Prepublished copy

Nature Environment & Pollution Technology

This is a peer-reviewed prepublished version of the paper

Purification and Characterization of Epidermin from *Staphylococcus epidermidis* and the Impact of Heavy Metals on their Antibacterial Properties

M. K. Hameed*, Z. R. Abbas* and S. H. Authman*
*Department of Biology, College of Science, University of Mustansiriyah, Baghdad, Iraq
Corresponding author: M. K. Hameed
Email: essamkarkh@gmail.com; maysam.khalil2021@gmail.com

ABSTRACT

Epidermin, a bacteriocin produced by *Staphylococcus epidermidis*, possesses antimicrobial and anti-enzymatic characteristics. It has been speculated that heavy metals could enhance the inhibitory activities of epidermin against clinical pathogens. This study aimed to extract and characterize epidermin from *Staphylococcus epidermidis* and investigate the impact of heavy metals on its antibacterial action on *Pseudomonas* species. *Staphylococcus epidermidis* was collected and isolated from clinical and environmental samples in Baghdad, Iraq. The bacterial isolates were identified through phenotypic, microscopic, and VITEK 2 compact system. Among the isolates, epidermin-producing strains were identified. Epidermin was extracted from the epidermin-producing isolates, purified, characterized and fractionated. The effect of three heavy metal ions on the inhibitory activity of epidermin was tested against *Pseudomonas* species. One of the thirteen epidermin-producing isolates was selected for epidermin extraction. The epidermin crude concentration was determined to be 0.43 mg/ml, exhibiting 70% activity. The results also showed that 100 mM of cadmium increased epidermin activity, while 50 mM of cadmium showed less activity. Cobalt and copper demonstrated a similar inhibitory-enhancement activity. The present study found that the inhibitory activity of epidermin against *Pseudomonas* species could be enhanced with heavy metals. Further investigations are encouraged and reccommended to explore the synergistic potential of epidermin and heavy metals as antimicrobial agents for controlling clinical pathogenic isolates.

Key Words	*Staphylococcus epidermidis*, Epidermin, Cd, Co, Cu, metal ions, *Pseudomonas* species
DOI	https://doi.org/10.46488/NEPT.2025.v24i03.D1726 (DOI will be active only after the final publication of the paper)
Citation of the Paper	M. K. Hameed, Z. R. Abbas and S. H. Authman, 2025. Purification and Characterization of Epidermin from Staphylococcus epidermidis and the Impact of Heavy Metals on their Antibacterial Properties. *Nature Environment and Pollution Technology*, 24(3), D1726. https://doi.org/10.46488/NEPT.2025.v24i03.D1726

INTRODUCTION

Staphylococcus epidermidis is a type of bacteria that occurs in clusters and has a characteristic round shape (cocci). It is characterized as coagulase-negative and gram-positive. The organism is a facultative anaerobe that can survive without oxygen and has catalase activity. In their natural environment, such as human skin or mucosa, *Staphylococcus*

epidermidis is typically harmless (Brown & Horswill, 2020; Foster, 2020). However, though harmless in their natural environment, these coagulase-negative *Staphylococcus* species can enter an individual's body through prosthetic devices, potentially introducing a small number of bacteria into the bloodstream. Once inside the body, these bacteria can form biofilms, which serve as protective structures against the host's immune defenses and antimicrobial treatments (Zheng *et al*., 2018).

The occurrence of bacteremia, caused by *Staphylococcus epidermidis* and other coagulase-negative staphylococci, is most commonly linked to medical device contamination (Severn & Horswill, 2023). When a prosthetic device is implanted in the human body, microorganisms from the skin can colonize the medical equipment. Some of these bacteria may enter the bloodstream, potentially leading to infection and health complications. *Staphylococcus epidermidis* is a major contributor to nosocomial (hospital-acquired) bloodstream infections (Liu *et al*., 2020). Additionally, *Staphylococcus epidermidis* infections are prevalent among infants (Cau *et al*., 2021). *Staphylococcus epidermidis* can produce and release epidermin, a tetracyclic peptide that belongs to the family of antibiotics.

Epidermin exhibits inhibitory effects on numerous Gram-positive bacteria, especially sensitive cells. Bacteriocin targets various cellular processes simultaneously, including RNA, DNA, polysaccharides, and protein synthesis. This leads to energy depletion and biosynthetic disruption in treated cells. Epidermin's primary biochemical target is the energy-transducing cytoplasmic membrane (Said-Salman *et al*., 2019). Through a barrel-stave mechanism, epidermin appears to impact membrane permeability by creating water-filled channels or gaps in the membrane structure (Alinaghi *et al*., 2022). Epidermin's classification as either an antibiotic or a bacteriocin sparked extensive debate. It was ultimately identified as a type-AI lantibiotic, satisfying the criteria for both antibiotics and bacteriocins, leading to it being categorized as neither (Alkhawaja *et al*., 2020).

Heavy metals are elements with a high atomic weight and density, typically greater than 5 g/cm^3, and are characterized by physical and chemical properties (Nriagu, 1996). While these elements are present in the Earth's crust, human activities such as mining, industrial processes, and agriculture have significantly increased their concentrations in the environment, leading to widespread contamination. Heavy metals, such as lead (Pb), mercury (Hg), cadmium (Cd), arsenic (As), and chromium (Cr), are naturally occurring elements with high atomic weights and densities. Excess of heavy metals can pose significant environmental (Alloway, 2013), and human health risks (Grandjean & Landrigan, 2014). In the environment, they can contaminate soil and water. Furthermore, heavy metals have been linked to neurological disorders, cardiovascular diseases, carcinogenesis, and reproductive and developmental consequences. However, some of these heavy metals are essential in trace amounts for biological functions. Heavy metals like cadmium, cobalt, and copper are essential for the physiological functioning of living organisms (Madhu *et al*., 2020). Heavy metal pollution exerts selective pressure on microbial communities, driving the evolution of metal-tolerant and antibiotic-resistant microorganisms through shared genetic mechanisms like efflux pumps (Seiler & Berendonk, 2012). Certain metals, such as silver and copper, exhibit natural antimicrobial properties, inspiring their use in medical applications like coatings and wound dressings (Lemire et al., 2013). Additionally, heavy metals can synergize with antibiotics, enhancing their effectiveness against resistant pathogens (Baker-Austin et al., 2006). Heavy metals can enhance the activity

of antimicrobial peptides (AMPs) like epidermin through various mechanisms. Metal ions such as zinc and copper can bind to AMPs, stabilizing their structure and increasing their charge, which strengthens their interaction with microbial membranes. Additionally, metal ions can facilitate the production of reactive oxygen species (ROS), further damaging microbial cells. These interactions make AMPs more potent against pathogens, even in resistant strains, offering therapeutic potential in combating infections (RSC Publishing, 2018).

The present study aimed to purify and characterize epidermin, a bacteriocin produced by *Staphylococcus epidermidis*, and to investigate the impact of various heavy metals (cadmium, cobalt and copper) on its antibacterial efficacy, focusing on understanding the mechanisms by which heavy metals may enhance or inhibit its activity against *Pseudomonas* species.

