# We are IntechOpen, the world's leading publisher of Open Access books Built by scientists, for scientists

**6,900**
Open access books available

**186,000**
International authors and editors

**200M**
Downloads

**154**
Countries delivered to

Our authors are among the

**TOP 1%**
most cited scientists

**12.2%**
Contributors from top 500 universities

CLARIVATE ANALYTICS
**BOOK CITATION INDEX**
INDEXED

**WEB OF SCIENCE**™

Selection of our books indexed in the Book Citation Index
in Web of Science™ Core Collection (BKCI)

## Interested in publishing with us?
Contact book.department@intechopen.com

Numbers displayed above are based on latest data collected.
For more information visit www.intechopen.com

# Bioinformatics Discovery of Vertebrate Cathelicidins from the Mining of Available Genomes

José Manuel Pérez de la Lastra,
Cristina Garrido-Orduña, Andrés A. Borges,
David Jiménez-Arias,
Francisco Javier García-Machado,
Mercedes Hernández, Concepción González and
Alicia Boto

Additional information is available at the end of the chapter

## Abstract

Due to the worrying increase in antimicrobial resistance to conventional antibiotics, the search for alternatives is becoming increasingly important. Antimicrobial peptides (AMPs), originating from natural resources, have been recognised as a novel class of antibiotics. An advantage of peptides over antibiotics is that the resistance is more difficult to attain than for conventional antibiotics. With the increasing number of genomes sequenced and available in the public domain, one alternative methodology to obtain novel AMPs is to analyse genes and proteins from genomic databases to predict and identify amino acid sequences that share similarities and molecular features with natural bioactive antimicrobial peptides. In this chapter, we summarise some of our recent results on the production of antimicrobial peptides, particularly, how we managed to identify a family of antimicrobial peptides: cathelicidins, through bioinformatics tools, from the genomes of two lower vertebrates (a reptile and a bird) available in public databases. We hope that our preliminary investigation with these novel peptides could be useful for the design of future strategies that pursue the production of antimicrobial peptides through biotechnology.

**Keywords:** cathelicidin, antimicrobial, peptide, antibiotics, genome mining

## 1. Introduction

Conventional drugs for the treatment of human diseases can be classified as small molecules, proteins and peptides. The cost of goods, products quality and scalability are always major factors when considering the development of any new class of drugs. Generally, in the drug development process, small molecules are still preferred over peptides primarily due to their ease of production, simplicity of administration (as oral pill), and superior pharmacodynamics properties. In addition to stability and delivery, these factors have historically been seen as hurdles for peptide drug development versus their small molecule counterparts [1].

In the case of antibiotics, due to the worrying increase in antimicrobial resistance to conventional antibiotics, together with the long-term decline of investment by pharmaceutical companies, the search for alternatives is becoming increasingly important [2]. Traditionally, soil bacteria (especially actinomycetes), fungi and higher plants were main sources for drug discovery. However, no new class of antibiotics has been discovered since 1987 [3]. Antimicrobial peptides, originating from natural resources, have been recognised as a novel class of antibiotics [4]. Cationic antimicrobial peptides neutralise target pathogens through deleterious interactions with membrane components, which relies on the difference in lipid composition between normal eukaryotic and prokaryotic (or transformed eukaryotic) cell membranes [5]. Therefore, microbial resistance to antimicrobial peptides is rare. Moreover, peptide antimicrobials are active against both metabolically active and non-metabolising microorganisms, whereas most antibiotics have specific targets that define their mechanism of action and are active only against replicating microbes. Antimicrobials with novel *modi operandi* are consequently in high demand in a world faced with an increasing number of multidrug resistant pathogens, as well as a changing panorama of infections [4, 6].

