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# Antimicrobial compounds from the mucus of garden snail *Cornu aspersa*

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**Abstract:** Natural products have long played a major role in medicine and science. The garden snail *Cornu aspersa* is a rich source of biologically active natural substances which might be an important source for new drugs to treat human disease. Based on our previous studies seven fractions containing compounds with Mw <3 kDa, <10 kDa, <20 kDa, >20 kDa, and between 3-5 kDa, 5-10 kDa, and 10-30 kDa were purified from the mucus of *C. aspersa* and analyzed by tandem mass spectrometry (MALDI-TOF/TOF). Seventeen novel peptides with potential antibacterial activity have been identified by *de novo* MS/MS sequencing using tandem mass spectrometry. The different fractions were tested for antibacterial activity against Gram<sup>−</sup> (*Pseudomonas aureofaciens* and *Escherichia coli*) and Gram<sup>+</sup> (*Brevibacillus laterosporus*) bacterial strains as well anaerobic bacterium *Clostridium perfringens*. These results revealed that the peptide fractions exhibit a predominant antibacterial activity against *B. laterosporus*, the fraction with Mw 10 – 30 kDa against *E. coli*, another peptide fraction <20 kDa against *P. aureofaciens*, and the protein fraction >20 kDa against the bacterial strain *C. perfringens*. The discovery of new antimicrobial peptides (AMPs) from natural sources is of great importance for public health due to their effective antimicrobial activities and low resistance rates.

**Keywords:** *Cornu aspersa* mucus; antimicrobial peptides, antibacterial activity; bacterial strains: *Pseudomonas aureofaciens* AP9, *Escherichia coli* NBIMCC 8785, *Brevibacillus laterosporus* strain BT-271, and *Clostridium perfringens* NBIMCC 8615

## 1. Introduction

Antimicrobial resistance is a major public health problem which requires scientists and clinicians to identify new efficient antimicrobial agents [1,2]. The World Health Organization (2017) composed a list of antibiotic-resistant bacteria for which the development of novel antimicrobial therapies is highly requested, with the global priority on *Acinetobacter baumannii*, *Pseudomonas aeruginosa*, and various species of the *Enterobacteriaceae* family [3].

AMPs are important components of the innate immune system providing immediate response to the large set of various pathogens such as bacteria, yeasts, fungi, viruses, and even cancer cells [4-8]. These naturally occurring evolutionarily conserved peptides have been found virtually in all

organisms ranging from prokaryotes to humans and display a remarkable structural and functional diversity [5]. Conventional AMPs with molecular masses of 1.5 to 8 kDa are positively charged, with ~15 to 60 amino acid residues and >30% hydrophobic residues [9,10]. AMPs are commonly classified based on their secondary structure into  $\alpha$ -helical,  $\beta$ -sheet, or extended/random-coil structure, and most AMPs belong to the first two groups [10].

Since invertebrates lack the adaptive immune system found in vertebrate species, they are reliant solely upon their innate immune systems to counteract invading pathogens. Considering the extraordinary evolutionary success of this group of organisms, it is evident that invertebrate innate immune mechanisms are extremely effective [11]. This has prompted intense studies of invertebrate species in last years. Most of the AMPs found in the hemolymph of invertebrates show activity against a mix of microorganisms including bacteria, viruses and protozoa [12-14].

Novel proline-rich antimicrobial peptides with molecular masses between 3000 and 9500 Da from the hemolymph of *Rapana venosa* snails were identified [15]. Some of them showed strong antimicrobial activities against *S. aureus* (Gram<sup>+</sup>) and low activity against *K. pneumonia* (Gram<sup>-</sup>). Several cysteine-rich peptides belonging to the defensin family also were purified from the Mediterranean mussels like myticin from *Mytilus galloprovincialis* [16-18]. They are expressed at high levels in hemocytes and are characterized by multiple disulfide bridges which ensure a precisely folded stable structure for the compact cationic and amphipatic mature peptides. Furthermore, it has been reported that several peptides from the hemolymph of the garden snail *H. lucorum* and *H. aspersa* exhibit a broad spectrum of antimicrobial activity against *S. aureus*, *S. epidermidis*, *E. coli*, *Helicobacter pylori*, and *Propionibacterium acnes* [19,20]. *Cornu aspersum* and *Helix aspersa* are two alternative names for the same species of snail. The taxonomically correct name is *C. aspersum*, but the previous name *H. aspersa* is used more widely.

Moreover, the mucus of land snails is a rich source of peptides and proteins with broad-spectrum antibacterial activity. Results indicated that the mucus fraction with Mw between 30-100 kDa from the common brown snail *H. aspersa* has a strong antibacterial effect against several strains of *P. aeruginosa* [14]. The identified proteins in *Cornu aspersum* with masses 37.4 kDa, 18.6 kDa, and 17.5 kDa appear also to have activity against *P. aeruginosa* [21]. Additionally, two proteins isolated from the mucus of the African giant land snail *Achatina fulica* were reported to display a broad spectrum of antibacterial activity against *S. aureus* and different strains of *P. aeruginosa* [22]. Furthermore, a novel cysteine-rich antimicrobial peptide mytimacin-AF with potent antimicrobial activity against Gram<sup>-</sup> and Gram<sup>+</sup> bacteria and the fungal strain *C. albicans* was isolated and purified from the mucus of the snail *A. fulica* [22]. Recently several peptide fractions with antibacterial activities [23,24] and antioxidant properties [25] were isolated from the mucus of the snail *C. aspersum*. Using mass spectrometry, the primary structures of a series of new antimicrobial peptides were determined.

In the present study the structure of novel peptides and protein fractions isolated from the mucus of garden snail *C. aspersum* with antibacterial activity are reported. Bacterial strains *Pseudomonas aureofaciens* AP9 and *Escherichia coli* NBIMCC 8785 (Gram<sup>-</sup>), *Brevibacillus laterosporus* BT-271 (Gram<sup>+</sup>) and positive anaerobic spore-forming rod-shaped bacterium *Clostridium perfringens* were chosen because of their antibiotic resistance.