MATERIALS AND METHODS

Sample Collection

A total of one hundred (100) samples, consisting of blood, skin, wound, and burn specimens, were obtained from Ibn Al-Baladi Hospital in Baghdad. Twenty (20) environmental samples were also collected from sewage water.

Isolation of Bacteria

Selective media (Himedia Company, India) were prepared and autoclaved for 15 minutes at 121°C. The samples were then inoculated on selective media and the cultures were incubated for 24 hours at 37°C to isolate *Pseudomonas* species and *S. epidermidis*. The experiment was replicated three times. Subsequently, the mixed cultures were subcultured onto freshly prepared nutrient agar plates through streaking, and the cultures were incubated at 37°C for another 24 hours. This process allowed the growth of distinct, isolated colonies, which were identified (Tille, 2015).

Identification of Bacterial Isolates

The characteristics of the bacterial isolates were determined using various phenotypic traits, such as form, odour, colour, diameter, and pigmentation on culture media. Microscopic examination was also conducted for this purpose. In addition, biochemical tests were performed using the VITEK 2 compact system.

Detection of Epidermin-Producing Isolates

The epidermin producing isolates were identified using the diffusion method according to Gupta (1996). Cultures of *S. epidermidis* isolates, aged 18-24 hours in brain heart infusion agar (BHIA), were prepared. These bacterial cultures were then centrifuged at 6,000 rpm for 30 minutes. The resulting filtrate was collected for each isolate, while the precipitates were discarded. The collected filtrate was heated to 70°C for 3 minutes. To perform the test, 100 µL of a suspension of *Pseudomonas* species, aged 18-24 hours, were transferred to the center of the solid BHIA plates. The bacterial suspension was adjusted using a 0.5 MacFarland solution. Using a sterile cork borer, wells with a diameter of 5 mm were made in the BHIA culture plates. Each well was then filled with 100 µL of each *S. epidermidis* suspension. The experiment was performed in triplicates for each isolate, with a negative control (PBS) and a positive control (100 µL of 6% acetic acid). The cultures were left at room temperature for 15 minutes,

incubating at 37°C for 18-24 hours. After the incubation period, the diameters of the *Pseudomonas* species growth inhibition zones around the wells were observed as evidence of a positive result, indicating epidermin production.

Extraction of Epidermin from the Epidermin-Producing *Staphylococcus epidermidis*

Epidermin extraction was conducted following the modified procedure outlined in Mirhosseini *et al*. (2010). An aliquot of 250 mL of BHIB medium was prepared and inoculated with *S. epidermidis* isolate No. 10 (the isolate with the most effective anti-growth activity against *Pseudomonas* species). The culture was allowed to grow for 18-24 hours at 37°C. Following the incubation period, the bacterial culture was centrifuged at 10,000 rpm for 30 minutes. This process separated the bacterial cells, forming sediment, which was discarded. The filtrate, containing the desired epidermin, was collected. The collected filtrate was then heated to 70°C for 3 minutes. Once the heated solution cooled, the supernatant, containing the crude epidermin, was carefully collected.

Purification of Epidermin using the Ammonium Sulphate Precipitation Method

To further purify the extracted crude epidermin, ammonium sulphate precipitation method was used as described by Al-Soufi *et al*., 2005. Ammonium sulfate was slowly added to the extracted crude epidermin at 4°C while stirring. This was done until it reached the desired saturation level of 70%. The solution was then kept overnight at 4°C to facilitate protein precipitation. Subsequently, the solution was centrifuged at 10,000 rpm at 4ºC for 15 minutes to separate the protein pellets from the remaining solution. To dissolve the protein pellets and remove most of the contaminated proteins and water, a small amount of 0.1 M phosphate buffer at pH 7 was used. This process resulted in the production of a partly purified epidermin. Several trials with gradual saturation of ammonium sulfate were performed to determine the optimal saturation ratio for epidermin precipitation. After obtaining the precipitated fraction, the number of proteins and epidermin activity in the dissolved precipitate was measured. Ammonium sulfate precipitate was filtered via an active dialysis membrane and then added to a buffer solution (0.01 M phosphate buffer) in a test tube.

Fractionation of Epidermin using Ion Exchange and Gel Filtration Chromatography

In the present study, ion-exchange and gel filtration chromatography were used to fractionate epidermin. Both methods are effective for purifying epidermin due to their ability to separate based on charge and size, respectively, offering high resolution and reproducibility. Ion-exchange chromatography selectively isolates proteins like epidermin by utilizing charge interactions, while gel filtration separates based on molecular size, reducing contaminants. These methods are less costly and simpler to implement compared to High-Performance Liquid Chromatography (HPLC), which may require more complex equipment and is typically reserved for smaller-scale or high-precision purifications (Qin et al., 2020; Sahu et al., 2019).

Epidermin was fractionated using the ion exchange chromatographic method described by Whitaker and Bernhard (1972). The DEAE-cellulose column was prepared by suspending 20 g of resin in 1 L of distilled water. After allowing the suspension to settle, it was washed multiple times with distilled water until clear. The suspension was filtered through a Whatman No. 1 filter paper using a Buchner funnel. The resin was resuspended in a solution containing 0.25 M sodium chloride and sodium hydroxide. After another round of filtration and rinsing with a 0.25 M hydrochloric acid solution followed by distilled water, the resin was equilibrated in a 0.05 M phosphate buffer at pH 7. The column was washed with an equal volume of the

same buffer to perform chromatography. The bound proteins were gradually eluted using increasing concentrations of sodium chloride (ranging from 0.1 to 1 M). The flow rate across the column was set at 30 ml/h, and each fraction's absorbance was measured using anultraviolet-visible (UV-VIS) spectrophotometer at 280 nm. The activity of each fraction of the epidermin was evaluated.

Sephadex G-150 (Pharmacia Fine Chemicals Company) was prepared following the manufacturer's instructions. First, Sephadex G-150 was suspended in a 0.05 M phosphate buffer at pH 7. Then, it was heated at 90°C for 5 hours to achieve bead swelling. After the beads swelled, the suspension was degassed to remove any trapped air bubbles. Next, the swollen Sephadex G-150 was packed into a glass column measuring 2x40 cm. The column was then equilibrated with the same 0.05 M phosphate buffer. The column was loaded with the concentrated sample obtained from the previous ion exchange chromatography stage. The elution flow rate was set at 30 mL/h, and the same 0.05 M phosphate buffer was used for equilibration and elution. As the samples passed through the column, the absorbance of each fraction was measured at 280 nm using a spectrophotometer. The protein concentration in each fraction was assessed using the Bradford method (Bradford, 1976). Additionally, epidermin activity was evaluated in each fraction to determine the presence and activity of purified epidermin.

Molecular Weight Determination

To determine the molecular weight of epidermin, gel filtration chromatography was employed using a Sephadex G-150 column measuring 2 x 40 cm. The column was equilibrated and eluted with 0.05 M phosphate buffer at pH 7. For calibration, several standard proteins with known molecular weights were used. These proteins included alcohol dehydrogenase, albumin, carbonic anhydrase, and lysozyme. Blue dextran was applied and measured at 600 nm to determine the volume of the void. The elution volume for each standard protein was then measured using a UV-Vis Bio-Rad spectrophotometer at 280 nm. By comparing the elution volume of epidermin with that of the known benchmark proteins, the molecular weight of epidermin was determined. This allowed researchers to determine the approximate size of the epidermin molecules compared to the standard proteins used as references.

