Characterization of antibacterial lipopeptide produced by *Bacillus mojavensis* 5RCN isolated from poultry guts and its activity against *C. perfringens*

Nadjib. CHERIF [*1], Hiba R. CHIBANI[1], Fatima Zohra. TAHARI[1]

1. Departement of Biology, University Of Ain Temouchent, Algeria

**e-mail:*nadjib.cherif@univ-temouchent.edu.dz

Abstract:

Members of the *Bacillus* genus are considered as the factories for the production of biologically active molecules that are potential inhibitors of growth of pathogens.Antimicrobial lipopetides have the potential to play an important role in preventing the rapid development in bacteria resistance to conventional antibiotics.Bacterial strains were isolated from poultry intestines and then screened to identify the *Bacillus* genus producing surfactins. These molecules were purified and characterized using ESI MS / MS.Finally, the antibacterial effectiveness of these surfactins against *C. perfringens* was assessed.A poultry intestinal *Bacillus mojavensis*5RCN with broad-spectrum antibacterial activity has been identified. The successful extraction of an effective antimicrobial compound from the fermentation media has been accomplished.During the primary screening, a strain that produced antimicrobial lipopetides and exhibited significant hemolytic activity, was isolated and identified. This identification was achieved through genotypic and morphological characterization. Moreover, ESI MS/MS analysis of the antibacterial molecule showed that it contained cyclic lipopeptides with C13, C14, C15, C16, and C17 acyl chains, and revealed the occurrence of most abundant surfactin homologues (1006, 1021, 1034, 1048, and 1062 m/z). The isolated surfactin displayed potent antibacterial activity against *Clostridium perfringens*. In light of this study, *Bacillus mojavensis*5RCN strain and its effective antibacterial surfactin lipopeptide could be utilized in a variety of food production and could have promising medical applications.

Keywords:*Bacillus mojavensis*, antimicrobial lipopeptide, surfactin, ESI MS/MS, *Clostridium perfringens*,

Introduction

Bacillus spp. produce the majority of the unique family of natural antimicrobial peptides known as lipopeptides (LPs) through NRPS pathways. It is clear that LPs have been extensively utilized in the domains of agriculture and medicine. For instance, the FDA has authorized the use of lipopeptides produced by *Paenibacillus polymyxa*, such as polymyxins and daptomycin, as antibiotics to treat diseases caused by multidrug-resistant superbugs [19].Surfactins, are the most studied and effective lipopeptide, due to their high ability to reduce the surface tension of water, and their antimicrobial effect.Surfactin isomers have received much attention during the last two decades since they exhibit numerous pharmaceutical activities including anticoagulation, anti-tumor, antiviral, anti-inflammatory, and immunosuppressive activities[17].Surfactin is a lipopeptide synthesized by the genus *Bacillus*. It consists of two types of acidic amino acids (glutamate and aspartate), five non-polar amino acids (leucine and valine) linked to a chain of β-hydroxy fatty acids C12-C19[1]. Surfactin also have interests in several fields, they are used as antimicrobial, antiviral and antitumor molecules and are widely used in the oil industry, agriculture as biopesticides, agri-food, pharmaceuticals and parapharmaceuticals. Antimicrobial lipopeptide have the potential to play a promising role in the fight against the rapid increase in microbial resistance to conventional antibiotics [13]. Other antimicrobial lipopeptides include fengycin, iturin, bacillomycins and mycosubtilins produced by *B. subtilis*. The other antimicrobial lipopeptides are lichenysin and pumilacidin, produced by *Bacillus licheniformis* and *Bacillus pumilus*respectively [4]. This work describes the production of surfactin by *B. mojavensis*isolated

from poultry intestines, then extracted, purified, and characterized using electrospray ionization MS (ESI MS/MS).The antimicrobial activity of surfactin against *Clostridium perfringens* was also demonstrated.