## 2. Materials and Methods

### 2.1. Mucus collection and separation of different fractions

The mucus was collected and purified from *C. aspersum* snails grown in Bulgarian farms using patented technology without any snail suffering [23]. After ultrafiltration using different membrane filters (10 and 20 kDa), the crude mucus extract was separated into two fractions: a peptide fraction with Mw below 10 kDa and a fraction containing compounds with Mw above 20 kDa. The peptide fraction with Mw below 10 kDa was additionally separated using Amicon® Ultra-15 centrifugal tube filters with 3 and 5 kDa membranes into three fractions. Finally, the following samples have been obtained:

Sample 1 – fraction with compounds of Mw<3 kDa

Sample 2 – fraction with compounds of Mw 3-5 kDa

Sample 3 – fraction with compounds of Mw 5-10 kDa

Sample 4 – fraction with compounds of Mw<10 kDa

Sample 5 – fraction with compounds of Mw 10-30 kDa

Sample 6 – fraction with compounds of Mw<20 kDa

Sample 7 – fraction with compounds of Mw>20 kDa

### 2.2. Molecular mass analysis and de novo sequencing of peptides by mass spectrometry

The isolated peptide fraction with Mw <3 kDa (Sample 1) was lyophilized and analyzed by MALDI-TOF-TOF mass spectrometry on an Autoflex™ III. High Performance MALDI-TOF&TOF/TOF System (Bruker Daltonics) which uses a 200 Hz frequency-tripled Nd-YAG laser operating at a wavelength of 355 nm. Analysis was carried out after mixing 2.0 µl of the sample with 2.0 µl of matrix solution (7 mg/ml of  $\alpha$ -cyano-4-hydroxycinnamic acid (CHCA) in 50% ACN containing 0.1% TFA), but only 1.0 µl of the mixture was spotted on a stainless steel 192-well target plate. The samples were allowed to dry at room temperature and analysed. A total of 3500 shots were acquired in the MS mode and a collision energy of 4200 was applied. The mixture of angiotensin I, Glu-1-fibrinopeptide B, ACTH (1–17), and ACTH was used for calibration of mass spectrometer. The MS/MS spectra were carried out in reflector mode with external calibration using fragments of Glu-fibrino-peptide B. Amino acid sequences of peptides were identified by precursor ion fragmentation using MALDI-MS/MS analysis.

### 2.3. Carbohydrate test

Seven isolated fractions from the mucus were analyzed by the orcinol-sulphuric test to determine the carbohydrate content. About 2 µl of the purified samples were applied to a thin layer plate and air dried. The plate was sprayed with orcinol/H<sub>2</sub>SO<sub>4</sub> and heated for 20 min at 100 °C. The orcinol/H<sub>2</sub>SO<sub>4</sub> solution contained 0.02 g of orcinol, 20% H<sub>2</sub>SO<sub>4</sub>, and H<sub>2</sub>O to a total volume of 10 ml.

### 2.4. SDS-PAGE electrophoresis

Protein fractions with antibacterial activity were analyzed by SDS-PAGE electrophoresis. DL-dithiothreitol acrylamide/bis-acrylamide (30% solution), bromophenol blue sodium salt (Sigma-Aldrich, Germany), *N,N,N',N'*-tetramethylethylenediamine (TEMED) and ammonium persulphate (APS) (GE Healthcare, Sweden) were used for SDS and 2D-PAGE electrophoreses. Equal volumes containing approximately 25 µg/lane of the samples dissolved in Laemmli sample buffer (Tris/HCl pH 6.8, glycerol 20%, SDS 4%, and bromophenol blue 0.02%) and protein standard

mixture (Precision Plus Protein™, All Blue, Bio-Rad) were separated by 12.5% SDS-PAGE (precast gels SERVAGel™ TG PRiME™) and visualized by staining with Coomassie Brilliant Blue G-250.

## 2.4. Antimicrobial assays

### 2.4.1. Microbial strains.

The Gram-positive bacterial strains of *Clostridium perfringens* NBIMCC 8615, *Brevibacillus laterosporus* strain BT-271, and Gram-negative bacterial strains of *Pseudomonas aureofaciens* AP9 and *Escherichia coli* NBIMCC 8785 were used in the assays of the antibacterial properties of the peptide fractions. These bacterial strains were chosen as models for pathogenic bacteria from different essential Gram-negative and Gram-positive groups with specific relations towards antibiotics and xenobiotics as well as specific permeability of their cell wall. Both strains *P. aureofaciens* AP9 and *Brevibacillus laterosporus* BT-271 were isolated by Topalova (1989) [26] and were characterized as resistant towards aryl-containing xenobiotics and aryl-containing antibiotics possessing the ability to degrade these compounds. *E. coli* NBIMCC 8785 is representative for bacteria from the *Enterobacteriaceae* family and was obtained from the National Bank of Industrial Microorganisms and Cell Cultures. The strain *Clostridium perfringens* (Veillon and Zuber 1898) Hauduroy et al. 1937, in Species 2000 & ITIS Catalogue of Life: 2019, NBIMCC 8615 was obtained from National Bank of Industrial Microorganisms and Cell Cultures too.

### 2.4.2. Nutrient media and culture conditions

Solid MPA (Meat-Peptide Agar) medium /Nutrient Agar/ was used to investigate the antibacterial activity through cultivation assays. A mesopeptone broth was used to multiply the microorganisms. A nutrient liquid media was used to multiply the microorganisms. Standard microbiological apparatus and glassware were also used following all the rules of good microbiological practice. The test strains used were stored as lyophilized microbial cultures in glucose media and peptone protector. After rehydration in saline the strains were maintained on slop agar in standard tubes.