Thermal Stability Evaluation

The optimal temperature for maximum epidermin production was identified by incubating cultures at various temperature ranges (30, 37, 42, 47, and 53 °C) for 30 minutes. After that, the epidermin was transferred immediately to an ice bath, according to the method described by Makky *et al*. (2013). The activity was determined, and the remaining activity (%) was plotted against temperature.

Determination of the Effect of pH on Purified Epidermin Stability

The purified epidermin was pre-incubated in a buffer solution of various pH (5, 6, 7, 8, and 9) for 30 minutes at 37 °C using 1M NaOH and 1M HCl. After that, the tubes were cooled in an ice bath, according to the method described by Makky *et al*. (2013). The activity was determined and the remaining activity (%) was plotted against pH.

Evaluation of the Effect of Heavy Metals on Epidermin Activity

The effects of different concentrations of heavy metal ions, such as Co ($CoCl_2$), Cu, and Cd ($CdCl_2.H_2O$) on epidermin activity were evaluated. Epidermin was pre-incubated alone at two different concentrations (50 and 100 mM). These concentrations were selected based on the results from preliminary experiments, which identified 50 and 100 mM as the optimal concentrations for the study. Heavy metal ions (Co, Cu, and Cd) at the test concentrations were added to the culture of *P. aeruginosa*. Then, epidermin activity and relative activity (the ratio of epidermin activity in the presence and absence of heavy metal ions) were calculated. The activity was assayed without metal ions and taken as 100% (Gandhi *et al*., 2019).

Statistical Analysis

The Statistical Packages of Social Sciences (SPSS; 2019) program was used to detect the effect of different factors in study parameters. Least significant difference (LSD) and analysis of variance (ANOVA) were used to compare between means. $P \leq 0.5$ was considered significant.

RESULTS AND DISCUSSION

Heavy metals have a variety of medical applications due to their unique chemical and physical properties (Prasad, 2008). Some heavy metals, such as zinc, barium, gadolinium, and technetium, have been utilized for various diagnostic purposes. Some cancer treatment procedures have used platinum and gold to suppress and inhibit the growth of cancerous cells (Kelland, 2007). This study examined the development and clinical use of platinum-based chemotherapy medicines like cisplatin, as well as their efficacy in treating a variety of malignancies. Some investigations reported copper, silver, cadmium, and cobalt possessing some antimicrobial properties. Valko (2005) reported the biological effects of various metals, including cobalt, and discussed their antimicrobial properties concerning toxicity and oxidative stress. Therefore, the present study extracted epidermin from *Staphylococcus epidermidis* and purified it. More so, the extracted epidermin was further characterized. The influence of three heavy metals (cobalt, cadmium, and copper) on the enhancement of the antibacterial activity of epidermin on a test pathogen, *Pseudomonas* species, was studied.

Epidermin Producing Isolates

Thirteen isolates were first identified (Figs. 1A and B) as *Staphylococcus epidermidis* based on their morphological, cultural, and biochemical characteristics, as well as the Vitek2 system confirmation. These isolates were obtained from various clinical sources. Simultaneously, thirty isolates were identified as *Pseudomonas aeruginosa*, originating from clinical sources, along with five isolates from the environment (sewage water). Out of the thirteen *S. epidermidis* isolates, three isolates (23.08%) produced epidermin. These particular isolates demonstrated a clear inhibitory zone against *Pseudomonas aeruginosa* isolates (Fig. 2). This result indicated that these epidermin-producing isolates of *S. epidermidis* possess antimicrobial properties, specifically inhibiting the growth of *Pseudomonas aeruginosa* (Fig. 1C).

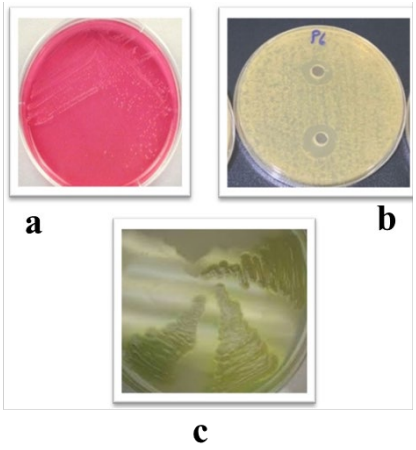

Fig. 1: (a) *Staphylococcus epidermidis* on mannitol salt agar; (b) Colonies of *Pseudomonas* species on *Pseudomonas* agar; (c) Epidermin production from *Staphylococcus epidermidis*

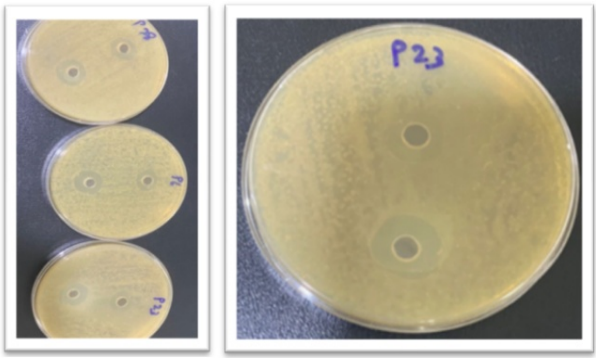

Fig. 2: Inhibitory effect of epidermin on the growth of *Pseudomonas aeruginosa's* epidermin fractions

Purified Epidermin

Ion exchange chromatography was employed to purify epidermin (Fig. 3A). During the washing stage, a single protein peak was obtained at varying sodium chloride concentrations, and similarly, another protein peak was obtained during the subsequent elution step. These protein peaks were further analyzed to assess their epidermin activity. Based on the results, the proteins that eluted within fractions 45 to 61 showed the highest concentration of epidermin activity. These fractions were combined, and, upon testing, exhibited an activity of 45 U/ml, a specific activity of 187.5 U/mg, a fold purification of 2.6, and a protein yield of 36%. The fractions containing epidermin were combined and subsequently further purified using a Sephadex G-150 column after the ion exchange chromatography. The Sephadex G-150 column has separation limits ranging from 5,000 to 600,000 Da, ensuring efficient separation and purification. This column is advantageous for protein separations due to its low maintenance requirements, ease of preparation, rapidity, and excellent recovery (Stellwagen, 1990). The results after using Tris-HCl buffer to wash the sample demonstrated that epidermin activity appeared as a single peak. The separate parts were then merged to form one protein peak. The

analysis of this peak (fractionation tubes 13-31) revealed an epidermin activity of 43 U/ml, a specific activity of 430 U/mg, and a purification fold of 6.1, with a protein yield of 34.4%.