Materialsandmethods

Isolation of bacteria and culture condition. The microorganism used in this study was isolated from the intestine of poultry from a production unit in the city of Remchi - Algeria and kept in the culture collection of the pedagogic laboratory of the University of Ain Témouchent (Algeria). Samples were collected from healthy chickens with good performance. After slaughtering the chickens under aseptic conditions, 10 grams of intestines were taken from various locations. These samples were then mixed with 90 ml of sterile physiological water and vigorously shaken for 5 minutes to dilute the mucus contents. The quantity of 1 ml or 1 g of each sample was diluted in sterile-distilled water. A volume of 1 ml from dilutions 10^{-9}, 10^{-8}, 10^{-7}, 10^{-6} and 10^{-5} were plated on nutrient agar. After incubation at 37°C for 24 hours, distinct colonies were selected based on colony morphology on a plate and were sub-cultured three times on fresh plates to obtain single colonies from one bacterial species.A screening process was conducted to identify surfactin-producing strains. The isolated bacteria were cultivated on blood agar plates containing 5% (v/v) human blood and then incubated at 37°C for 48 hours. Hemolytic activity was observed as a clear zone around the bacterial colonies, indicating the presence of surfactin production.

Strain characterization. After morphological identification as a gram-positive bacillus and some biochemical tests such as catalase, oxidase, hemolysis and other enzymatic tests (cellulase, amylase, protease), the phylogeny of the strain was determined by sequence analysis of the 16S rRNA gene amplified by PCR.

The 16S rRNA gene of *B. subtilis* was amplified by PCR using the following universal primers: FP RW01 5'AACTGGAGGAAGGTGGGGAT3' and RP DG74 5'AGGAGGTGATCCAACCGCA3'. The PCR program was set to one initial cycle of denaturation at 94°C for 5 minutes, 35 cycles of: 30 seconds denaturation at 94°C, 30 seconds annealing at 59°C, and 90 seconds extension at 72°Cand finally 10 min of extension at 72°C for one cycle.

Production of lipopeptide. For pre-culture, a colony of a pure culture was inoculated in a flask with 10 ml LB medium (5 g/Ltryptone, 10 g/LNaCl, 10 g/Lyeast extract) overnight then the young cells were used for inoculation of the main cultures, the inoculum was adjusted to an initial optical density of 0.1 (600 nanometres), 2 ml of this preculture was added to 25 ml of the following medium, (g/L): $NaNO_3$ (2.0), KCl (0.5), $Na_2HPO_4H_2O$ (1.0), KH_2PO4 (1.0), $CaCl_2$(0.025); $MgSO_4$ (0.1), $FeSO_4.7{\cdot}H_2O$ (0.001) and 2 ml/L trace element solution containing the following ingredients (mg/L): $FeCl_3{\cdot}6H_2O$ (60), $ZnSO_4{\cdot}7H_2O$ (600), $MnSO_4{\cdot}H_2O$ (200), $CuSO_4{\cdot}5H_2O$ (590), $CoCl_2{\cdot}6H_2O$ (60), pH 7.0,and incubated at 37° C for 48 hours on a rotary shaker 150 rpm.

Extraction and recovery of lipopeptide. Lipopeptide were extracted by centrifugation at 10.000 rpm and 4°C for 20 min. The supernatant was filtered with a 0.50 mm filter and then acidified to pH 2 with 1M H_2SO_4. Grey white pellets formed by precipitation were collected by centrifugation at 10.000 rpm at 4°C for 20 min. Then a volume (2:1) of chloroform:methanol was added, surfactin was recovered in the organic layer. The extraction was performed twice, and the organic layers were pooled and evaporated. The residue was re-dissolved in water and shaken well.

Purification of Lipopeptides. Lipopeptide extracts from our *Bacillus*strain were chromatographed on silica gel 60 columns (particle size 0.063-2mm), and eluted with 9:1 chloroform-methanol and methanol. Using thin layer chromatography (TLC), fractions were developed with $CHCl_3/CH_3OH/NH_4OH$ 2M (40:10:1 v/v), and spots were visualized using 0.2% ninhydrin in acetone followed by heating at 110 °C for peptides, and the lipophilic nature of the peptide was

confirmed by exposing the TLC plate with iodine vapour.

Electrospray ionization-mass spectrometry (ESI-MS/MS). Lipopeptide were subjected to analysis using mass spectrometer with electrospray ionization (ESI)quadrupole time of flight (Q-TOF) in positive ion mode, in a capillary column (Agilent Zorbax, 1.8 µm particle diameter), 10 µl of the prepared lipopeptide solution dissolved in methanol was absorbed. Two mobile phases were used for separation, mobile phase 1 containing 0.1% trifluoroacetic acid in water and mobile phase 2 containing 0.1% trifluoroacetic acid in 90% acetonitrile. The flow rate was set to 2 µl/min. For the electrospray ionization mass spectroscopy (ESI-MS/MS) spectra, the positive ion mode was used with a capillary voltage of 3 kV, a dry gas of 8.0 l/min, and a dry gas temperature of 250°C. Analysis of MS spectra was programmed to a full scan of the injected sample and recorded in positive ion mode in the mass range of 180 to 1700 m/z.