### 2.4.3. Studies of antibacterial activities, using different methods

Cultivation methods were applied in mesopeptone (nutrient) agar as follows: Two types of inoculation were used to test the microorganisms.

Inoculation is carried out by mixing the standardized microbial suspension with liquid agar at a temperature below 40°C. With this approach, microorganisms penetrate deep inside the nutrient agar. This procedure is modeling the case in which bacteria develop deeply in the skin. For inoculation the standardized microbial suspension (50 µl with a density of 10<sup>9</sup> cell/mL) was spread over the surface of the nutrient solid agar.

Cultivation were performed at 36°C for 48-72 hours for *Pseudomonas aureofaciens* AP9 and *Brevibacillus laterosporus* BT-271. *Clostridium perfringens* NBIMCC 8615 was cultivated in an anaerobic camera /Merck/ in a thermostat at 36°C for 72 hours. After culturing, the diameter of the sterile areas was measured obtained after interaction of the antibacterial agents and test cultures. The results are presented as mm<sup>2</sup>/mgP/µMol.

## 2.5. Electron Microscopic Assays

The impact of active fractions isolated from the mucus of garden snail *C. aspersum* on the cell structure of bacteria was examined by scanning electron microscopy (SEM). Samples were prepared by treatment with ethyl alcohol in a series of increasing concentrations. All data are arithmetic averages of three independent repeats.

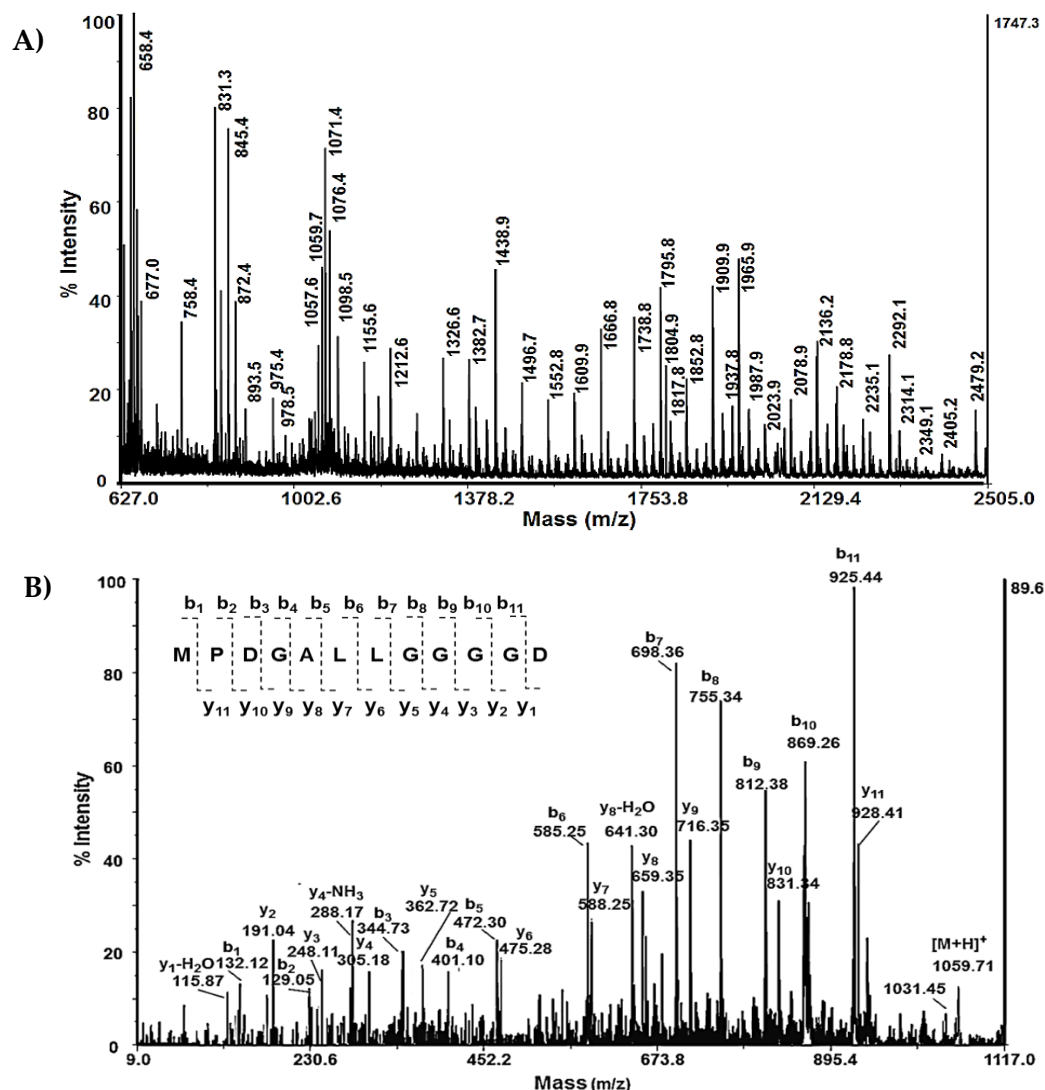
### 3. Results

#### 3.1. Purification and characterization of different fractions from mucus

The purified crude mucus extract was separated into various fractions by ultrafiltration under pressure with membrane filters of different pore sizes of 1, 10, and 20 kDa and centrifugal tube filters with 3 and 5 kDa membranes. As a result, 7 fractions have been obtained (Sample 1 – Mw <3 kDa; Sample 2 – Mw 3-5 kDa; Sample 3 – Mw 5-10 kDa; Sample 4 – Mw <10 kDa; Sample 5 – Mw 10-30 kDa; Sample 6 – Mw <20 kDa, and Sample 7 – Mw >20 kDa).