Our emphasis in this chapter will be on the discovery of naturally occurring antimicrobial peptides for use in development of new antimicrobial drugs. Discovery of natural bioactive molecules is, of course, only the first step in the biodiscovery pipeline. The spectrum of available candidate peptide drugs is by far not limited to the human peptide pool. Through evolution, numerous peptides have evolved to exhibit their natural bioactivity outside of the producing organisms [5]. The cationic peptides polymyxin B and gramicidin S have been used in the clinic as topical over-the-counter medicines for a long time, and the cationic lantibiotic nisin is used as an antimicrobial food additive. In general, antimicrobial peptides in different species are surprisingly different. For example, magainins found in the skin of the frog *Xenopus laevis* were initially thought to be universal antimicrobial agents. However, it turns out that every frog investigated has its own peptide antibiotics [7]. It has been argued that that the immense diversity of cationic peptides arises from their antimicrobial function as well as the different pathogenic microbe challenges they face in each host organisms. We believe that nature still harbours a virtually infinite array of potential peptidic medications that await human pharmacological characterisation. Particularly, organisms living in germ-filled environments could be an abundant source of antimicrobials and can likely provide us with superior templates for use in development of new antimicrobial drugs to help solve the medical problems that exist today [8].

With the advent of facile genome sequencing, new antibiotics are being found by the techniques of genome mining, offering hope for the future [9]. The aim of this study is to illustrate the identification of novel antimicrobial peptides from exotic vertebrates through refined computational methods. Genome mining involves looking at the sequenced genome of organisms to determine if gene cluster involved in the production of new antimicrobial peptides can be found in these organisms. The cathelicidins are a family of antimicrobial peptides encoded in the genome of vertebrates. These peptides are characterised by being formed by a pre-propeptide, which in neutrophils is processed by the enzyme elastase to originate the active peptide. There is a great variability between the sequences of the active peptides of cathelicidins [10]. However, the pre-pro peptide is highly conserved, which is very useful for the mining of vertebrate genomes to identify putative genes of this family of antimicrobial peptides. The genes coding for cathelicidins typically consist of four exons, the fourth exon being the one that codes for the active peptide and, therefore, the least conserved. The number of cathelicidin genes is different among species. For example, cows have 11 genes, humans have only one, and sheep have eight [10, 11]. This structural and/or functional diversity within the cathelicidin family could reflect the spectrum of microbicide and immunomodulatory activities of cathelicidins in different species exposed to different microbial load [12].

We have previously described the characterisation of peptides derived from the genomes of mammals [13]. We here illustrate a simple method for the identification of two cathelicidin genes in the genome of lower vertebrates (birds and reptiles) living in different environments, and thus surrounded by different pathogens. We also perform the bioinformatic analysis of the corresponding active peptides to detect possible antimicrobial properties and describe the functional characterisation of the two identified cathelicidins against a panel of reference microorganisms. Finally, we describe our current attempt for the biotechnological production of these peptides in microalgae.

## 2. Materials and methods

### 2.1. Obtaining of the cathelicidin sequences

Access to the genomes of the different species studied was carried out in September 2012, through the genebank public databases (http://www.ncbi.nlm.nih.gov/genome). At these dates, different genomes were still assembled in contiguous, or contigs, that were accessible for tracing by nucleotide BLAST (http://blast.ncbi.nlm.nih.gov/Blast.cgi).

For the screening of animal genomes, a nucleotide sequence of 83 base pairs (bp) was used, based on a highly conserved region between bird cathelicidins, which corresponds to the following sequence:

cccaggctgtggactcctacaaccaacggcctgaggtgcagaatgccttccggctgctcagcgccgaccccgagcccggcccg

P R L W T P T T N G L R R M P S G C S A P T P S P A

The search for sequences homologous to this sequence was made using the BLAST application, using the values of the default parameters, which returned us the contiguous (contigs), or scaffolds, of genomic DNA where sequences homologous to our sequence and, therefore, probable coding genes for cathelicidins.