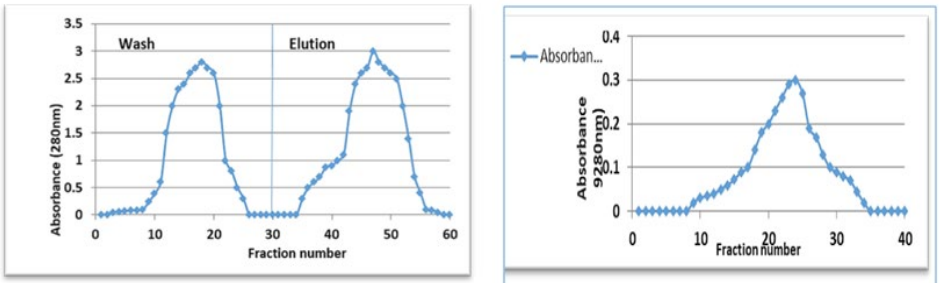

Fig. 3: (A) Ion exchange chromatography on DEAE cellulose; (B) Gel filtration chromatography on Sephadex

Antibacterial Potential of Epidermin

Thirteen filtrates from *S. epidermidis* were evaluated for their inhibitory activity using a diffusion assay against an indicator isolate of *Pseudomonas* species. The results indicated significant inhibition of these isolates. However, there was a noticeable variation in the inhibition effectiveness, as evidenced by the differences in the diameters of the inhibition zones compared to the control. One of the isolates displayed moderate anti-bacterial activity, with inhibition zones measuring 10, 12, and 15 mm, respectively as presented in Fig. 2. On the other hand, other isolates did not exhibit any activity against *Pseudomonas* species. A crude extract derived from *S. epidermidis* isolates inhibited the growth of *Pseudomonas* species. Unlike lysostaphin, epidermin does not possess lytic properties and has a narrow spectrum of actions. It does not inhibit Gram-negative bacteria, like other bacteriocins produced by Gram-positive microorganisms. However, epidermin exhibits inhibitory activity against other Gram-positive bacterial species, as reported by Maisnier-Patin *et al*. (1995).

Molecular weight of Purified Epidermin

The inhibitory activity of epidermin on *Pseudomonas* species provided valuable insights into the biological properties and potential applications of this molecule. The molecular weight of epidermin was determined by the Sephadex G-150 column (Fig. 4).

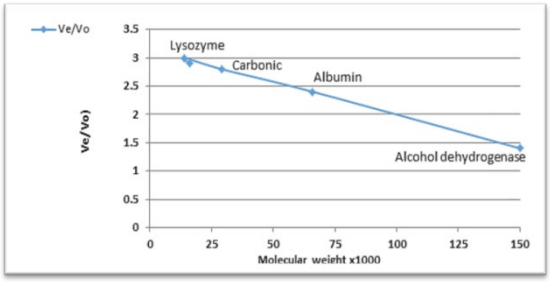

Fig. 4: Molecular weight determination of epidermin by Sephadex G-150 column

Thermal Stability of Purified Epidermin

As indicated in Fig. 5, the epidermin maintained 89% of its activity when incubated at temperatures ranging from 30, 37, 42, and 47. However, above this temperature, the epidermin's activity decreased, with residual activity reaching 30-40% at 53°C ($P \leq 0.05$). The activity of epidermin, as an antibacterial agent against *Pseudomonas* spp. could be affected by temperature. Generally, higher temperatures enhance epidermin's antimicrobial efficacy by less than 45%, while lower temperatures may reduce its activity by more than 30%. The epidermin's inhibitory activity rose at 37°C (Fig. 5), and this property decreased with higher temperatures until it lost its antibacterial potential at 53°C without an inhibition zone in the medium (Fig. 6A-C).

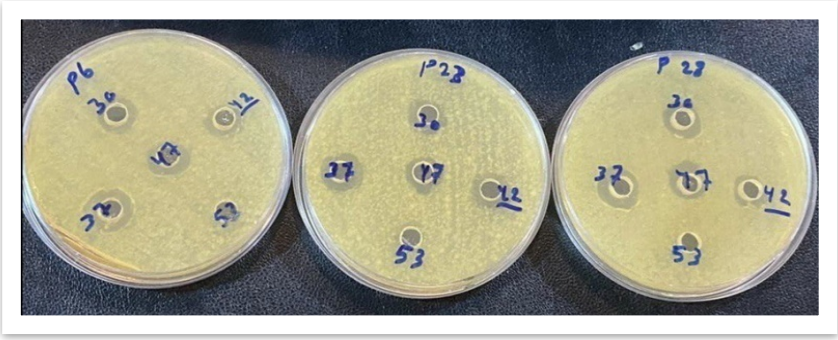

Fig. 5: Effect of temperature on epidermin activity as antibacterial agent against *Pseudomonas* species

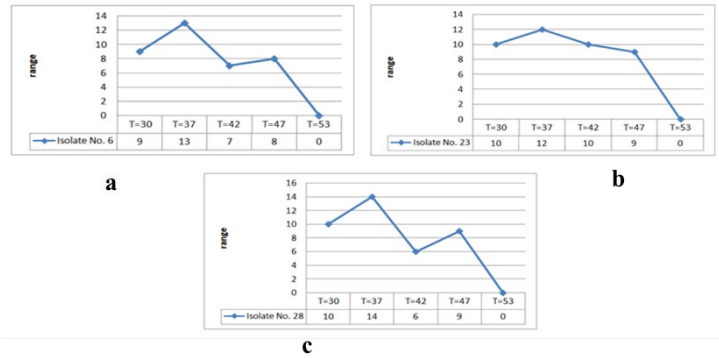

Fig. 6: Effect of temperature on the inhibitory effect of epidermin against *Pseudomonas* species. a: Isolate No. 6; b: Isolate No. 23; c: Isolate No. 28

Elevated temperatures, within a certain range, can increase epidermin activity against *Pseudomonas* species. This is because higher temperatures promote epidermin diffusion into the bacterial cells, facilitating its interaction with the microbial membrane. The increased fluidity of the bacterial cell membrane at higher temperatures also enhances the binding and disruption of the membrane by epidermin, leading to more effective antimicrobial action (Yao *et al*., 2022). Lower temperatures can diminish epidermin's antimicrobial activity. At colder temperatures, epidermin mobility and diffusion may be reduced, limiting its ability to effectively reach and interact with bacterial cells. This reduced diffusion can impede the

binding and disruption of the bacterial cell membrane, leading to decreased antibacterial efficacy against *Pseudomonas* species (Guo*et al*., 2022). It is imperative to note that the specific temperature range at which epidermin exhibits optimal antimicrobial activity against *Pseudomonas* species may vary and can be influenced by factors, such as the specific strain of *Pseudomonas* species, the concentration of epidermin, and the duration of exposure. Additionally, extreme temperatures (either too high or too low) can denature or degrade epidermin, rendering it less effective or inactive (Darbandi *et al*., 2022).

Optimal pH for Epidermin Stability

The optimal pH for the stability of epidermin, an antimicrobial peptide, can vary depending on the specific characteristics of the peptide and the experimental conditions. However, studies have shown that epidermin exhibits increased stability and activity in a slightly acidic to neutral pH range. The pH ranges from 5 to 9 affect epidermin stability, with the highest inhibition ($P \leq 0.05$) observed at pH 7 for three isolates. The lowest inhibition was observed at pH 6, with no resistance isolates identified (Table 2; Figs. 7 and 8A-C).

