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Identification of the first bacteriocin isolated in Lebanon extracted via a modified adsorption-desorption method and its potential food application

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Abstract

Introduction: The raw goat milk is considered as a good source of novel bacteriocinogenic lactic acid bacteria (LAB) strains.

Method: The bacteriocin, which named enterocin CMUL20-2 was secreted by *Enterococcus faecium* CMUL20-2. This bacterial strain was originally isolated from raw goat's milk, was extracted by using a modified adsorption-desorption method and purified via RP-HPLC. antimicrobial activity was tested against several pathogenic and spoilage microbes.

Results: The enterocin CMUL20-2 showed a strong adsorption on cell wall of producer strain even in acidic environment which facilitate its extraction in only two simple steps. The recovered purified enterocin has decreased procedure time and diminished the number of undesirable molecules present in Rogosa and Sharpe (MRS) broth. The recovered enterocin showed antimicrobial activity against several foodborne pathogenic and spoilage microbes.

Conclusion: The recovered enterocin was able to tolerate a variety of food chain conditions such as high temperature, pH and storage stability, and it can be a good candidate to protect food from spoilage microbes.

Keywords
Dairy product; Enterocin; Food preservation; Lactic acid bacteria; *Listeria*.

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Introduction

Lactic acid bacteria (LAB) are generally recognized as safe (GRAS) and widely used as starter cultures for dairy, meat, and vegetable fermentations [1]. When LAB present in human or animal ecosystems, they play an important role in the prevention and treatment of bacterial and viral diseases by different mechanisms [2, 3]. In food sector, one of the most important contributions of these microorganisms is the extended of the shelf life of the fermented products. Also, they play an important role in food preservation due to the ability to produce several antimicrobial substances, including organic acids, hydrogen peroxide, bacteriocins, and bacteriocin-like substances [4, 5]. Many biological hazards are present in dairy product food chain, these "critical points" usually controlled by pasteurization and chemical preservatives. Some dairy products based on unpasteurized milk are considered as a high risk products as regard as to food borne intoxication such as Lebanese traditional raw cheese. *Listeria monocytogenes (L. monocytogenes)*, the most common pathogen transmitted in food, particularly in dairy products, can cause serious diseases such as enteritis, meningitis and abortion[6]cell wall-associated internalins and one small, secreted internalin (InlC. The mortality rate caused by listeriosis is between 15 and 30% [7]. This pathogenic bacterium can be present in all dairy products especially in the unpasteurized one.

The raw goat milk microbiota is considered a good source of novel bacteriocinogenic LAB strains that can be exploited as an alternative for use as biopreservative in foods as observed in previous studies [8-10].

The aim of the present study is to characterize and purify the first Lebanese antimicrobial peptide secreted by *E. faecium* CMUL20-2 isolated from Lebanese goat's raw milk, and to usea modified adsorption-desorption method which can facilitate the purification of potential enterocin.

Materials and Methods

LAB isolation from goat's milk and assessment of their anti-listerial activity

A total of twenty-four samples of goat's milk were collected from individual households in rural areas of different regions in North Lebanon. Samples were collected aseptically in sterile bottles (100 ml), kept in an ice-box container (4°C) and transported to the laboratory for microbiological analyses. The samples were serial diluted, plated and streaked into DeMan, Rogosa and Sharpe (MRS) agar (Bio-Rad, Germany). 150 µl of each LAB working culture were added to 15 ml of MRS broth and incubated at 37 °C for 24 h. The culture broth was centrifuged at 15364 × g for 15 minutes at 4 °C. The supernatant was adjusted to pH 6.0 with 2 M NaOH (Sigma-Aldrich, Germany) and sterilized using 0.22µm syringe filter, the cell-free supernatant was used as crude bacteriocin [11]. The assessment of anti-listerial activity of crude supernatant was carried out using Agar Well Diffusion method by using *L. monocytogenes* ATCC® 19115™ as indicator strain as described by [12]. To determine the nature of the antimicrobial compound, the crude supernatant was treated by proteinase K, lipase and amylase (Sigma-Aldrich, Germany) as describe previously by [13].

Strain identification

A bacterial Strain CMUL20-2 which exhibits the largest inhibition zone against *L. monocytogenes* was selected for further studies. This strain was identified accordingto the physiological and biochemical characteristics [14]. The identification was confirmed by using the pyrosequencing of V1 and V3 of 16S rDNA as previously described [15].