## 2.2. Prediction of cathelicidin genes

Once the different contigs of homology were obtained with this sequence, we proceeded to study if they contained genes corresponding to cathelicidins. For this, two types of programs were used: GenScan (http://genes.mit.edu/GENSCAN.html) and GeneMark (http://exon. gatech.edu/GeneMark/), with the default values of the different parameters. Both programs predict putative genes within these contigs or scaffolds of previously selected sequences. The criteria adopted for the identification of cathelicidins in these contigs was that the predicted genes contained four exons and that in addition the amino acid sequence of the first three exons was homologous to the already known cathelicidins, with a percentage of residue identity of more than one 30%, provided that the position of some key residues of the pre-pro-peptide, such as cysteines, essential for the maintenance of its structure, is preserved.

## 2.3. Prediction of antimicrobial peptides

The fourth exon of the predicted cathelicidins was then translated into amino acids and, to identify the possible cleavage site by the neutrophil elastase enzyme, the amino acid sequence of the fourth exon was subjected to the online Peptidecutter tool (http://web. expasy.org/peptide_cutter/), taking as an active peptide that generated after the theoretical cut by this enzyme in its leftmost position, towards the C-terminal end.

## 2.4. Bioinformatic analysis of the cathelicidin sequences

The percentage of hydrophobicity and the Boman index of the derived cathelicidins were calculated by the ADP3 tool (http://aps.unmc.edu/AP/prediction/prediction_main.php). The Boman index estimates the potential of a protein to bind to another protein. Thus, if a protein has a high Boman index, it means that said peptide would be multifunctional and, once inside the cell, it would have more or less capacity to bind to several proteins and perform different functions within the cell, given its capacity to interact with a wide range of proteins. The antimicrobial activity of the peptides was evaluated in silico with the AMPA tool (http://tcoffee.crg.cat/apps/ampa/do) described in [14] Torrent et al. and the APD3 tool (http://aps. unmc.edu/AP/prediction/prediction_main.php), described in [15], using the parameters that both have defined by default. The secondary structure of the peptides was calculated using the PSIPred tool (http://bioinf.cs.ucl.ac.uk/psipred/). The alpha-helicity of the regions present in the peptides was visualised in 2D by the helical projection tool available on the website http://rzlab.ucr.edu/scripts/wheel/wheel.cgi.

## 2.5. Peptide synthesis

After performing the bioinformatic study, peptides were synthesised (Caslo, Lyngby, Denmark) on a 10 mg scale and with a purity of more than 90% (analysed by HPLC). To avoid

degradation of peptides during storage, their N-terminal end was acetylated and their C-terminal end amidated. For the performance of the different assays, aliquots of each of the polypeptides (2 mg) were dissolved in pure water to a stock concentration of 100 μM.

## 2.6. Antimicrobial activity

The *in vitro* antimicrobial activity of the polypeptides was tested against the following pathogenic microbes, including bacteria and fungi: *Staphylococcus aureus* ATCC 6538, *Salmonella* sp. CECT 456, *Klebsiella pneumoniae* ATCC 23357, *Escherichia coli* ATCC 9637, *Bacillus cereus* ATCC 21772, *Proteus mirabilis* CECT 170, *Enterococcus faecalis* ATCC 29212, and *Candida albicans*. The minimum peptide concentration necessary to inhibit the growth of the tested microorganisms was evaluated by MIC assay, as described elsewhere [16]. Pathogenic microorganisms tested to evaluate the antimicrobial activity of the peptides were preserved at a temperature of −80°C in Luria-Bertani medium (LB) supplemented with 20% volume/volume glycerin. To determine the MIC, the peptides were solubilised in sterile milli-Q water to a final concentration of 100 μM, after which serial dilutions were carried out. 50 μl of each dilution of each polypeptide was mixed with 50 μl of the corresponding bacterial pointer suspension in Mueler-Hilton medium to a total volume of 100 μl in each well of a microplate. Thus, the effective concentrations to which the bacterial suspensions were subjected were 50, 25, 12.5, 6.5, 3.2, 1.6, 0.8, 0.4, 0.2 and 0.1 μM. Two replicates were carried out for each strain, concentration and peptide under test. As positive controls water was used in place of the specific polypeptide, and as negative controls each of the polypeptides was used without the corresponding bacterial suspension. In addition, two non-antimicrobial peptides were used as negative controls. Such peptides had a structure similar to the peptides of the invention (α-helix), but their sequence is totally different and shows no antimicrobial activity. The microplates were incubated at 37°C for 12–16 h with a shaking of 20 s prior to the measurement of the absorbance. To evaluate microbial growth, the optical density at 600 nm was measured using an EZ Biochrome plate reader. Each experiment was performed twice. As MIC, the lowest peptide concentration was taken at which no bacterial growth occurred at the end of the experiment.