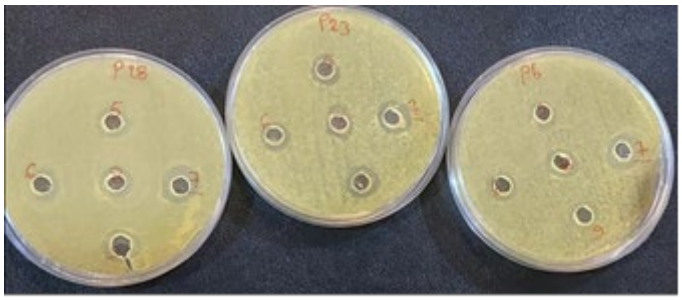

Fig. 7: Effect of pH on epidermin stability as antibacterial agent against *Pseudomonas* species

Table 1: Effect of pH on epidermin stability as an antibacterial agent against *Pseudomonas* species

Isolate No.	Diameter of inhibition zone (mm)					L.S.D. value
	pH 5	pH 6	pH 7	pH 8	pH 9	
6	10 ±0.52	8 ±0.37	12 ±0.64	9 ±0.51	10 ±0.57	1.749 *
23	8 ±0.37	6 ±0.22	11 ±0.58	8 ±0.45	9 ±0.51	2.057 *
28	6 ±0.21	7 ±0.29	12 ±0.61	10 ±0.52	8 ±0.37	2.166 *
L.S.D. value	2.071 *	1.894 *	1.447 NS	1.791 *	1.791 *	---
* ($P \leq 0.05$).						

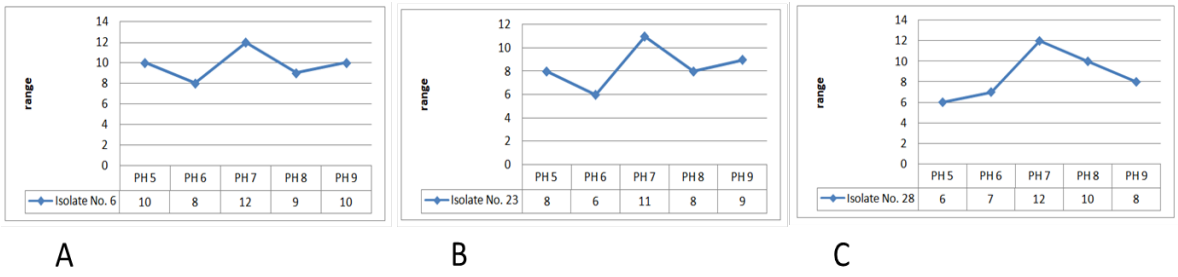

Fig. 8: Effect of pH on epidermin stability as antibacterial agent against *Pseudomonas* species A: Isolate No. 6; B: Isolate No. 23; C: Isolate No. 28

Epidermin demonstrated high stability and activity at neutral pH values, around pH 7. At this pH range, the peptide's structural integrity and antibacterial potential are generally maintained. The neutral pH range is relevant for applications where maintaining epidermin stability and activity are crucial (de Szalay & Wertz, 2023). It is critical to note that extreme pH values (extremely acidic or highly alkaline) can negatively impact epidermin stability and activity. Very low pH values can lead to peptide denaturation or degradation, while highly alkaline conditions can cause structural changes that reduce epidermin's effectiveness as an antimicrobial agent (Kuo *et al*., 2020). The optimal pH for epidermin may also depend on the specific bacterial target or the physiological conditions of the environment where the peptide is employed.

Effects of Heavy Metal Ions on Epidermin Activity

The impact of heavy metals on bacterial resistance and public health is a growing concern worldwide, particularly as it relates to the spread of antibiotic resistance and its implications for human health. Heavy metals, such as mercury, lead, cadmium, arsenic, and chromium, are toxic elements that persist in the environment due to industrial activities, agricultural practices, and improper waste disposal. These metals can accumulate in water, soil, and food chains, posing a significant risk to both ecosystems and human health (Pal et al., 2017). The results (Table 3) showed that in the presence of 50 mM Cu, isolate 23 demonstrated resistance against the test pathogen, *Pseudomonas* species, while all isolates at 100 mM Cu had reduced activity. A concentration of 100 mM Cd increased epidermin antimicrobial activity against the test isolates of *Pseudomonas* species, while less activity was observed at 50 mM Cd. There was resistance of isolates 28 and 23 when 50 and 100 mM of Co were used to supplement epidermin, respectively. The results of the present study are in agreement with the findings of Ahmed *et al*. (2015), who reported that Co and Cu inhibited epidermin activity. Copper ions (Cu^{2+}) have antimicrobial properties and are often used in various applications to control bacterial growth. When it comes to copper's effects on epidermin, it is expected that copper ions can enhance epidermin's antimicrobial activity in several ways. Epidermin exerts its antimicrobial effects by interacting with bacterial cell membranes and disrupting their integrity. Copper ions can synergize with epidermin by binding to bacterial membranes and destabilizing them further. This enhanced membrane disruption can increase epidermin efficacy against bacteria.

Table 2: Effect of various metal ions on epidermin activity as antibacterial agent against *Pseudomonas* species

Isolate No.	Diameter of inhibition zone (mm)					
	Cu (50 mM)	Cu (100 mM)	Cd (50 mM)	Cd (100 mM)	Co (50 mM)	Co (100 mM)
6	11 ±0.47	16 ±0.59	22 ±1.06	17 ±0.72	15 ±0.67	19 ±0.85
23	R	14 ±0.71	16 ±0.82	13 ±0.56	12 ±0.55	R
28	13 ±0.61	11 ±0.54	18 ±0.89	16 ±0.82	R	14 ±0.71
L.S.D. value	1.945 *	2.702 *	2.510 *	1.966 *	1.718 *	2.052 *
* ($P \leq 0.05$).						

Copper ions can also bind to the epidermis peptide chain, promoting structural stability. This stabilization may improve epidermin's overall activity and effectiveness as an antimicrobial agent (Tomić & Vuković, 2022). Copper ions can bind to the peptide and enhance its membrane-disrupting properties, resulting in improved antibacterial action, which is concentration-dependent (Libardo*et al*., 2014). While lower concentrations of copper can enhance the antimicrobial activity of epidermin, higher concentrations may become toxic to the peptide itself or the host cells. Therefore, careful consideration of copper concentrations is essential for optimizing epidermin's antimicrobial efficacy in the presence of copper ions (Rajput *et al*., 2021). Generally, copper ions can play a beneficial role in enhancing the antimicrobial activity of epidermin, making it a promising combination for antimicrobial applications.

Cadmium ions (Cd^{2+}) are heavy metallic ions with toxic effects on biological systems. The specific effects of cadmium on epidermin have not been extensively studied. Cadmium can inhibit enzymatic activities within bacterial cells, which may indirectly affect bacteria's susceptibility to antimicrobial peptides like epidermin. Cadmium ions can enter bacterial cells and disrupt various cellular processes. It is possible that cadmium accumulation within bacterial cells could alter their response to epidermin, making them more resistant to its antimicrobial effects. It should be taken into consideration that the specific effects of cadmium on epidermin can vary depending on the concentration of cadmium, exposure duration, and other factors (Li *et al*., 2021). Given the toxic nature of cadmium, high concentrations are likely to have a more pronounced negative impact on the antimicrobial activity of epidermin. However, further research is needed to fully understand the effects of cadmium on epidermin and its implications for antimicrobial efficacy.

