) (Fayed *et al*., 1997), necrosis of muscle tissue, haemorrhagic septicemia and reddish pigmentation on fish lateral line and head parts (etiological agent: *S. marcescens*) (Baya *et al.*, 1992). In contrary to *P. fluorescens* and *S. marcescens, L. lactis* subsp. *lactis* demonstrated a weaker inhibitory effect against *P. aeruginosa, K. pneumoniae, A. hydrophila* and *E. coli.* All of these opportunistic pathogens can cause serious epidemic throughout a geographical region, and consequently resulting a significant economy losses in fish production. Our result showed lactic acid produced by *L. lactis* subsp. *lactis* was bacteriostatic towards all the pathogenic bacteria tested in the study. This is of particularly important to aquaculture industry, as farmed fishes are currently protected from bacterial disease through vaccination and chemotherapeutic agents. The use of probiotic would able to reduce the incidence of disease or lower the severity during disease outbreaks (Balcázar *et al*., 2008). Since, this probiotic strain *L. lactis* subsp. *lactis* CF4MRS were isolated from fish intestines they are believed able to colonize the GIT and deliver its beneficial effects more effectively as compared to terrestrial strains.

Conclusion

Our present study shows that *L. lactis* subsp. *lactis* CF4MRS isolated from gastrointestinal samples of catfish, *Clarias batrachus* have a wide range of antimicrobial activity which could be potentially useful in controlling important fish pathogens. This inhibitory substance is mostly attributed to an extracellular metabolite by-product - lactic acid. Based on the large inhibitory spectrum and strong inhibitory activity, *L. lactis* subsp. *lactis* CF4MRS can be applied as a probiotic bacterium in the protection of fish health. Our study highlights fish diseases can be potentially controlled and managed by using these beneficial bacteria.

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