## 2.7. Haemolytic activity

The haemolytic activity of the peptides is an indicator of the toxicity of peptides in eukaryotic cells, and a characteristic that is generally determined for those compounds that may come into contact with the human body. A lower percentage of haemolysis could be correlated with a lower cytotoxic activity of the peptide. Briefly, the haemolytic activity of the peptides was evaluated by determining the release of haemoglobin that is produced by contacting a solution comprising each of the peptides with a suspension of 10% volume from fresh human blood. Collection of blood samples was performed under aseptic conditions using a Vacutainer® K2E (Belliver, Great Britain) system with EDTA, and stored in Alsever medium at 4°C. When the assays were performed, blood was collected centrifuged at 6000 g for 5 min to separate the erythrocytes. After the erythrocytes were collected, they were washed three times with PBS buffer and resuspended in PBS buffer until a suspension containing 10% by volume of erythrocytes was obtained. In parallel, the peptides were solubilised in PBS buffer to obtain a battery of concentrations for each of them ranging from 12.5, 6.25, 3.13, 1.56, 0.78, 39, 0.2, 0.1,

0.05 and 0.02 μM. Three replicates were used for each peptide and concentration tested. Briefly, 50 μl of the 10% by volume erythrocyte suspension was mixed with 50 μl of the aliquots comprising each of the different concentrations of the peptide to be tested in each well of a 96-well plate, and the mixture for 45 min at 37°C under agitation. The plates were then centrifuged at 3500 g for 5 min, and 80 μl aliquots of the supernatant were transferred to 100-well flat bottom microplates, which were diluted with 80 μl of milli-Q water. The degree of haemolysis was determined from the absorbance at 540 nm with a plate reader. As a positive control for haemolysis assays, a PBS buffer solution containing 1% Triton X-100 (Sigma-Aldrich, Spain) was used. The percentage of haemolysis (H) was determined using the equation: $H = 100 \times [(Op - Ob)/(Om - Ob)]$ where Op is the optical density measured for one of the concentrations of the peptide tested; Ob is the optical density of the buffer solution; and Om is the optical density for the positive control with Triton X-100.

## 2.8. Molecular cloning of cathelicidins

Synthetic DNA (Genscript, Piscataway, NJ, USA) corresponding to amino acid sequence of the peptides was designed so that they contained the signal peptide from the Chlamydomonas aryl sulphatase protein, the codon optimisation for *C. reinhardtii* and they incorporated restriction sites for correct positioning into the multiple cloning sites (MCS) of the vector pChlamy_4. (Thermofisher, Madrid, Spain). For the transformation of the cyanobacteria *S. elongatus*, primers were designed to incorporate restriction sites for the correct positioning of the amplified inserts into the vector pSyn_6 (Thermofisher). The TAP and BG11 media and all restriction enzymes and primers were purchased from Thermofisher. The correct cloning of the inserts was verified by PCR (for colony screening) and by sequencing (Secugen, Madrid, Spain).

## 2.9. Transformation of microalga

For the cloning and transformation of the eukaryotic and prokaryotic microalga *C. reinhardtii* and *S elongatus*, we followed the recommendation of the manufacturer (Thermofisher). Antibiotics zeocin and spectinomycin were purchased from Thermofisher. The anti-V5 epitope antibody used for the immunodetection of recombinant peptides was purchased from Invitrogen.