Extraction of enterocin CMUL20-2

Adsorption-desorption of enterocin CMUL20-2

According to Yang et al., 1992, enterocin CMUL20-2 have been extracted by adsorption-desorption method [16].

Stress condition as a new strategy of adsorption-desorption method

After 24 hrs of *E. faecium* CMUL20-2 culture, the pH of MRS broth was 6.0. A centrifugation of CMUL20-2 broth was performed directly at 15364 × g. The pellet was inoculated in sterile PBS (pH= 7.0) without any nutriments, for 15 min and 18 hrs followed by a centrifugation step (15364 × *g* for 15 minutes at 4 °C) and the supernatants were tested.

Total purification and determination of the molecular weight of enterocin CMUL20-2 by using LC-ESI-MS/MS

The purification of enterocin CMUL20-2 was performed by using RP-HPLC (Agilent/Bruker, Germany) using C18 column (Supelco-58419, Sigma-Aldrich, Germany). The direct supernatant of "PBS-CMUL20-2 strain" – obtained from adsorption-desorption section- was introduced into RP-HPLC which achieves few different fractions. Each fraction was collected and tested for antilisterial activity. In order to determine the molecular weight of enterocin CMUL20-2, Mass spectrometry was performed by using LC-ESI-MS/MS techniques (Bruker, Germany).

Characterization of enterocin CMUL20-2

Enterocin CMUL20-2 stability at different temperature, pH and during storage

The influence of temperature on purified enterocin CMUL20-2 stability was determined at 37, 50, 75 and 100 °C by using temperature-controlled water bath for 4, 15, 30, 60, 90 and 120 min. In addition, the enterocin was assessed under autoclaving condition (121 °C, 1 atmosphere, 30 min) [17]. The effect of pH on bacteriocin stability was determined by adjusting the pH of purified enterocin CMUL20-2 to various pHs (2 to 12) using 2 M HCl and/or 2 M NaOH. The samples were then incubated at 30 °C for 4 hrs, the pH was readjusted back to 7.0. For storage stability, the enterocin was tested under different storage temperatures (-20, 4 and 37°C) [18].

Inhibitory spectrum of the purified enterocin CMUL20-2

In order to determine the antimicrobial spectrum of enterocin CMUL20-2, Gram positive (*L. monocytogenes* and *S. aureus*) and Gram negative bacteria (*Escherichia coli (E. coli), Salmonella Typhimurium* (*S. Typhimurium*) were tested using the agar well diffusion method. While for fungal procedure, few conidia (without mycelium) were taken from culture plate and then poured in 0.9% NaCl suspension. After dilution, the conidia concentration was determined by using a cell counter under microscope. Once the suspension was ready (10^6 conidia/ml), a sterile swab was immersed in suspension and then the excess was removed by pressing the swab at the edges.

Time-kill curve assay of the purified enterocin CMUL20-2 against L. monocytogenes in BHI broth and pasteurized milk

The time-kill curve assay was carried out in order to study the bactericidal activity of purified enterocin CMUL20-2 against *L. monocytogenes* during different intervals time (0, 2, 4, 8, 10 and 12 h). 150 µl of purified enterocin CMUL20-2 were added to 15 ml of BHI broth with an initial concentration of 3.2×10^4 CFU/ml of *L. monocytogenes*. In another test, an initial concentration of 2.0×10^4 CFU/ml of *L. monocytogenes* were added to 15 ml of pasteurized milk, and then 150µl of purified bacteriocin were added. The optical density was measured at 660nm at each time point. A co-culture between *L. monocytogenes* and CMUL20-2 strain (binary system 1:1) without pH regulation was performed. The initial concentration for each bacterium was 2.0×10^4 CFU/ml. This mixture was incubated aerobically at 35 °C, and the duration of incubation was: 0, 2, 4, 8, 10 and 12 h. Colony counts were performed by plating

100 µl of serial dilution of culture on differential agar medium RAPID L. Mono™ Chromogenic Media (Bio-Rad, Germany) followed by incubation at 37 °C for 24 h. CFUs on each plate were counted and average CFU/ml was calculated.

Strain safety assessment

Hemolysis test

Ten microliters of overnight liquid culture were added in MRS broth and incubated overnight at 37°C. A loopful of the new liquid culture was streak on Columbia base agar amended with sheep blood, then incubated anaerobically at 37°C for 16 hours. A strain of *Staphylococcus aureus* ATCC ® 25923™ was used as positive control.