Cobalt's specific effects on epidermin have not been extensively studied, and the information available on its direct impact on epidermin is limited. However, considering the characteristics of cobalt ions and their potential interactions with antimicrobial peptides, the possible effects of cobalt on epidermin can be speculated. Cobalt ions could bind to epidermin's peptide chain and induce structural changes. This interaction might affect the peptide's stability and conformation, potentially compromising its antimicrobial activity (El Ali *et al*., 2020). Cobalt exhibits cytotoxic effects, particularly at high concentrations. This toxicity could affect both the bacteria and potentially the host cells involved (Claudel *et al*., 2020). The exact effects

of metal ions on epidermin can depend on various factors, including the concentrations of the metal ions, pH, and the specific bacterial strains being targeted. Moreover, epidermin's mechanism of action may involve multiple factors beyond metal ions alone. Further research is needed to understand the intricate interactions between metal ions and epidermin's antimicrobial activity.

The findings of the present study demonstrated a significant antibacterial activity, particularly against Gram-negative pathogens like *Pseudomonas species*. Similar research on bacteriocins shows comparable findings, as several bacteriocins (like nisin and mutacin) also displayed potent activity against multidrug-resistant (MDR) and biofilm-forming bacteria (Gänzle and Hammes, 2009). Combining bacteriocins with antibiotics, such as chloramphenicol or ciprofloxacin, has been shown to enhance efficacy, suggesting a potential synergy (Kumar and Bhattacharya, 2014). Furthermore, heavy metals' influence on these antibacterial properties is notable, as certain metals can either enhance or inhibit bacteriocin function, much like their impact on antibiotic resistance (Matias, 2013). Heavy metals drive bacterial resistance through co-selection, stress-induced mutations, and horizontal gene transfer (HGT). Co-selection occurs when resistance genes for heavy metals and antibiotics coexist on mobile genetic elements, enabling bacteria to acquire multidrug resistance. Heavy metal exposure triggers stress responses, increasing mutation rates and activating efflux pumps that expel both metals and antibiotics, enhancing resistance. Additionally, oxidative stress damages bacterial DNA, fostering resistance evolution. Horizontal gene transfer further spreads resistance traits across bacterial populations, complicating the control of infections with standard antibiotics (Hobman and Crossman, 2015; Fashola et al., 2016).

Combining bacteriocins like epidermin from *Staphylococcus epidermidis* with heavy metals could have significant clinical and environmental applications. In clinical settings, bacteriocins are known for their antibacterial activity, and heavy metals can enhance this effect by disrupting bacterial cell walls and increasing the permeability of bacterial membranes. This combination could be particularly useful in overcoming antibiotic resistance by synergizing bacteriocins with metals to target resistant pathogens, a potential new treatment for resistant infections in hospital settings. In environmental applications, this combination could be used in wastewater treatment or soil remediation to control microbial contamination, especially in environments polluted with heavy metals.

The combination of heavy metals like cadmium with bacteriocins, such as epidermin, enhances antibacterial activity by disrupting bacterial membranes and boosting bacteriocin efficacy. However, cadmium's toxic effects, including oxidative stress, DNA damage, and chronic health risks like kidney damage and cancer, pose significant concerns. Prolonged cadmium exposure and tissue accumulation amplify these risks, raising questions about its safety in clinical applications (Klaassen, 2008). While cadmium may broaden bacteriocin effectiveness, its potential toxicity necessitates careful evaluation to balance therapeutic benefits against environmental and health hazards. To mitigate the risks associated with cadmium toxicity, several strategies could be explored. For instance, using lower concentrations of cadmium in combination with bacteriocins, or targeting specific delivery mechanisms that reduce systemic exposure, could help balance the therapeutic benefits and toxic risks. Additionally, bioremediation techniques, which use microbes or plants to detoxify environments contaminated with cadmium, could potentially help reduce the environmental

impact of such combinations (Foley, 2018). Overall, while the combination of bacteriocins with heavy metals offers exciting possibilities, further research into their safety profiles, especially concerning heavy metal toxicity, is essential to ensure their safe and effective use in both medical and environmental applications.

CONCLUSIONS

The purification and characterization of epidermin from *Staphylococcus epidermidis* confirm its potential as a natural antibacterial agent, particularly against gram-positive bacteria. Also, heavy metals enhanced the inhibitory activities of epidermin. Understanding the anti-enzymatic activity of purified epidermin and heavy metal interaction will help to develop novel strategies for combating *Pseudomonas* infections in clinical and environmental situations. Further investigations are required to explore the potential applications of the synergistic effects of epidermin and heavy metals as antimicrobial agents in the future.

ACKNOWLEDGEMENTS
The authors would like to thank the authority of Mustansiriyah University (www.uomustansiriyah.edu.iq), Baghdad, Iraq, for supporting this research.

CONFLICT OF INTEREST
The authors declared no conflict of interest.

REFERENCES

Abbas Ahmed, M., Dahad, F., Taha, M. T. and Hassan, S. F. 2015. Production, purification and characterization of L-asparaginase from marine endophytic Aspergillus sp. ALAA-2000 under submerged and solid-state fermentation. J Microb. Biochem Technol., 7(3):165-172. DOI: 10.4172/1948-5948.1000199

Ali, H., Khan, E., & Sajad, M. A. (2013). Phytoremediation of heavy metals—Concepts and applications. Chemosphere, *91*(7), 869-881. doi: 10.1016/j.chemosphere.2013.01.075.

Alinaghi, A., Macedo, A., Cheruvu, H. S., Holmes, A. and Roberts, M. S., 2022. Human epidermal in vitro permeation test (IVPT) analyses of alcohols and steroids.Int J Pharm., 627:122114. doi: 10.1016/j.ijpharm.2022.122114.

Alloway, B. J., 2013. Heavy metals in soils: trace metals and metalloids in soils and their bioavailability. Springer. 236p.

Al-Soufi, W., Reija, B., Novo, M., Felekyan, S., Kühnemuth, R. and Seidel, C. A. M., 2005. Fluorescence correlation spectroscopy, a tool to investigate supramolecular dynamics: inclusion complexes of pyronines with cyclodextrin.J Am Chem Soc.,127(24): 8775-8784. doi: 10.1021/ja0508976

Alkhawaja, E., Hammadi, S., Abdelmalek, M., Mahasneh, N., Alkhawaja, B. and Abdelmalek, S. M., 2020. Antibiotic-resistant Cutibacterium acnes among acne patients in Jordan: a cross-sectional study. BMC Dermatol., 20(1):1-9. doi: 10.1186/s12895-020-00108-9.