##### 3.1.1. Molecular mass analysis and *de novo* sequencing of peptides by mass spectrometry

MS analysis of Sample 1 shows several peptides with Mw <3 kDa (Figure 1 A).



**Figure 1.** Mass spectrometric analysis of peptide fraction with MW < 3000 Da by Autoflex<sup>TM</sup> III, High Performance MALDI-TOF&TOF/TOF Systems (Bruker Daltonics): A) MALDI-MS spectrum; B) MALDI-MS/MS spectrum of peptide [M+H]<sup>+</sup> at m/z 1059.71 Da.

Matrix-assisted laser desorption/ionisation time-of-flight mass spectrometry (MALDI-TOF/MS), sequencing the protonated molecular ions [M+H]<sup>+</sup>, was applied to determine the molecular mass and amino acid sequences of the peptides. The amino acid sequences of peptides with low molecular weight were identified by *de novo* sequencing experiments (MALDI-TOF-MS/MS) of the protonated molecule ions [M+H]<sup>+</sup>. Following b- and y-ions in MS/MS spectrum of peptide at m/z 1059.71 [M+H]<sup>+</sup>, the amino acid sequence MPDGALLGGGGD was identified (Figure 1 B).

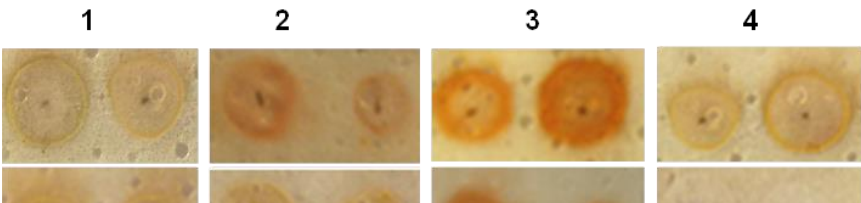
Using the same method, the amino acid sequences of 17 novel peptides in Sample 1 with molecular masses between 1000-2800 Da were identified (Table 1). The isoelectric points (pI) and grand average of hydropathicity (GRAVY) of the peptides were predicted by the ExPASy MW/pI tool program and ExPASy ProtParam tool (Table 1).

**Table 1.** Amino acid sequences of peptides from the mucus of garden snail *C. aspersum*, identified by *de novo* sequencing on MALDI-MS/MS.

No	Amino acid sequence of peptides	Exper. mass [M+H] <sup>+</sup> , Da	Calcul. monoisotop. mass, Da	pI	Grand average of hydropathicity (GRAVY)	Net charge
1	DLTLNGLSPK	1057.58	1056.58	5.84	-0.300 ( hydrophilic)	-1/+1
2	MPDGALLGGGGD	1059.71	1058.47	3.56	+0.058 (hydrophobic)	-2/0
3	DGPADNAQGAVG	1071.44	1070.46	3.56	- 0.600 (hydrophilic)	-2/0
4	SLEERDIQS	1076.44	1075.49	4.14	- 0.980 (hydrophilic)	-3/+1
5	GGLLAAGAGGGGAHV	1098.53	1097.58	5.52	+1.200 (hydrophobic)	0/0
6	LGLGNGGAGGGLVGG	1155.57	1154.60	5.52	+ 0.687(hydrophobic)	0/0
7	LNLGLDAGGGDPGG	1212.57	1211.58	3.56	- 0.093 (hydrophilic)	-2/0
8	FNHKSLPKLEN	1326.64	1325.64	8.60	-1.227 (hydrophilic)	-1/+2
9	NLVGGLSGGGRGGAPGG	1382.70	1381.71	9.75	- 0.024 (hydrophilic)	0/+1
10	LGGLGGGAGGGGLVGEFG	1438.86	1437.72	4.00	+0.439 (hydrophobic)	-1/0
11	NLVGSGGGGRGGANPLG	1496.73	1495.75	9.75	- 0.217 (hydrophilic)	0/+1
12	NGPNGGLGGSLVNGDPK	1552.76	1551.76	5.84	- 0.735 (hydrophilic)	-1/+1
13	GLLGGGGAGGGGLVGGLLNG	1609.94	1608.86	5.52	+0.776 (hydrophobic)	0/+1
14	MGGLLGVNNGGKGGGGPGAP	1666.83	1665.83	8.50	+0.005 (hydrophobic)	0/+1
15	MLLNAKWAPHSTGPPNA	1804.91	1803.91	8.52	- 0.400 (hydrophilic)	0/+1
16	LPFLGLVGGLLGGVGGGGGGGGPAL	2136.20	2135.17	5.52	+1.023 (hydrophobic)	0/0
17	DVESLPVGGLGGGGGAGGGGLVGGNLGGGAG	2479.20	2478.21	3.67	+0.353 (hydrophobic)	-2/0

3.1.2. A glycosylation screening

A screening of the fractions from mucus performed by orcinol/H<sub>2</sub>SO<sub>4</sub> assay identified several glycosylated fractions. As is shown in Figure 2 all fractions except the control fraction water (position 8) change the colour in the orcinol - sulfuric acid test applied to the silica gel plate. The peptide fraction with compounds with Mw 3-10 kDa (Figure 2, position 3), and protein fraction 7 with compounds with Mw above 20 kDa (Figure 2, position 7) show very intensive brown colour, probably more of containing compounds in these fraction are glycosylated.