Antibacterial assay. To evaluate the antimicrobial effect of *Bacillus mojavensis* derived lipopeptide, well diffusion method was performed to determine the sensitivity pattern of *Clostridium perfringens* to this metabolite.*C. perfringens* strain was plated on Columbia blood agar, and incubated anaerobically overnight at 37°C. The resulting colonies of were suspended in 0.9% NaCl to achieve 0.5 McFarland standard. 0.1 ml of bacterial suspension was spread on Wilkins Chalgren agarWCA, *Bacillus mojavensis* derived lipopeptide were serially diluted (10^{-1} to 10^{-4}) and distributed on the wells of WCA and incubated anaerobically at 41°C for 24 hours. The diameter of the inhibition zone was measured and the sensitivity was determined.

Results

Screening and isolation of lipopeptide producing microorganisms. In primary screening, 70 bacterial isolates with distinct morphology were isolated as pure culture from guts chicken's samples and screened for hemolytic activity. The results revealed that out of all the isolates tested, only 21 isolates displayed significant hemolytic activity on blood agar (fig.1). Among these, isolate 5RCN, which was confirmed to be *Bacillusmojavensis*, exhibited the largest zone of hemolytic activity.

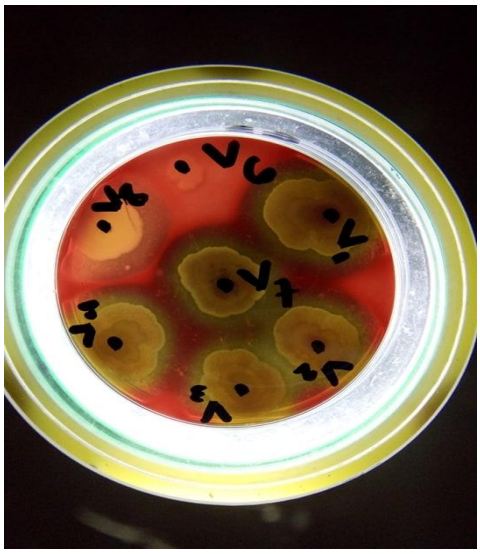

Fig. 1. Hemolytic activity of *Bacillus sp* isolates from chicken's guts.

Identification and taxonomic affiliation of the *Bacillus mojavensis*.The strain 5RCN was selected and identified as the producer of the highest amount of biosurfactants. This bacterial isolate is characterized as a rod-shaped, Gram-negative bacterium with negative oxidase and positive catalase properties.BLAST analysis was conducted using bioinformatics tools, which revealed a significant identity match with *Bacillus mojavensis*. This confirms that the strain 5RCN belongs to the species *Bacillus mojavensis* and suggests its potential role as a prominent producer of biosurfactants in the chicken gut.To determine the phylogenetic position of the 5RCN strain, a comparison of its 16S rRNA gene sequence with some other *Bacillus* strains available in the database was performed. According to Fig 02, the phylogenetic tree showed that the 5RCN strain was placed in a monophyletic group with *Bacillus axarquiensis*, *Bacillus mojavensis*, and *Bacillus subtilis*. The 16S rRNA gene sequence similarity between 5RCN and the type strains of *Bacillus*

axarquiensis, *Bacillus mojavensis*, and *Bacillus subtilis* was 99.8, 99.8, and 99.5%, respectively.