Antibiotic susceptibility testing

Disc diffusion method was used to screen for the antibiotic susceptibility of *E. faecium* CMUL20-2 by using 24 antibiotics (Bio-Rad, France). Tests were done according to the criteria of the National Committee of Clinical Laboratory Standards (NCCLS) using Müller-Hinton agar. Diameters of inhibition zones were measured after anaerobic incubation at 37°C for 24 hrs [19].

Screening for the presence of virulence genes

In order to evaluate the safety of the CMUL20-2 strain, several virulence genes have been targeted using conventional PCR according to [20]. The total DNA extraction was carried out byusing QIAamp DNA minikit (Qiagen, Germany) according to the manufacturer's instructions. The target genes were *gelE* (gelatinase), *esp* (enterococcal surface of protein), *asal* (aggregation substance) and *hyl* (hyaluronidase) [21]. Three clinical strains from "Collection Microbiologique Université Libanaise (CMUL)" were used as positive control for the presence of virulence genes.

Results

Isolation of LAB and screening of enterocin activity

A total of hundred bacterial isolates were obtained from twenty four samples of goat's milk from different geographic sites in north of Lebanon. All of these bacteria shared characteristics of LAB; they were Gram positive, catalase negative and oxidase negative. All LAB were tested for their antimicrobial activity against *Salmonella Typhimurium* ATCC® 14028™, *L. monocytogenes* ATCC® 19115™, *E. coli* ATCC® 8739™ and *S. aureus* ATCC® 25923™ using agar well diffusion method as described before [22]. Only four LAB isolates (6-10, 14-1, 14-2 and CMUL20-2) have shown an antimicrobial activity against *L. monocytogenes* ATCC® 19115™. The CMUL20-2 isolate showed the strongest anti-listerial activity (23 mm of diameter of inhibition zone).

Characterization of antimicrobial substances in crude supernatant

Proteinase K treated supernatant lost its anti-listerial activity, whereas in presence of lipase and amylase the activities persisted indicating the proteinous nature of this substance.

Strain identification

The strain CMUL20-2 was Gram positive cocci and didn't exhibit a catalase activity. Other parameters were evaluated such as the ability of growth at different temperatures. The CMUL20-2 strain was able to grow at 6.8% of NaCl but not at 10 and 18% of NaCl. Moreover, this strain could grow at various pH values (4, 5, 6, 7, 8 and 9) but not at pH 2 and 3. Based on the route for identification of LAB, the strain CMUL20-2 was identified as *Enterococcus* spp., [23]. The molecular identification identified the active strain as *E. faecium*by using pyrosequencing method as described before [15].

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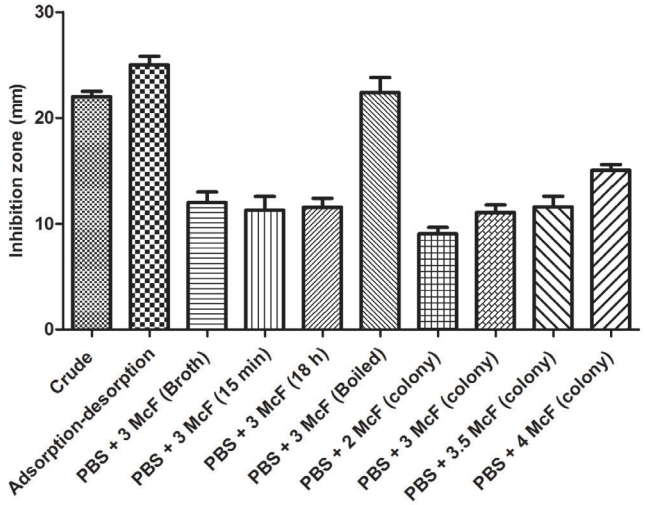
Figure 1: Inhibition zone diameters resulted from different conditions of extracted enterocin CMUL20-2.