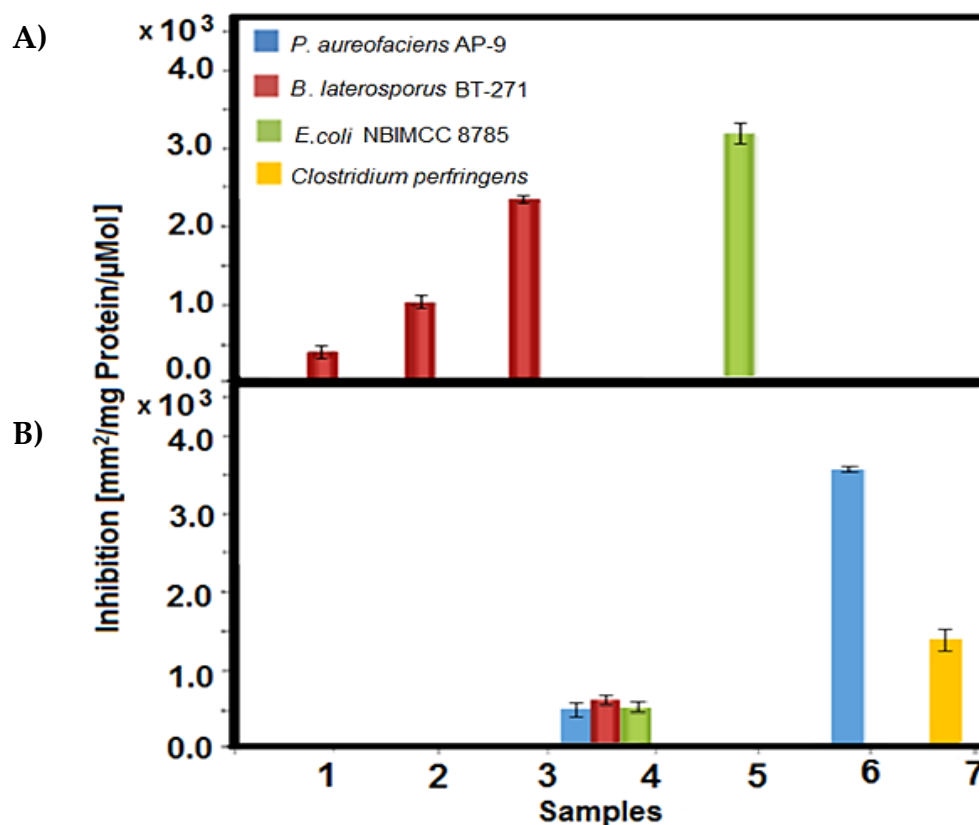
**Figure 2.** Orcinol – sulphuric acid test applied on to a silica-gel plate of different fractions isolated from the mucus of garden snail *C. aspersum*. The spots on: position 1) fraction with Mw < 1 kDa; position 2) fraction with Mw < 3 kDa; position 3) fraction with Mw 3-10 kDa; position 4) fraction with Mw 5-10 kDa; position 5) fraction with Mw <10 kDa; position 6) fraction with Mw < 20 kDa; position 7) fraction with Mw above 20 kDa; position 8) control - contains only water.

### 3.2. Antibacterial activity of different fractions from the mucus of garden snail *C. aspersum*

It is known that the Gly and Pro content in peptides plays a crucial role in the activity against different bacteria. Understanding the mechanism of these new antibacterial compounds from the mucus of *C. aspersum* may contribute to the potential of anti-infection therapeutics. Therefore, the antibacterial activity of Sample 1 (compounds with Mw <3 kDa), Sample 2 (compounds with Mw 3-5 kDa), Sample 3 (compounds with Mw 5-10 kDa), Sample 4 (compounds with Mw <10 kDa), Sample 5 (compounds with Mw 10-30 kDa), Sample 6 (compounds with Mw <20 kDa), and Sample 7 (compounds with Mw >20 kDa), isolated from mucus of garden snail was analysed against Gram<sup>+</sup> bacterial strains of *Clostridium perfringens*, *Brevibacillus laterosporus* BT-271 and Gram<sup>−</sup> bacterial strains of *Pseudomonas aureofaciens* AP9 and *Escherichia coli* NBIMCC 8785.

Two cultivation approaches were used that differ according to the way of inoculation of the bacteria into and on the solid media respectively described as investigation of antibacterial effect *in-depth* and on surface. The surface application gives information about the antibacterial effect when bacteria are located on the surface of the tissue. The inoculation of the bacteria into media gives information about antibacterial effect of the AMPs when infection is spread *in-depth* of tissue.

The results of tracing the antibacterial activity of seven fractions isolated from mucus against *Clostridium perfringens* NBIMCC 8615 compared to the antibacterial activity against other test cultures (strains) are shown in Figures 3A,B. No surface inoculation of the bacterial material is observed in Figure 3A. Antibacterial activity of all tested peptide and protein fractions is consistent with the anaerobic nature of the bacterium. Even though the cultivation is in anaerostatic chambers, under anaerobic conditions, neither of the five fractions isolated from the mucus inhibits bacterial strain growth. The obtained results reveal that three peptide fractions with Mw <3 kDa (Sample 1), Mw 3-5 kDa (Sample 2), and Mw 5-10 kDa (Sample 3) have antibacterial activity against Gram<sup>+</sup> bacterial strain *B. laterosporus* BT-271 and the most active one is Sample 3 (Figure 3A), which is one of the two fractions with the highest carbohydrate content (orcinol/H<sub>2</sub>SO<sub>4</sub> test, Figure 2). The compounds with Mw 10-30 kDa (Sample 5) possess a very high inhibition effect against *Escherichia coli* NBIMCC 8785 (Figure 3A).