Allgaier, H., Jung, G., Werner, R. G., Schneider, U. and Zähner, H. J., 1985. Elucidation of the structure of epidermin, a ribosomally synthesized, tetracyclic heterodetic polypeptide antibiotic.PLos One., 24(12):1051-1053. https://doi.org/10.1002/anie.198510511

Baker-Austin, C., Wright, M. S., Stepanauskas, R., & McArthur, J. V. 2006. Co-selection of antibiotic and metal resistance. *Trends in Microbiology, 14*(4), 176–182. Doi: 10.1016/j.tim.2006.02.006.

Bradford, M. M. J. A. B. 1976. A rapid and sensitive method for the quantifiction of microgram quantities of protein utilizing the principle of protein-dye binding.*Anal Biochem*., 72(1-2): 248-254. doi: 10.1006/abio.1976.9999

Brown, M. M.andHorswill, A. R. J. P. P. 2020. Staphylococcus epidermidis—Skin friend or foe? PLoS Pathog.,16(11): e1009026. https://doi.org/10.1371/journal.ppat.1009026

Cau, L., Williams, M. R., Butcher, A. M., Nakatsuji, T., Kavanaugh, J. S., Cheng, J. Y. et al. . (2021). Staphylococcus epidermidis protease EcpA can be a deleterious component of the skin microbiome in atopic dermatitis. J. Allergy Clin. Immunol.,147(3): 955-966. doi: 10.1016/j.jaci.2020.06.024

Chakraborty, N., Joshi, A., Ahuja, K., Vashisht, A., Basu, A., Purty, R. S. and Chatterjee, S. 2023. Immunogenicity studies on lantibiotics. In *Lantibiotics as Alternative Therapeutics. Elsevier* (pp. 255-275). DOI:10.1016/B978-0-323-99141-4.00009-6

Claudel, M., Schwarte, J. V. and Fromm, K. M., 2020. New antimicrobial strategies based on metal complexes. Chemistry. 2(4): 849-899. https://doi.org/10.3390/chemistry2040056

Darbandi, A., Asadi, A., Mahdizade Ari, M., Ohadi, E., Talebi, M., Halaj Zadeh, M. et al. 2022. Bacteriocins: Properties and potential use as antimicrobials. J. Clin. Lab. Anal., 36(1): e24093. doi: 10.1002/jcla.24093.

de Szalay, S. and Wertz, P. W. 2023. Protective barriers provided by the epidermis.Int. J. Mol. Sci., 24(4): 3145. doi: 10.3390/ijms24043145

El Ali, Z., Ollivier, A., Manin, S., Rivard, M., Motterlini, R., and Foresti, R. 2020. Therapeutic effects of CO-releaser/Nrf2 activator hybrids (HYCOs) in the treatment of skin wound, psoriasis and multiple sclerosis. Redox Biol., 34: 101521. doi: 10.1016/j.redox.2020.101521

Fashola, M. O., Ngole-Jeme, V. M., &Babalola, O. O. (2016). Heavy metal pollution from gold mines: Environmental effects and bacterial strategies for resistance. Int J Environ Res Public Health., 13(11): 1047. doi: 10.3390/ijerph13111047

Foster, T. J., 2020. Surface proteins of *Staphylococcus epidermidis*.*Sec.* Front Microbio.*,*11: 1829. doi: 10.3389/fmicb.2020.01829

Foley, R. H., et al. 2019. Bioremediation strategies for reducing cadmium toxicity in the environment. Environmental Toxicology and Chemistry.

Gandhi, K. A., Sunmathi, D., and Nanthavanan, P. 2019. Extraction, characterization and partial purification of L-Asparaginase from the leaves of *Arachis hypogaea* L., Biosci., Biotech. Res. Asia, 18(3): 681-691. http://dx.doi.org/10.13005/bbra/2783

Gänzle, M. G., & Hammes, W. P. (2009). Lactic acid bacteria and bacteriocins in fermented foods. Food Research International, 42(8): 826-832. Doi: 10.1016/j.foodres.2009.04.004.

Grandjean, P., Landrigan, P. J., 2014. Neurobehavioural effects of developmental toxicity. Lancet Neurol, 13(3): 330-338.doi: 10.1016/S1474-4422(13)70278-3

Guo, C., Cheng, F., Liang, G., Zhang, S., Jia, Q., He, L., *et al*., 2022. Copper-based polymer-metal–organic framework embedded with Ag nanoparticles: Long-acting and intelligent antibacterial activity and accelerated wound healing, Chem. Eng. J*.,* 435(8): 134915. DOI:10.1016/j.cej.2022.134915

Gupta, S., 1996. The Short Textbook of Pediatrics (7th ed.). Jaypee Brothers Medical Publishers.

Hobman,, J. L., & Crossman, L. C., 2015. Bacterial antimicrobial metal ion resistance. J. Med. Microbiol., 64(5): 471-497. doi:10.1099/jmm.0.023036-0

Kelland, L., 2007. The resurgence of platinum-based cancer chemotherapy. Nature Rev. Cancer, 7: 573–584. doi: 10.1038/nrc2167

Klaassen, C. D., & Liu, J., 2008. Toxicology of cadmium: Mechanisms of toxicity, mutagenesis, and carcinogenesis. In C. D. Klaassen (Ed.), Casarett and Doull's Toxicology: The Basic Science of Poisons (7th ed.). McGraw-Hill Education.

Kumar, A., & Bhattacharya, M., 2014. Synergy between bacteriocins and antibiotics in enhancing antimicrobial activity. J. Antibiotics, 67(5): 183-188. doi: 10.1038/ja.2013.114.

Kuo, S.-H., Shen, C.-J., Shen, C.-F., and Cheng, C.-M. 2020. Role of pH value in clinically relevant Diagnostics (Basel)., 16;10(2):107. doi: 10.3390/diagnostics10020107

Leite, E. L., Oliveir.JR, A. F., Carmo, F. L., Berkova, N., Barh, D., Ghosh, P., and, Azevedo, V. 2020. Bacteriocins as an alternative in the treatment of infections by *Staphylococcus aureus*.*An Acad. Bras.Cienc*., (suppl 2):e20201216. doi: 10.1590/0001-3765202020201216

Lemire, J. A., Harrison, J. J., & Turner, R. J. (2013). Antimicrobial activity of metals: Mechanisms, molecular targets, and applications. *Nature Reviews Microbiology, 11*(6): 371–384. doi: 10.1038/nrmicro3028.

Li, Z., Wang, Y., Liu, J., Rawding, P., Bu, J., Hong, S., and Hu, Q.2021. Chemically and biologically engineered bacteria-based delivery systems for emerging diagnosis and advanced therapy. Adv. Mater*.,* 33(38):e2102580. doi: 10.1002/adma.202102580

Libardo, M. D., Cervantes, J. L., Salazar, J. C., and Angeles-Boza, A. M. 2014. Improved bioactivity of antimicrobial peptides by addition of amino-terminal copper and nickel (ATCUN) binding motifs.ChemMedChem., 9(8): 1892-1901. doi: 10.1002/cmdc.201402033

Liu, J., Goyer, R. A., & Waalkes, M. P., 2008. Toxic effects of metals. In Casarett & Doull's Toxicology: The Basic Science of Poisons (7th ed.). McGraw-Hill, 468p.