# 3. Results

## 3.1. Identification of cathelicidins

Cathelicidins are found in varying numbers in numerous different species, including reptiles and birds. A remarkable degree of molecular diversity has been noted within this gene family. However, a well-conserved feature across evolutionary distant species is an N-terminal cathelin-domain. Using this domain to search into genome databases, we have found two novel cathelicidins from the genome mining of a reptile (painted turtle) and a bird (budgerigar). Predicted cathelicidins were identified by screening genome databases using the BLAST tool for DNA sequences. The painted turtle cathelicidin of 154 amino acids was found in contig number 974.21, whereas a bird cathelicidin of 151 amino acids was found in contig 900159920384 of the

newly sequenced genomes of these species, which from September 2012 were available in the genome database (http://www.ncbi.nlm.nih.gov/genome/).

The complete sequence of cathelicidins from these two species was assembled by joining the four exons identified in abovementioned contigs using GeneScan and GeneMarK programs and located in the following positions:

| Species | Exon | Begin | End | Length |
|---|---|---|---|---|
| Turtle | 1 | 799 | 933 | 135 |
|  | 2 | 2556 | 2663 | 108 |
|  | 3 | 3485 | 3568 | 84 |
|  | 4 | 4442 | 4579 | 138 |
| Bird | 1 | 125 | 298 | 174 |
|  | 2 | 499 | 606 | 108 |
|  | 3 | 712 | 792 | 81 |
|  | 4 | 877 | 969 | 93 |

The DNA sequence thus assembled was translated into amino acids and the resulting protein was subjected to PSI-BLAST to confirm that this protein was indeed a cathelicidin (not shown). The cathelicidin of the painted turtle, thus obtained, had the following sequence:

LRKDVLSFRDFPAEIPDEPPLVTPTDPSRCRVGSNLGPQRMSLYCDMTSNPRQELKFMV-KETVCPVSENLNGTECDFRDNGVVRDCSGFFSTQQESPIVIINCNTVTKED-PHIRRSRSPRRSRWPRRWYLPGSYTLIAHGGGKGKGKGSRLQMA

Whereas for the bird, the sequence of the corresponding cathelicidin was the following:

MPSSWALVLVVLGGACALPAPAPLAYTQALAQAVASYNQRPEVQNSFRLLSADPEPAP-SIQLSSLQLLNFTIMETQCPARARIHPDACEFKEDGLVKDCSAPVPQHGGPVLGVTCVD-STADPVRVKRFWPLLVTAIRTVAAGVSMFKSSKG

The coding sequence of exon 4 (in blue) is that which corresponds to the active peptide, while the remaining portion corresponds to the conserved pre-pro-peptide.

The amino acid sequence of exon 4 from both reptile and bird cathelicidin was then subjected to the prediction of the cut site for neutrophil elastase and it generated the following antimicrobial peptides:

**Cc_SP-37**: SPRRSRWPRRWYLPGSYTLIAHGGGKGKGKGSRLQMA

**Mm_KR-26**: KRFWPLLVTAIRTVAAGVSMFKSSKG

## 3.2. Bioinformatic analysis of peptides

Peptides Cc_SP-37 and Mm_KR-26 were subjected to *in silico* analysis of their antimicrobial activity using several bioinformatic tools. For example, the online tool APD3 calculated the different parameters related to the possible antimicrobial activity of the peptides. The APD

database has a unique peptide prediction program. For example, the program will calculate select properties of the peptide (e.g., net charge, length, percentage of hydrophobic residue and amino acid composition). The *in silico* structural analysis of the peptides indicated that both of them were alpha helical and had a positive net charge. However, the cathelicidin Mm_KR-26, from the bird, had a lower net charge, higher hydrophobicity and lower Boman index than the cathelicidin Cp_SP-37, from the reptile (**Table 1**).