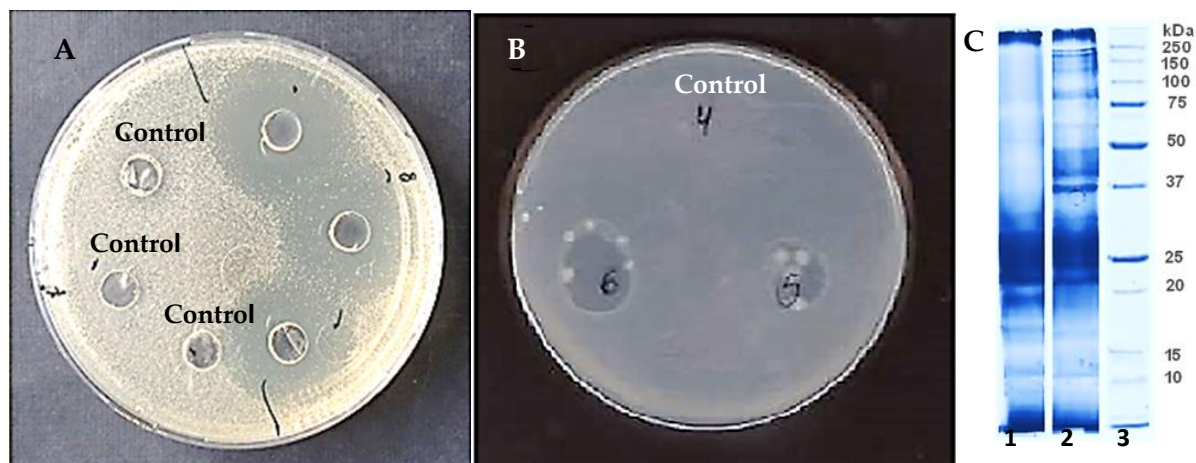


**Figure 3.** Comparative analysis of the antibacterial activity of Sample 1 (compounds with Mw <3 kDa), Sample 2 (compounds with Mw 3-5 kDa), Sample 3 (compounds with Mw 5-10 kDa), Sample 4 (compounds with Mw <10 kDa), Sample 5 (compounds with Mw 10-30 kDa), Sample 6 (compounds with Mw <20 kDa) and Sample 7 (compounds with Mw >20 kDa), isolated from mucus of garden snail against Gram<sup>+</sup> bacterial strain of *Clostridium perfringens*, *Bacillus laterosporus* strain BT-271 and Gram<sup>-</sup> bacterial strains of *Pseudomonas aureofaciens* AP9 and *Escherichia coli* NBIMCC 8785 upon: A) surface inoculation of bacteria; B) deep inoculation of bacteria. Antibacterial activity was measured in Inhibition [mm<sup>2</sup>/mg Protein/μMol sample].

Comparative analysis of the antibacterial activity of different samples, isolated from mucus of garden snail *C. aspersum* upon deep inoculation of bacteria reveals that Sample 6 with Mw <20 kDa has highest antibacterial activity against Gram<sup>-</sup> bacterial strains of *Pseudomonas aureofaciens* AP9 (3510.00 mm<sup>2</sup>/mg protein/μMol) in comparison to the other tested fractions, whereas Sample 4 with Mw <10 kDa shows insignificant antibacterial activity against *Bacillus laterosporus* strain BT-271 (Gram<sup>+</sup>) and Gram<sup>-</sup> bacterial strains of *Pseudomonas aureofaciens* AP9 and *Escherichia coli* NBIMCC 8785 (Figure 3B). Furthermore, only one tested fraction, with high carbohydrate content (Sample 7 with Mw >20 kDa) (Figure 3B) showed high antibacterial activity against *Clostridium perfringens* NBIMCC 8615. This anticlostridial activity is relatively high - 1400.17 mm<sup>2</sup>/mg protein/μMol in comparison to the other tested peptide and protein fractions. These results indicate that upon deep inoculation of the microbial material, the protein fraction is of interest for therapeutic purposes against clostridia-induced infections.

The large sterile zones formed due to a strong inhibition effect of Fraction 7 against *Clostridium perfringens* NBIMCC 8615 is illustrated with three repetitions in Figure 4A due to the deep inoculation of the bacteria in comparison to the control. For comparison, the antibacterial effect of AMCs (Sample 5) against *Escherichia coli* NBIMCC 8785 at surface inoculation of the bacteria is illustrated in Figure 4B. It clearly shows that fraction 7 of AMCs displays an antibacterial effect against *Clostridium perfringens* NBIMCC 8615. This allows to speculate that this fraction is may become a candidate for medical treatment of anaerobic infections caused by clostridia.

These two fractions with antibacterial activity were subject to SDS-PAGE in order to determine the approximate size of the antimicrobial substances. The electrophoresis (Figure 4 C) revealed several compounds in region 10–30 kDa (visibly bands at ~12 kDa, 17–20kDa kDa, ~22 kDa, and between 25–30 kDa) and proteins in fraction >20 kDa ( bands at ~37 kDa, ~ 42 kDa, between 45–50 kDa, ~65kDa, between 80–90 kDa and between 150–250kDa).



**Figure 4.** A) Illustrations of sterile areas in investigation of antibacterial activity against model bacteria antibacterial effect of Sample 7 against *Clostridium perfringens* at deep anaerobic cultivation; B) Antibacterial activity of Sample 5 against *Escherichia coli* NBIMCC 8785 at surface cultivation. C) 12.5% SDS–PAGE with Coomassie Brilliant Blue G-250 staining of protein fractions: position 1 (Samples 5 with Mw 10–30 kDa), position 2 (Samples 7 with Mw >20 kDa), and position 3 (standard protein marker, Protein Prestained Standards, Biorad).

To shed light on the mechanism of antibacterial action, we studied the effect of the active fraction with Mw 10–30 kDa on the cell structure of the Gram- bacterial strain *E. coli* NBIMCC 8785 by scanning electron microscopy (SEM).



**Figure 5.** Illustration of antibacterial effect of peptide fraction 5 on the cells of *Escherichia coli*: A) Control 18-hour culture of *E. coli* in nutrient broth; B) Damaged membranes and deformation of cell of 18 hour culture of *E. coli* in nutrient broth after 1 hour action of peptide fraction 5 - 10000X; C) Damaged membranes and deformation of cell of 18 hour culture of *E. coli* in nutrient broth after 1 hour action of peptide fraction 5 . Magnification 30000X.