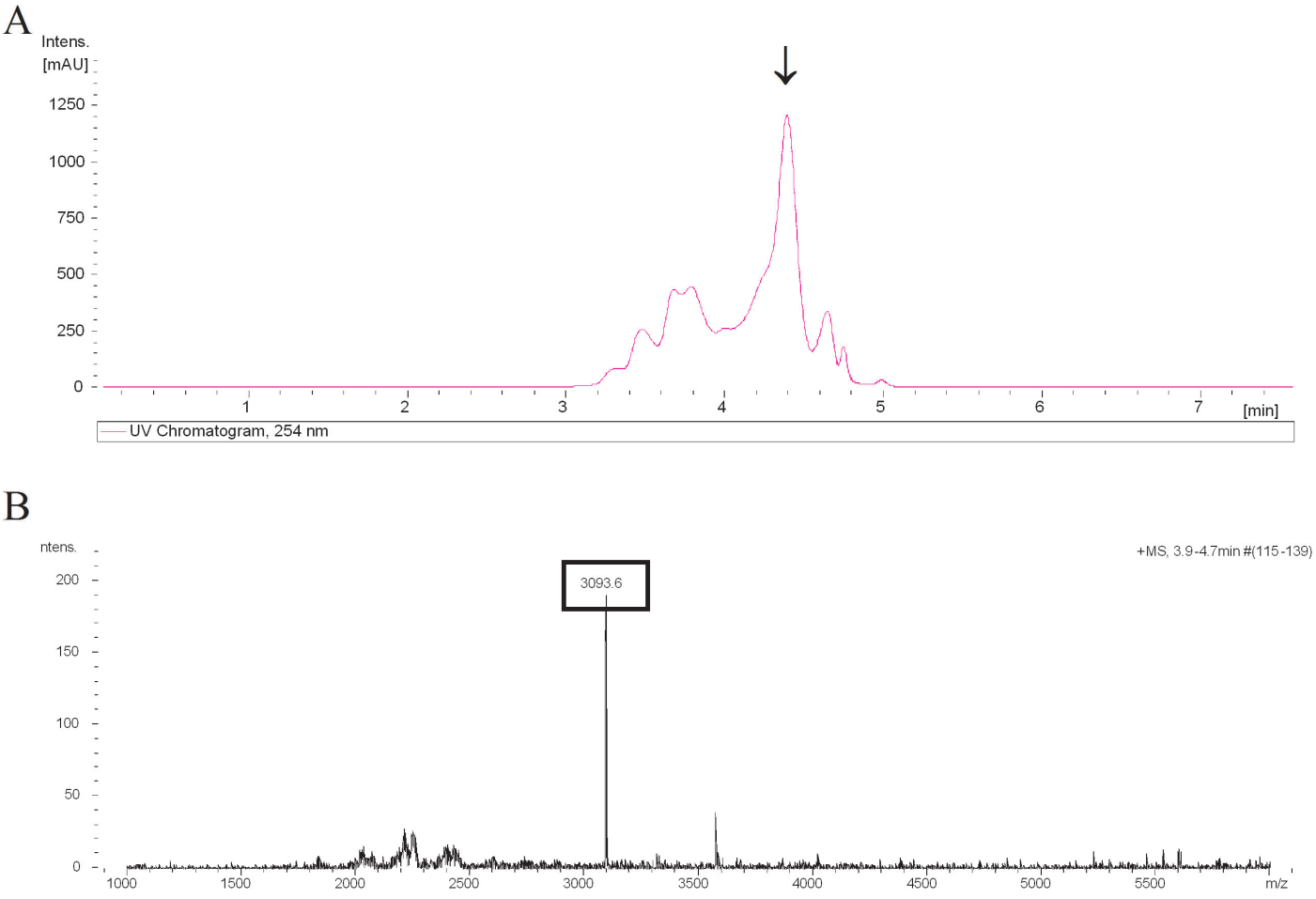
New strategy of enterocin CMUL20-2 purificationand determination of the mass and charge

After the modified method of extraction, the inhibition zone diameter increased from 22 mm recorded for crude enterocin CMUL20-2 to 25 mm and the activity units increased from 5120 AU/ml to 10240 AU/ml.