To predict and calculate the regions of the protein with possible antimicrobial activity, the AMPA bioinformatics tool was used, which predict antimicrobial regions on any protein by assigning an antimicrobial index to each residue (**Figure 1**).

The AMPA algorithm also uses an antimicrobial propensity scale to generate an antimicrobial profile by means of a sliding window system. The probability values displayed correspond to misclassification probability that is the probability to find the predicted stretch in a non-antimicrobial protein. Using this tool, a single stretch, spanning residues 2–15, was found for the turtle cathelicidin Cc_SP-37, which confirmed the possible antimicrobial character of the predicted cathelicidin. No stretch was found for the bird cathelicidin Mm_KR-26 (**Figure 2**).

| Peptide | Specie | Net charge | Hydroph | Boman index |
|---------|--------|-----------|---------|-------------|
| Cp_SP-37 | *Chrysemys picta bellii* | +9 | 24% | 2.59 kcal/mol |
| Mm_KR-26 | *Melopsittacus undulatus* | +5 | 50% | 0.65 kcal/mol |

**Table 1.** Structural analysis of genome-derived cathelicidins from painted turtle (*Chrysemys picta bellii*) and budgerigar (*Melopsittacus undulatus*).
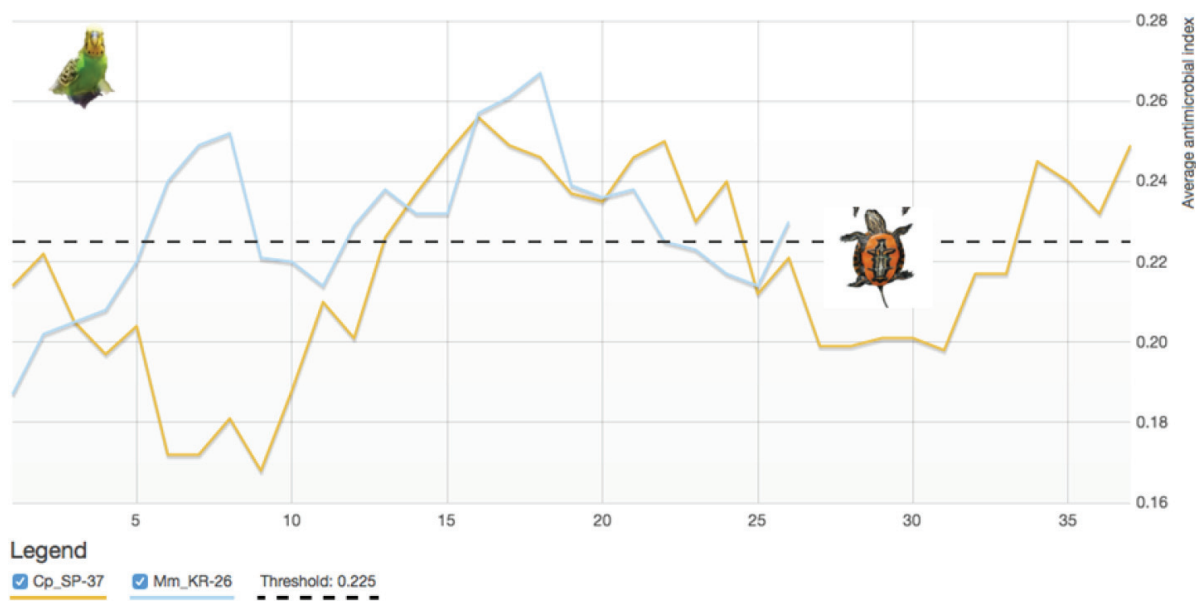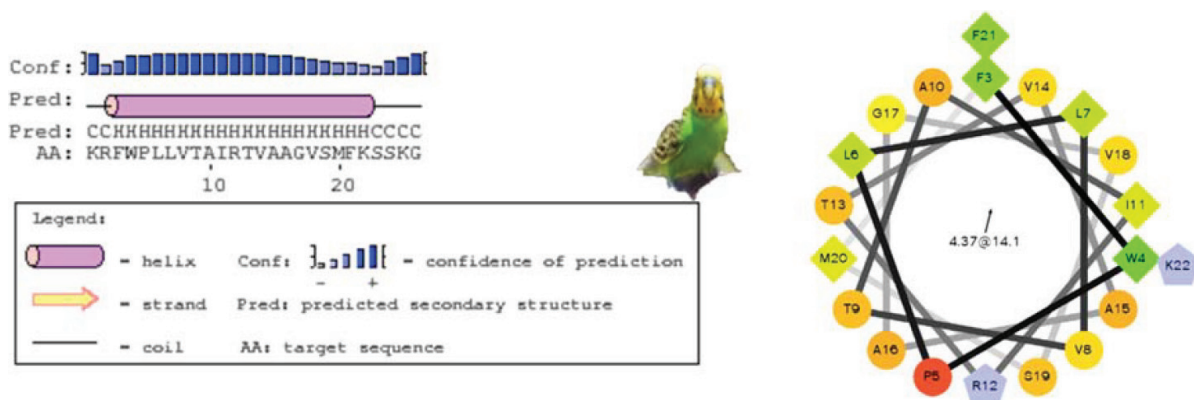