As is shown Figure 5, the results obtained by SEM from control - 18-hour culture of *E. coli* in nutrient broth (Figure 5A) and the damaged membranes of cell of 18 hour culture of *E. coli* in nutrient broth after 1 hour action of peptide fraction 5 with at magnification 10000X (Figure 5B) and 30000X (Figure 5C).

#### 4. Discussion

The discovery of new antimicrobial compounds (AMCs) from natural sources is of great importance for public health, since these molecules are pharmacological candidates due to their effective antimicrobial activity and low resistance rates. The resistance against them is not prevalent although AMPs have been exposed to microbes for millions of years [27]. Besides, mutations in the microbes during the course of evolution led to the diversification of AMPs [28]

In our previous work we have determined the primary structure and their antimicrobial activity of only 9 peptides produced by the mucus of the garden snail *H. aspersa*, in particular against Gram<sup>−</sup> *Pseudomonas aureofaciens* AP9 and Gram<sup>+</sup> *Brevibacillus laterosporus* BT271 bacteria [24]. We hypothesized that other peptides from the mucus would also be effective against bacterium *Clostridium perfringens* NBIMCC 8615.

In certain primary structures (Table 1), many peptides contain amino acid residues such as, glycine (G), proline (P), leucine (L), valine (V), tryptophan (W), aspartic acids (D), phenylalanine (F), arginine (R), which are typical for peptides with antimicrobial activity. Many AMPs are unstructured in free solution and fold into their final conformation upon partitioning with biological membranes. Generally, these proteins could attain diverse conformations such as  $\alpha$ -helices,  $\beta$ -sheets, mixed conformations, loops, and extended structures [28,29]. Analyses using the ExPASy ProtParam tool indicate that the mucus fraction with Mw<3kDa contains both cationic and anionic AMPs, but is dominated by cationic AMPs. Most of the peptides, identified in fraction with Mw<3kDa, are characterized by an amphipathic structure and display generally hydrophobic surfaces (Table 1). This fact is considered as a prerequisite for the disruption of biological membranes and direct cell lysis [29]. It is known that cationic AMPs kill microbes *via* mechanisms that predominantly involve interactions between the peptide's positively charged residues and anionic components of target cell membranes. These interactions can then lead to a range of effects including membrane permeabilization, depolarization, leakage or lysis resulting in cell death [30]. Generally, cationic antimicrobial peptides act on the membrane of microorganisms by an electrostatic difference, but there are AMPs that act internally and can interact on ribosomes, internal proteins, or nucleic acids. Some of the identified peptides in Table 1 have a primary structure similar to glycine-rich linear antimicrobial peptides, as Ctenidin1-3 with activity against the Gram-negative bacterium *E. coli*, isolated and characterized from hemocytes of the spider *Cupiennius salei* [31]. Also acanthoscurrins isolated from the hemocytes of the spider *Acanthoscurria gomesiana* act against Gram-negative bacteria, *Escherichia coli*, and the yeast *Candida albicans* [32], are characterized as cationic peptides with high glycine content. Using alignment of amino acid sequences presented in Table 1 with CAMPSing (<http://www.campsign.bicnirrh.res.in/blast.php>), the peptides Nos 5 and 6 showed ~72% identity with Ctenidin 1 and Ctenidin 3, and peptides Nos 9, 10, 13, and 17 have about 73-76% identity with two isoforms of acanthoscurrin.

Some of the identified peptides shown in Table 1 belong to anionic antimicrobial peptides (AAMPs) which have been increasingly identified in invertebrates, vertebrates, and plants over the

last decade. Previous research also identified AAMPs in mucus fraction with Mw below 10 kDa, isolated from garden snail *H. aspersa* [23, 24]. Usually AAMPs show antibacterial activity, but a number of them are multifunctional, variously showing antifungal, anticancer, a neuropeptide activity, and the probability to become potential conventional antibiotics [29]. Antimicrobial mechanisms proposed for these peptides include toroidal pore formation and membrane interaction according to the Shai-Huang-Matsuzaki model along with pH-dependent amyloidogenesis and membranolysis *via* tilted peptide formation [33]. AAMPs generally adopt amphiphilic structures. For a number of these peptides, post-translational modifications are essential for antimicrobial activity. Membrane interaction appears a key to the antimicrobial function of AAMPs. The architectures of AAMPs vary from alpha-helical peptides from some amphibians to cyclic cysteine knot structures observed in some plant proteins. But in many cases, the mechanisms underlying the antimicrobial action of these peptides are unclear or have not been elucidated [30].

The detected peptides (shown in Table 1, Nos 6, 10, 13, 14, 16, and 17) containing high levels of glycine and leucine residues belong to a new class of Gly/Leu-rich antimicrobial peptides. High homology (above 70.0%, identified from alignment with CAMPSing) was found between amino acid sequences of peptides Nos 13, 14, 16, and 17 (Table 1) and leptoglycin (GLLGLLGPLLGGGGGGGGLL, pI 5.52, GRAVY 1.073) [34] which inhibits the growth of Gram<sup>−</sup> *P. aeruginosa*, *E. coli*, and *Citrobacter freundii* strains, but it did not show antimicrobial activity against Gram<sup>+</sup> bacteria. Our previous studies have also shown the presence of peptides with similar amino acid sequences in the extracts of the garden snail [23,24].

Proline-rich AMPs, with a high content of Pro, Gly and Arg residues, are an important group of AMPs predominantly active against Gram-negative bacteria. Previous studies have shown, if Pro residues are inserted into the sequences of  $\alpha$ -helical AMPs, their ability to permeabilize the bacterial cytoplasmic membrane decreases substantially along with the number of Pro residues incorporated, what could explain our results. Proline-rich peptides, previously known to bind to heat shock proteins, are shown to inhibit protein synthesis [35,36]. Due to our results that the identified sequences, 12 peptides of the identified sequences contain 1 to 3 Pro amino acid residues in the polypeptide chain. In nine peptides Pro residues are incorporated in the C-terminal region of the polypeptide chain, but only in 5 peptides Pro is located in the N-terminal region. Two peptides contain proline residues both in the N-terminal and C-terminal polypeptide chain. Moreover, one proline residue was found in the center of the polypeptide chain for two peptides (Nos 8, 15).