Purification and molecular weight determination

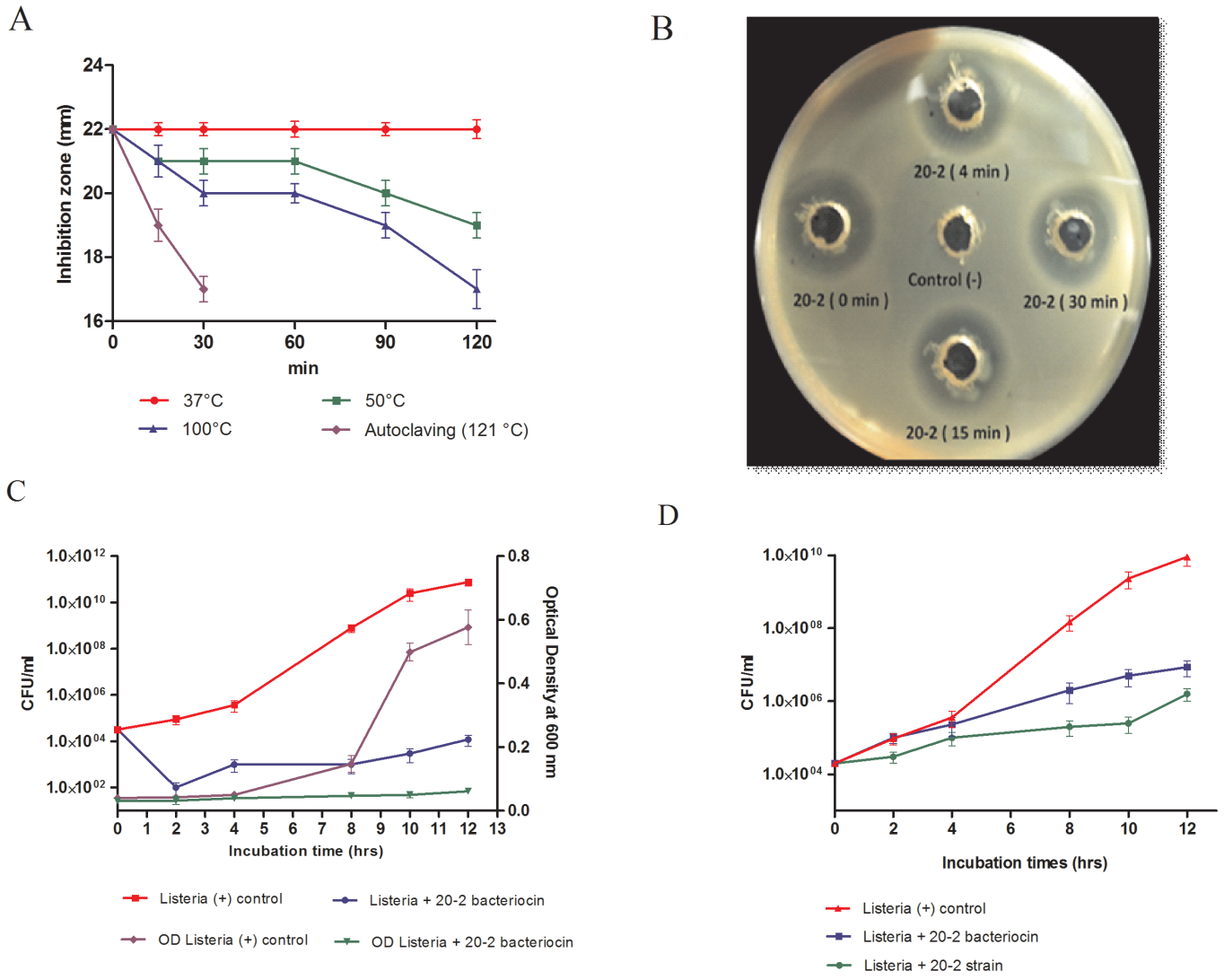
The enterocin CMUL20-2 has been eluted at 25 % of acetonitrile concentration by RP-HPLC. As shown in **Figure 2A**, the retention time was at 4.7 min. The molecular weight of enterocin CMUL20-2 was 3093.6 Da **(Figure 2B)**.

Figure 2.



A. RP-HPLC profile indicating the retention time of enterocin CMUL20-2. B. molecular weight determination of enterocin CMUL20-2 by LC-ESI/MS.

Figure 3.



A. Enterocin CMUL20-2 stability under different temperature with time; B. Enterocin CMUL20-2 stability under autoclaving (121 °C) with time; C. Time kill curve of L. monocytogenes ATCC® 19115™ treated with CMUL20-2 bacteriocin in BHI broth; D. Time kill curve of L. monocytogenes ATCC® 19115™ treated with CMUL20-2 strain and it's bacteriocin in pasteurized milk.