**Figure 1.** Graphical result of the in silico study of the cathelicidin sequences Mm_KR-26 (blue line) and Cp_SP-37 (yellow line), using the AMPA bioinformatic tool. The calculated average antimicrobial index is represented along the amino acid sequences. The threshold value of this index is indicated by a dashed line.
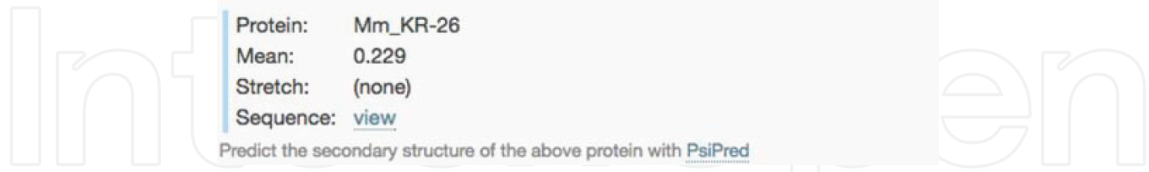
**Figure 2.** Result of the in silico study of the cathelicidin sequences, using the AMPA bioinformatic tool. The antimicrobial stretches are shown with a mean and a propensity and probability value.

The secondary structure of the bird and turtle cathelicidins was then computed with the PSIPred tool, which for the bird cathelicidin Mm_KR-26, predicted a single helical region comprised between residues 2 and 23 (**Figure 3**). However, no helical portion was predicted for the turtle cathelicidin Cc_SP-37 (not shown).

To further study the amphipathic character of the helices predicted by the PSI-Pred tool, these residues were represented in helical projection (**Figure 3**). For the helix of the peptide Mm_KR-26, comprised between the residues 2 and 23, it was observed that the two positively charged residues (R12 and K22) were disposed towards the same side, thus generating an amphipathic helix, characteristic of the antimicrobial peptides, with a hydrophobic moment of 4.37 units at 14.1°C.



**Figure 3.** Result of the in silico study of the cathelicidin Mm_KR-26, using the PSIPred bioinformatic tool for the prediction of the secondary structure. The predicted helical content of the bird cathelicidin Mm_KR-26 was then subjected to a helical projection and calculation of the hydrophobic moment, using the on line viewer available at http://rzlab.ucr. edu/scripts/wheel/wheel.cgi. Hydrophilic residues are presented as circles, hydrophobic residues as diamonds, potentially negatively charged as triangles, and potentially positively charged as pentagons. Hydrophobicity is colour-coded: the most hydrophobic residue is green, and the amount of green is decreasing proportionally to the hydrophobicity, with zero hydrophobicity coded as yellow. Hydrophilic residues are coded red with pure red being the most hydrophilic (uncharged) residue, and the amount of red decreasing proportionally to the hydrophilicity. The potentially charged residues are light blue. The scalar value of the hydrophobic moment of the helix and the angle of its direction are shown inside the helix.