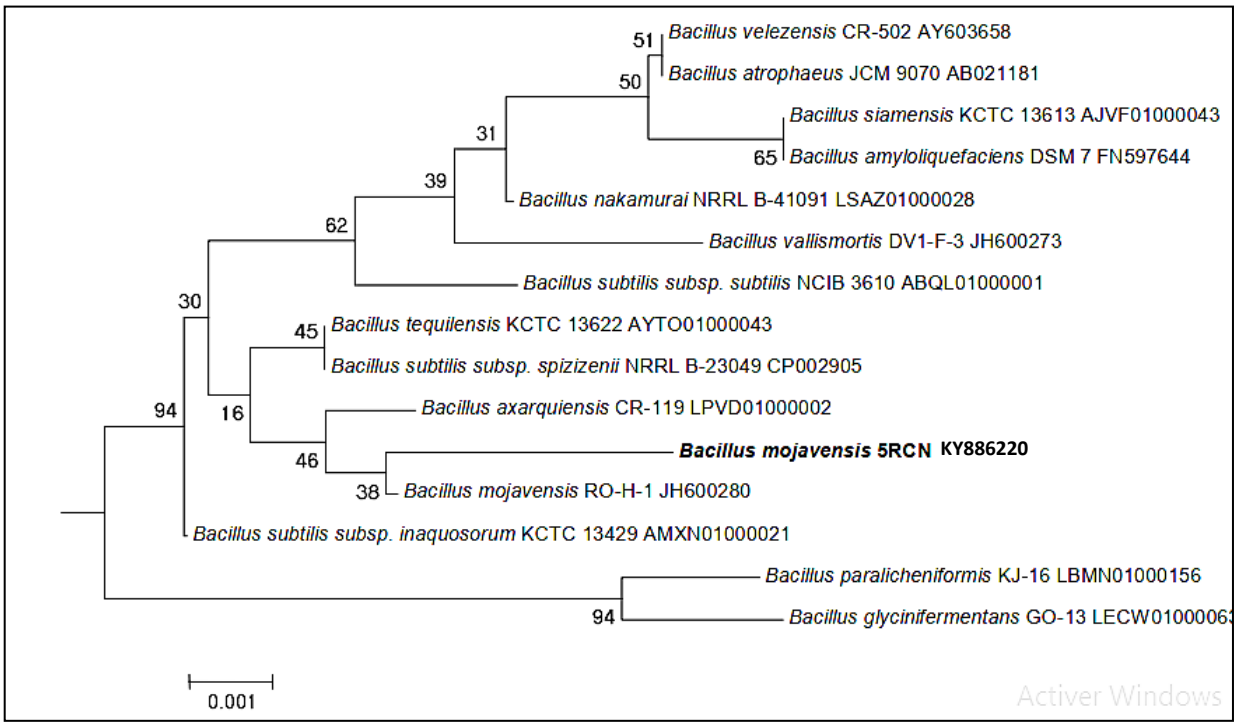

Fig. 2. 16S rRNA gene based phylogenetic tree showing affiliation of the *B. mojavensis* 5RCN strain with related species of the genus *Bacillus*.

ESI–MS analysis of lipopeptide extract. The surfactin isoforms were eluted based on their hydrophobicities using mass spectrometric data. Direct injection into an ESI-MS/MS was used for confirmation of the production of surfactin by *Bacillus mojavensis* 5RCN strain in both negative and positive ionization modes (fig.3.a, b). Full negative scan mode was applied to detect deprotonated molecules of surfactin A at m/z 1006, 1020, 1034, 1048 and 1062 (fig.3.b). Peaks of sodiated surfactin isomers with a difference of one CH_2 group and a mass of 14 Da were observed in full positive scan mode at m/z 1030, 1044, 1058, 1072 and 1086 (fig.3.a). Table 1 presents all observed surfactin homologue product ions and their m/z values.This analysis confirmed the presence surfactin in the extract of fermentation medium of *Bacillus mojavensis* 5RCN.This analysis confirmed the presence of three main surfactins, which were respectively, C13 surfactin, C14 surfactin, C15 surfactin, C16 surfactin and C17 surfactin. The first three molecules correspond to the same surfactin isoform, denoted by Leu at position 7 of the amino acid sequence, as evidenced by the equal product ions of the peptidic product ions series. The primary parent ions identified at m/z, 1030, 1044, 1058, 1072 and 1086 were attributed to a little quantity of the sodium adduct of a valine-7 surfactin for the huge peak appearing in fractions in front of the five mainpeaks. The surfactin peptide sequence was determined by analyzing the ESI-MS/MS spectrum of the precursor ions m/z 1006, 1021, 1034, 1048, and 1062, assuming preferential breaking of the lactone bond in the collision chamber.