Characterization of enterocin CMUL20-2

Effect of different temperatures on purified enterocin CMUL20-2 stability

As shown in **Figure. 3A**, the effect of different temperature on purified enterocin CMUL20-2 activity was evaluated by measuring the inhibition zones diameters. It has been found that the tested enterocin was thermostable; it withstands high temperature up to 121°C, although a partial loss in the activity was observed with a continuous increase in the time of exposure to high temperature **(Figure 3A)**. Antibacterial activity of enterocin CMUL20-2 was detected after 15 and 30 min at 121°C (autoclave), indicating that the bacteriocin is highly heat stable **(Figure 3A, 3B)**.

Stability of purified enterocin CMUL20-2 during storage and at different pH

The storage of purified enterocin produced by CMUL20-2 strain showed a high stability at –20 and 4°C for at least six months. The antimicrobial

activity of bacteriocin expressed as inhibition zone diameter was 20 mm after 2 months at -20 °C; however, it was 18 mm after 2 months at 4 °C. Enterocin CMUL20-2 was stable at different pH values except pH 11 and 12.

Inhibitory spectrum of the purified bacteriocin

The susceptibility of various microorganisms to enterocin CMUL20-2 were presented in **Table 1**. Enterocin CMUL20-2 showed an antibacterial activity against *L. monocytogenes* ATCC® 19115™ and *L. monocytogenes* isolated from food (hotdog), *Streptococcus bovis* isolated from clinical sample and *Clostridium perfringens* isolated from meat sample. Furthermore, this enterocin showed high antifungal activity against *Penicillium* spp, however it didn't show any activity against *Aspergillus flavus*.

Table 1. Inhibitory spectrum of the purified enterocin CMUL20-2.

Indicator organisms	Strain N°	Purified enterocin CMUL20-2
L. monocytogenes	ATCC® 19115™	++
L. monocytogenes	Food strain	+++
L. innocua	ATCC® 33090™	++
Streptococcus bovis	CMUL 425	+
Staphylococcus aureus	ATCC® 25923™	-
Staphylococcus aureus	CMUL 052	-
Enterococcus faecalis	CMUL 624	-
Enterococcus faecium	CMUL 613	-
Clostridium perfringens	ATCC® 13124	+++
Bacillus cereus	ATCC® 11778™	-
Lactobacillus acidophilus	CIP 7613	-
Lactobacillus brevis	CIP102806	-
Escherichia coli	ATCC® 8739™	-
Salmonella typhimurium	ATCC® 14028™	-
Pseudomonas aeruginosa	CMUL 297	-
Brucella spp.	CMUL 031	-
Campylobacter jejuni	CMUL 016	-
Penicillium spp.	CMUL 127	++
Aspergillus flavus	CMUL 051	-
Candida albicans	CMUL 15	-

Time-kill curve of *L. monocytogenes* ATCC® 19115™ treated with the purified enterocin CMUL20-2 in BHI broth and pasteurized milk

The time course of killing activity using time kill curve assay was performed. The viable count recorded as CFU/ml for *L. monocytogenes* ATCC® 19115™, was plotted against the exposure time of the bacterial cells to purified enterocin CMUL20-2. The changing in CFU/ml was compared to a positive control (untreated bacterial cells). Time-kill curve analysis for the bacterium treated with purified enterocin CMUL20-2 showed that the count of *L. monocytogenes* was rapidly reduced from 3.2×10^4 CFU/ml to 1×10 CFU/ml after 2 hrs of incubation and then increased slowly to 1.2×10^4 CFU/ml after 12 hrs comparing to positive control (7.60×10^{10} CFU/ml). On the other hand, the $OD_{660\ nm}$ of the *L. monocytogenes* test tubes was 0.06 after 12 hrs of incubation which was lower than the positive control (OD_{660nm}=0.58) **(Figure 3C)**. Time-kill curve analysis for the bacterium treated with purified bacteriocin in pasteurized milk showed that the count of viable *L. monocytogenes* in test tube increase of 2 log, from 2.20×10^4 CFU/ml to 1.60×10^6 CFU/ml after 12 hrs incubation. However, in the positive control an increase of 5 log was observed to rich a final concentration of 9.0×10^9 CFU/ml. The same result was obtained when a co-culture between *L. monocytogenes* and CMUL20-2 strain in pasteurized milk was carried out. The concentration of *L. monocytogenes* in co-culture was increased slowly from 2.2×10^4 CFU/ml to 8.7×10^6 CFU/ml after 12 hrs of incubation in comparison with the positive control (9.0×10^9 CFU/ml) **(Figure 3D)**.

After 12 hrs of incubation, the CMUL20-2 strain, has slightly acidified the co-cultured milk (pH from 6.5 to 5.5).