The alignment in BLAST has shown, that five peptides (Nos 1 and 4) demonstrate high homology with hemocyanins isolated from snails *Helix aspersa*, *Helix pomatia*, and *Helix lucorum* (<https://blast.ncbi.nlm.nih.gov>). Peptide No 1 shows 100% identities with hemocyanin subunit  $\beta$ -HaH [*H. aspersa*, Sequence ID: AYO86685.1], subunit  $\beta$ -HiH [*H. lucorum* Sequence ID: AEO51766.1], and  $\beta$ -HpH [*H. pomatia*, Sequence ID: AYO86688.1]. Peptide 4 is 100% identical with the sequence from hemocyanin  $\beta$ -HaH [*H. aspersa*, Sequence ID: AYO86685.1], subunit  $\beta$ -HiH [*H. lucorum*, Sequence ID: AEO51766.1],  $\beta$ -HpH [*H. pomatia*, Sequence ID: AYO86688.1], and subunit  $\alpha$ D-HaH [*H. aspersa*, Sequence ID: AYO86683.1]. Peptide No 8 shows 60% identity with beta-C chain unit D of *Helix pomatia* hemocyanin (P12031 (HCYB\_HELPO)). Probably proteolytic processes may have led to the appearance of these peptides in the mucus. Furthermore, the majority of known AMPs originate from processing of larger inactive proteins, and some studies suggest that biologically active proteins, such as hemocyanins [37] or hemoglobins [38] can be processed to produce bioactive

compounds [12]. Some of the identified peptides contain high levels of glycine and leucine residues, as well as up to three proline residues, which is probably important for the stability of their antimicrobial activity. A comparison of the alignment of amino acid sequence of the peptides from the mucus of garden snail *H. aspersa* (presented in Table 1) with databases by software CAMPsing revealed high identification (above 70 %) with known AMPs. This fact confirms their affiliation of the AMPs family.

The results obtained reveal that peptide fractions (Samples 1, 2, and 3) exhibit a predominant antibacterial activity against the Gram<sup>+</sup> bacterial strain *B. laterosporus* BT-271, whereas peptide fraction with Mw <20 kDa has significant antibacterial activity against Gram<sup>−</sup> bacterial strains *P. aureofaciens* AP9 upon deep inoculation of the bacterium. The presented results show that the higher carbohydrate content of the peptide fractions (Samples 1, 2, and 3) leads to higher antibacterial activity against *B. laterosporus* BT-271. We hypothesize the presence of a synergistic effect of peptides in a fraction below 3 kDa, a fraction of 3–10 kDa and polypeptides with a molecular weight between 10–20 kDa (at ~12 kDa, between 17–20 kDa), which are due to strong antibacterial activity against *P. aureofaciens* AP9 in deep inoculation of the bacterium. The fraction with Mw 10–30 kDa exhibits highest antibacterial activity using surface inoculation of bacterial strain *E. coli* NBIMCC 8785. From electrophoresis (Figure 4C) it was clearly that the compounds active against *P. aureofaciens* AP9 in the *H. aspersa* mucus are some of identified at ~12 kDa, between 17–20 kDa, ~22 kDa, and between 25–30 kDa. Recently, two peptides (one 17.5 kDa and one 18.6 kDa) were identified in the mucus of *H. aspersa* which appear to have activity against *Ps. aeruginosa* [21].

Results from comparative analysis of the antibacterial activity of fractions against *C. perfringens* NBIMCC 8615 (Gram<sup>−</sup>) and other tested bacterial strains by surface and deep inoculation of the bacteria reveal that a protein fraction with Mw >20 kDa (one of the two fractions with the highest carbohydrate content) is the most effective against the bacterial strain *C. perfringens* at deep anaerobic cultivation. Probably the proteins determined at ~37 kDa, ~42 kDa, 45–50 kDa, ~65 kDa, 80–90 kDa and between 150–250 kDa (electrophoresis, Figure 4C) are responsible for the anticlostridial effect. Some of the found proteins may be related to example to a new protein named Aspermin with a molecular weight 37.4 kDa and protein with a molecular weight ~50 kDa, determined previously in fractions from the mucus of *H. aspersa* with anti-pseudomonal properties [21], as well as protein with Mw 50.81 kDa from *A. fulica* mucus [39]. Furthermore, the protein determined between 80–90 kDa probably corresponds of protein with MW of 83.67 kDa (achacin) isolated from *A. fulica* mucus, active against *Streptococcus mutans* and *Actinobacillus actinomycetemcomitans* [39]. Our new data are in agreement on the antimicrobial properties of the mucus from *H. aspersa*, and *A. fulica* [14,21,39].

The combination of two vectors of action of protein fraction >20 kDa against clostridial infections, an antibacterial and a regenerative effect, will be the basis for the development of synergistic therapeutic agents.

Our results show that the antibacterial activity of fraction with Mw 10–30 kDa (Fraction 5 with Mw 10–30 kDa) induces serious damaging of the bacterial membrane changing of the shape, activity and metabolism of the bacteria strain *Escherichia coli* NBIMCC 8785 (Figures 5 A, B, C). These results will be extended by investigations on the mechanism of the antibacterial effect against *Clostridium perfringens*.

## 5. Conclusions

In conclusion, the presents work gives an example on isolation and structural identification of new biomolecules from a complex mixture of *H. aspersa* mucus using advanced sophisticated instrumentation. Du to different biotests, several of the mucus fractions have considerable antimicrobial activity which could probably add to the arsenal of antibiotics as candidates with low resistance rates.

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