Liu, Q., Liu, Q., Meng, H., Lv, H., Liu, Y., Liu, J.,*et al*., 2020. *Staphylococcus epidermidis* contributes to healthy maturation of the nasal microbiome by stimulating antimicrobial peptide production.Cell Host Microbe*.,* 27(1): 68-78.e5. doi: 10.1016/j.chom.2019.11.003

Łoboda, A., Sobczak, M., Król, J., & Targosz-Korecka, M. (2018). Role of metal ions in the structural stabilization and activity enhancement of antimicrobial peptides. *Journal of Biological Chemistry*, 293(22), 8575–8586. https://doi.org/10.1074/jbc.RA117.000895

Madhu, P. M., Sadagopan, R. S., 2020. Effect of heavy metals on growth and development of cultivated plants with reference to cadmium, chromium and lead–a review. J. Stress Physiol. Biochem*.,*16(3): 84-102.

Maisnier-Patin, S., Richard, J. 1995. Activity and purification of linenscin OC2, an antibacterial substance produced by Brevibacterium linens OC2, an orange cheese coryneform

bacterium. Appl. Environ. Microbiol., 61(5): 1847-1852. doi: 10.1128/aem.61.5.1847-1852.1995

Matias, E. A., et al. (2013). Heavy metals and their impact on antibiotic resistance in bacteria: A review. *Environmental Toxicology and Pharmacology*, 36(2): 343-351. Doi: https://doi.org/10.1016/j.etap.2013.06.007

Makky, E., Ong, J. J., Karim, M. R., and Lee, C. J. 2013. Production and optimization of L-asparaginase by Bacillus sp. KK2S4 from corn cob. Afr. J. Biotechnol., 12(19): 2654-2658 DOI: 10.5897/AJB2013.12231

Mirhosseini, M., Nahvi, I., Emtiazi, G., & Tavassoli, M. 2010.Characterisation of anti-Listeria monocytogenes bacteriocins from *Enterococcus faecium* strains isolated from dairy products. Int. J. Dairy Technol., 63(1): 55-61. DOI:10.1111/j.1471-0307.2009.00543.x

Nriagu, J. O., 1996. History of global metal pollution. Science, 272(5259): 223-224 DOI: 10.1126/science.272.5259.223.

Pal, C., Asiani, K., Arya, S., Rensing, C., Stekel, D. J., Larsson, D. G. J., & Hobman, J. L. 2017. Metal resistance and its association with antibiotic resistance. Adv. Microb. Physiol., 70: 261-313. doi:10.1016/bs.ampbs.2017.02.001

Prasad, A. S., 2008. Zinc in human health: effect of zinc on immune cells. Mol. Med., 14(5-6): 353–357.doi: 10.2119/2008-00033.Prasad.

Rajput, V., Chaplygin, V., Gorovtsov, A., Fedorenko, A., Azarov, A., Chernikova, N.,*et al*., 2021. Assessing the toxicity and accumulation of bulk-and nano-CuO in *Hordeum sativum* L.Environ Geochem Health, 43(6): 2443-2454. doi: 10.1007/s10653-020-00681-5

Qin, J., Zhang, L., Li, J., & Wang, Q., 2020. Ion-Exchange Chromatography and Gel Filtration for Purification of Proteins. Journal of Chromatography B, 1144, 121982. https://doi.org/10.1016/j.jchromb.2020.121982

RSC Publishing. 2018. Metal ion interactions with antimicrobial peptides: Mechanisms of action and enhancement of activity. *Chemical Science Reviews*, 7(5): 332-349. https://doi.org/10.1039/C7CS00568G

Said-Salman, I., Jebaii, F., Yusef, H., Moustafa, M.E. 2019. Evaluation of Wi-Fi radiation effects on antibiotic susceptibility, metabolic activity and biofilm formation by *Escherichia coli* 0157H7, *Staphylococcus aureus* and *Staphylococcus epidermis*. J. Biomed. Phys. Eng., 9(5): 579-586. doi: 10.31661/jbpe.v0i0.1106

Sahu, S., Mishra, S., & Kumar, P. (2019). Purification of epidermin from *Staphylococcus epidermidis* using ion-exchange and gel filtration chromatography. J. Chromatog. Sci., 57(5): 417-424.

Schnell, N., Entian, K.-D., Schneider, U., Götz, F., Zähner, H., Kellner, R., and Jung, G. 1988. Prepeptide sequence of epidermin, a ribosomally synthesized antibiotic with four sulphide-rings. *Nature.,* 333(6170): 276-278. doi: 10.1038/333276a0.

Seiler, C., & Berendonk, T. U., 2012. Heavy metal driven co-selection of antibiotic resistance in soil and water bodies impacted by agriculture and aquaculture. Front. Microbio*,* 3:399. Doi: https://doi.org/10.3389/fmicb.2012.00399

Severn, M. M., & Horswill, A. R. 2023. *Staphylococcus epidermidis* and its dual lifestyle in skin health and infection. Nat. Rev. Microbiol., 21(2): 97-111. doi: 10.1038/s41579-022-00780-3

SPSS (2019). Statistical Packages of Social Sciences-SPSS/ IBM Statistics 26 step by step. 16th Edition.https://doi.org/10.4324/9780429056765.

Stellwagen, E., 1990. Gel filtration. Methods in enzymol, Elsevier, 182: 317-328. https://doi.org/10.1016/0076-6879(90)82027-Y

Steunou, A. S., Durand, A., Bourbon, M.-L., Babot, M., Tambosi, R., Liotenberg, S., and Ouchane, S. 2020. Cadmium and Copper Cross-Tolerance. Cu+ Alleviates Cd2+ Toxicity, and Both Cations Target Heme and Chlorophyll Biosynthesis Pathway in *Rubrivivax gelatinosus*. Front. Microbiol*.,* 11: 1-12.doi: 10.3389/fmicb.2020.00893

Tille, P., 2015. Bailey & Scott's diagnostic microbiology-E-Book. Elsevier Health Sciences.

Tomić, S. L., and Vuković, J. S. 2022. Antimicrobial Activity of Silver, Copper, and Zinc Ions/Poly (Acrylate/Itaconic Acid) Hydrogel Matrices Inorganics.,10(3): 38. https://doi.org/10.3390/inorganics10030038

Valko, M., Morris, H., Cronin, M. T. D. 2005. Metals, toxicity and oxidative stress. Curr. Med. Chem, 12(10): 1161-1208. doi: 10.2174/0929867053764635

Whitaker, J. R., and Bernhard, R. A., 1972. Experiments for: an introduction to enzymology. Whiber Press.

World Health Organization (WHO). (2021). *Guidelines for Drinking-water Quality* (4th ed.).

Yao, S., Hao, L., Zhou, R., Jin, Y., Huang, J., Wu, C., 2022. Multispecies biofilms in fermentation: Biofilm formation, microbial interactions, and communication. Comp. Rev. Food Sci. Food Saf., 21(4): 3346-3375. doi: 10.1111/1541-4337.12991

Zheng, C. X., Ma, X. F., Zhang, Y. H., Li, H. J., and Zhang, G. F., 2018. Research progress in the mechanism of translation initiation of eukaryotic mRNAs. Yi Chuan*.,* 40(8): 607-619. doi: 10.16288/j.yczz.17-393.