Strain safety assessment

Hemolysis activity, resistance to antibiotics and detection of virulence genes

E. faecium CMUL20-2 didn't exhibit any hemolytic activity. Moreover, *E. faecium* CMUL20-2 was susceptible to ciprofloxacin, amoxicillin, vancomycin, doxycycline, gentamicin, imipenem and moxifloxacin and resistant to rifampin, bacitracin and optochin. The PCR detection of virulence gene revealed that *E. faecium* CMUL20-2 didn't harbor any of the virulence genes tested.

Discussion

The new strategy of extraction and purification was based on the adsorption-desorption capacity of some bacteriocinogenic strains. According to the study of Yang *et al.,* 1992, the neutralization of broth facilitate the adsorption of bacteriocin on the producing strain cell wall. In contrast, the acidic environment allows desorption of the adsorbed bacteriocins. A remarkable increasing of antimicrobial activity of enterocin CMUL20-2 was shown after extraction of crude bacteriocin by classical adsorption-desorption method. In the present work (modified method), the pellets issued from MRS broth (pH=4.5) were inoculated directly in neutral PBS (adjusted to 3 McFarland) without neutralization and then, the supernatant of inoculated PBS was taken without acidification step. As shown in **Figure 1**, the aforementioned supernatant showed 12 mm of inhibitory zone diameter. These results suggested that the enterocin CMUL20-2 have been adsorbed on the producing strain (in acidic MRS broth) and then desorbed in the neutral PBS which is slightly different to the classical method. In order to determine the possibility of producer strain to secrete its enterocin in PBS solution, the producer strain was cultured in neutral PBS and incubated for 15 min and 18 h separately. The anti-listerial activity showed that no significant difference of enterocin concentration between the two incubation times (11.2 mm and 11.5 mm respectively). Another hypothesis is that the enterocin didn't desorbed from producer strain, but the producer strain cells was lysed in PBS. However, the supernatant of boiled PBS containing producer strain showed an increasing of inhibitory zone diameter = 22.4 mm. This increasing of antilisterial activity may be related to the increasing of active enterocin. This can be due to the additional activity of pre-enterocin (intracellular enterocin) which may be active as reported before by Ray et al., [24]. They showed that the pre-pediocine Ach was also active. The additional activity can be due also to pre-enterocin which has been heat cleaved and activated after boiling. In order to confirm the adsorption of enterocin on the producer strain's cell wall, different concentrations of producer strain (2, 3, 3.5 and 4 McFarland) were prepared in neutral PBS from colonies cultured in MRS agar and not from broth. As shown in **Figure 1**, the activity of PBS supernatants varied from 9 to 15 mm. These results indicated that the activity is proportional to the cell concentration of producer strain. This present work suggests that CMUL20-2 strain strongly adsorbed the enterocin at acidic and neutral pH.

Enterocin desorption was not related to pH value only, but the lack of nutriment seems to be a main condition to desorb this enterocin which is different to the classical hypothesis[16]. In general, the producer strain which can adsorb its bacteriocin will release it in a stressful environment such as acidic environment and thus, it is an indicator of the presence of live microorganisms which take nutriments and secrete organic acid. In our case, the CMUL20-2 strain releases its enterocin in neutral PBS. Therefore, the only explanation is that the producer strain was also present in stressful envi-

ronment lacking nutriments which is also an indicator of microorganism presence. Thus, the use of direct colonies of producer strain from MRS agar seems to be very effective strategy to purify the enterocin in only two steps: PBS-colonies followed by RP-HPLC.

Besides the modified strategy of extraction and purification, the present study also showed that enterocin CMUL20-2 exhibit a good activity against some foodborne pathogens as well as spoilage micro-organisms. In addition, this enterocin can resist to several stressful conditions such as high temperature, pressure and acidic environment.

Conclusions

This study proved that the present purified enterocin CMUL20-2 is very active AMP which can inhibit pathogenic foodborne bacteria and fungi. Besides his antimicrobial activity, enterocin CMUL20-2 showed a high stability under several industrial conditions such as high temperature, acidic environment and storage conditions and also in a complex food product like pasteurized milk. Moreover, the producer strain showed a safety status and thus has a potential for food application especially in food preservation. We suggest that this new discovered Lebanese enterocin with its producing strain can be used as safe biopreservative in many food processes.

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Conflict of Interest

No conflict of interest.

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