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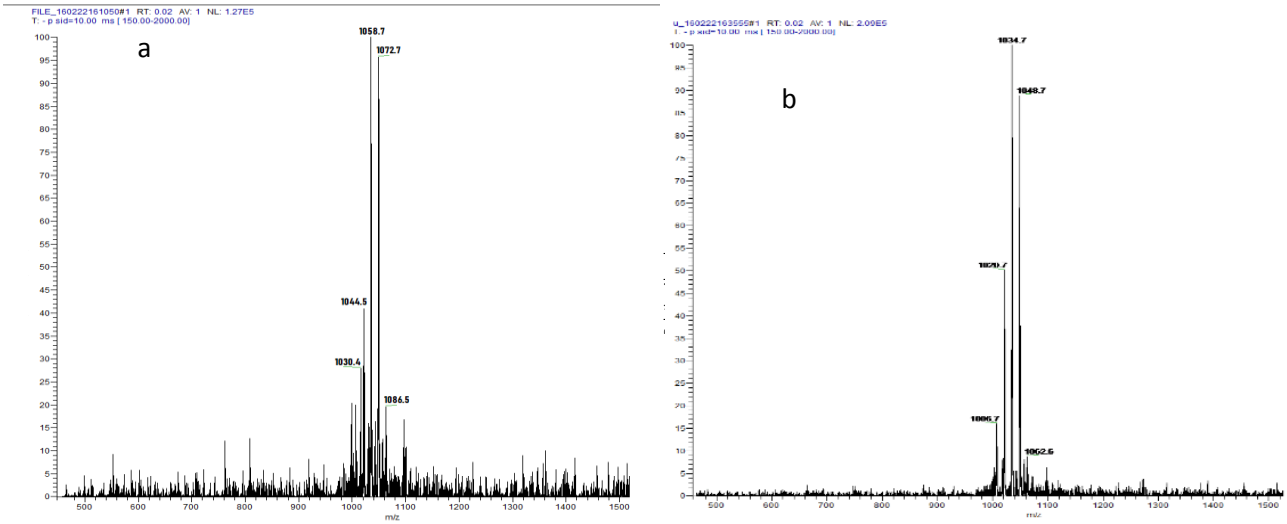

Fig. 3.ESI/MS analysis of the extract from *B. mojavensis*5RCNstrain. **a.** Chromatograms of the surfactin, positive ion mode. **b.** Chromatograms of the surfactin positive ion mode.

Table 1. Surfactin isomers produced by *B. mojavensis* 5RCN strain detected by ESI- MS/MS

Surfactin acyl chain length	**C-13**	**C-14**	**C-15**	**C-16**	**C-17**
Exact Mass	1007	1021	1035	1049	1063
$[M+Na]^+$	1030	1044	1058	1072	1086
$[M-H]^-$	1006	1021	1034	1048	1062

Antimicrobial Activity of surfactin. In order to further clarify the activities of antimicrobial agents produced by *Bacillus mojavensis* 5RCN, the extract of surfactin were tested against *C. perfringens*. According to the results obtained, as shown in fig.4, the *Bacillus mojavensis* 5RCN derived lipopeptide was effective against *C. perfringens*.The surfactin extract was able to inhibit the pathogens with a zone of inhibition diameter of 2.4 cm. Moreover, dissolving lipopeptide extracted from *B. mojavensis* 5RCNin methanol increased its antibacterial activity, as evidenced by an increase in the zone of inhibition against*C. perfringens*. The zone of inhibition was the largest for the surfactin dissolved in methanol, with 2.9 cm. Methanol increased the antibacterial activity of this kind of lipopeptide. The agar well diffusion method revealed no antibacterial activity of methanol or ethyl acetate against the bacterium.

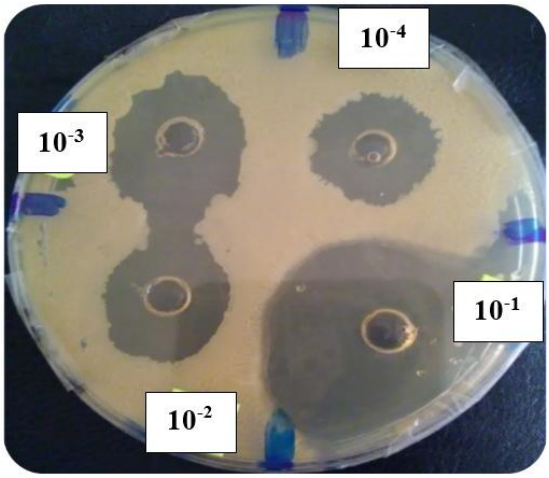

Fig.4. Antimicrobial activity of*Bacillus mojavensis* 5RCN derived lipopeptide (surfactin) against *C. perfringens*.

Discussion

One of the most major enteric problems in chicken, necrotic enteritis that has a significant financial impact on the global poultry business. It is caused by *C. perfringens*, which produce toxins [8,16]. Lipopeptides have a well-known capability for preventing infections, and *Bacillus* has been widely acknowledged as a bio-control agent that may be used safely in the food sector. [6,9,18]. In the fight against the sharp rise in microbial resistance to traditional antibiotics, antimicrobial peptides AMPs have a promising future [13]. First discovered in *B. subtilis*, surfactin is a secondary metabolite that has a structure consisting of a seven-amino-acid peptide loop and a hydrophobic fatty acid chain. It exists in a variety of isoforms. Among them, *B. subtilis* and *B. amyloliquefaciens* are often added to poultry feed, which can promote gut health for better growth performance of broilers by competitive exclusion and producing antimicrobial substances to reduce pathogens and coccidiosis[10].

The aim of this study was to identify potent antimicrobial peptides (AMPs) producing bacteria. In a primary screening, we isolated and identified an antimicrobial lipopeptide producing strain *B. mojavensis* 5RCN by genotypic, morphological characterization as it displays an important hemolytic activity. Due to their amphiphilic character, surfactins are capable of causing hemolysis, on blood agar plates, surfactin-producing bacteria are confirmed using minimum hemolytic concentration (MHC) values [15]. In this study, about 3% of SPB strains' hemolytic activity was present.

According to research of Hong et al. 2021, there are six isoforms of the commercial surfactin (Sigma-Aldrich) derived from *B. subtilis*. The results from ESI- MS/MS analysis demonstrate that this strain produces isomers of surfactin with, C13, C14, C15, C16 and C17 acyl chains. Five possible isoforms of the surfactin from *B. mojavensis* 5RCN were found, and they had different structures when combined with the ions Na+, K+, and Ca2+. Their protonated masses were 1006, 1021, 1034, 1048, and 1062 m/z. (table 01; fig.3) which is in accordance with previous studies [1,12,15]. These results demonstrate that surfactin purified from *B. mojavensis* 5RCN was identical to surfactin produced by *B. subtilis* and *B. amyloliquefaciens*, according to information from the examination of the amino acid composition [7,20].

Necrotic enteritis is caused by *Clostridium perfringens* and primarily affects chickens. Recently, the search for antibiotic alternatives capable of improving the gut health and immune status of poultry has intensified [5]. According to several studies, *B. subtilis*-fermented products with the highest surfactin concentration exhibit antibacterial activity in vitro against *Escherichia coli*, *Staphylococcus aureus*, *Salmonella typhimurium*, and *C. perfringens*. Surfactin produced by various strains of *B. subtilis* also exhibited a spectrum of antibacterial activity [3,14]. Jia Lv et al, 2020 report that three strains of *C. difficile* were shown to be antagonistic by *Bacillus* produces lipopeptide with an inhibition zone varied from 7.05 mm to 22.00 mm, the same result obtained in our study from where the *Bacillus mojavensis* 5RCN derived lipopeptide was effectiveagainst *C. perfringens*.

The surfactin extract was able to inhibit the pathogens with a zone of inhibition diameter of 2.4 cm. While being purified, organic solvents such as methanol, ethanol, acetone, chloroform, and ethyl acetate had no effect on the lipopeptid's antibacterial activity, indicating its solvent stability [2]. Surfactin may have an effect on cell membranes similar to that of a detergent because in previous studies, its

biological activities involved interactions with cellular membranes [7]. Systematic analysis of the antibacterial mechanism revealed that the surfactin damages the permeability barrier and integrity of the cell wall and cell membrane, which results in the death of *Clostridium* cells [11].Surfactins have many benefits over chemically created antimicrobial substances, including being less toxic, extremely biodegradable, and maintaining antagonistic activity against many microorganisms at a range of pH levels and high temperatures [15].

Conclusion

Bacillus isolated from poultry intestines has a significant capacity to produce lipopeptides. Wefind that *B. mojavenss* 5RCN and their surfactin formulations have strong antibacterial action against *C. perfringens*.ESI MS/MS showed five possible isoforms of the surfactin from *B. mojavensis* 5RCN with different acyl chains (C13 to C17). More research is needed to determine surfactin's *in vivo* cytotoxic effects and the precise chemical mechanism(s) of action.

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