### 3.3. Antimicrobial activity of peptides

For the analysis of the antimicrobial activity of cathelicidins, we used the minimal inhibitory concentration assay over a panel of different microorganisms of reference (**Table 2**). For simplicity, we show some figures with the microorganisms *B. cereus, E. coli, E. faecalis, C. albicans, P. corrugata* and *Salmonella* sp. In these experiments, it was found that both the bird peptide Mm_KR-26 and the painted turtle Cc_Sp-37 were particularly effective at a low dose to inhibit the growth of *B. cereus*, with MIC values of 1.6–3.1 and of 3.1–6.3 μM, respectively. However, both peptides were not effective, at the higher dose tested, to inhibit the growing of *S. aureus* and *P. mirabilis* and both were not very effective against *C. albicans*, with MIC values of 25–50 μM (**Table 2**).

To visualise the relative efficacy of the inhibition of the growing of microorganism, we estimated the percentage of inhibition of the growing of microorganisms at different concentrations of peptide, as well as the effective concentration of peptide to inhibit at 50% the growing of the microorganism, the $EC_{50}$ values. **Figures 4** and **5** (top and bottom) show these values obtained with a few representative microorganisms for the bird peptide Mm_KR-26 and the painted turtle Cc_Sp-37, respectively.

As can be seen from the results shown in **Figure 4**, the cathelicidin Mm_KR-26 was particularly effective against *E. faecalis*, which required less concentration of peptide for inhibition of the growth of this microorganism, (with an $EC_{50}$ value of 1.3 μM) and, in less extent, against *C. albicans* with an $EC_{50}$ value of 34.6 μM (**Figure 4**).

As can be seen from the results shown in **Figure 4**, the cathelicidin Mm_KR-26 was particularly effective against *E. faecalis*, which required less concentration of peptide for inhibition of the growth of this microorganism, (with an $EC_{50}$ value of 1.3 μM) and, in less extent, against *C. albicans* with an $EC_{50}$ value of 34.6 μM (**Figure 4**).

Similarly, the painted turtle peptide Cc_SP-37 was particularly effective against *E. faecalis*, which required less concentration of peptide for inhibition of the growth of this microorganism, (with

| Reference microorganism | Mm_KR-26 | Cc_SP-37 | Kanamycin |
|---|---|---|---|
| *S. aureus (ATCC6538)* | >50 μM | >50 μM | 0.8–1.6 μg/ml |
| *Salmonella* sp. *(CECT456)* | 3.1–6.3 μM | 12.5–25 μM | 0.4–1.6 μg/ml |
| *K. pneumoniae (ATCC23357)* | 1.6–3.1 μM | 3.1–6.3 μM | 0.4–0.8 μg/ml |
| *E. coli (ATCC9637)* | 3.1–6.3 μM | 6.3–12.5 μM | 0.4–1.6 μg/ml |
| *B. cereus (ATCC21772)* | 1.6–3.1 μM | 3.1–6.3 μM | 0.8–3.1 μg/ml |
| *P. mirabilis (CECT170)* | >50 μM | >50 μM | 0.4–0.8 μg/ml |
| *E. faecalis (ATCC29212)* | 0.8–1.6 μM | 6.3–3.1 μM | 0.4–0.8 μg/ml |
| *C. albicans* | 25–50 μM | 25–50 μM | – |
| *P. corrugata* | 6.3–12.5 μM | 25–50 μM | – |

**Table 2.** Minimum inhibitory concentration of the bird peptide Mm_KR-26 and the painted turtle Cc_Sp-37 compared with the antibiotic kanamycin.
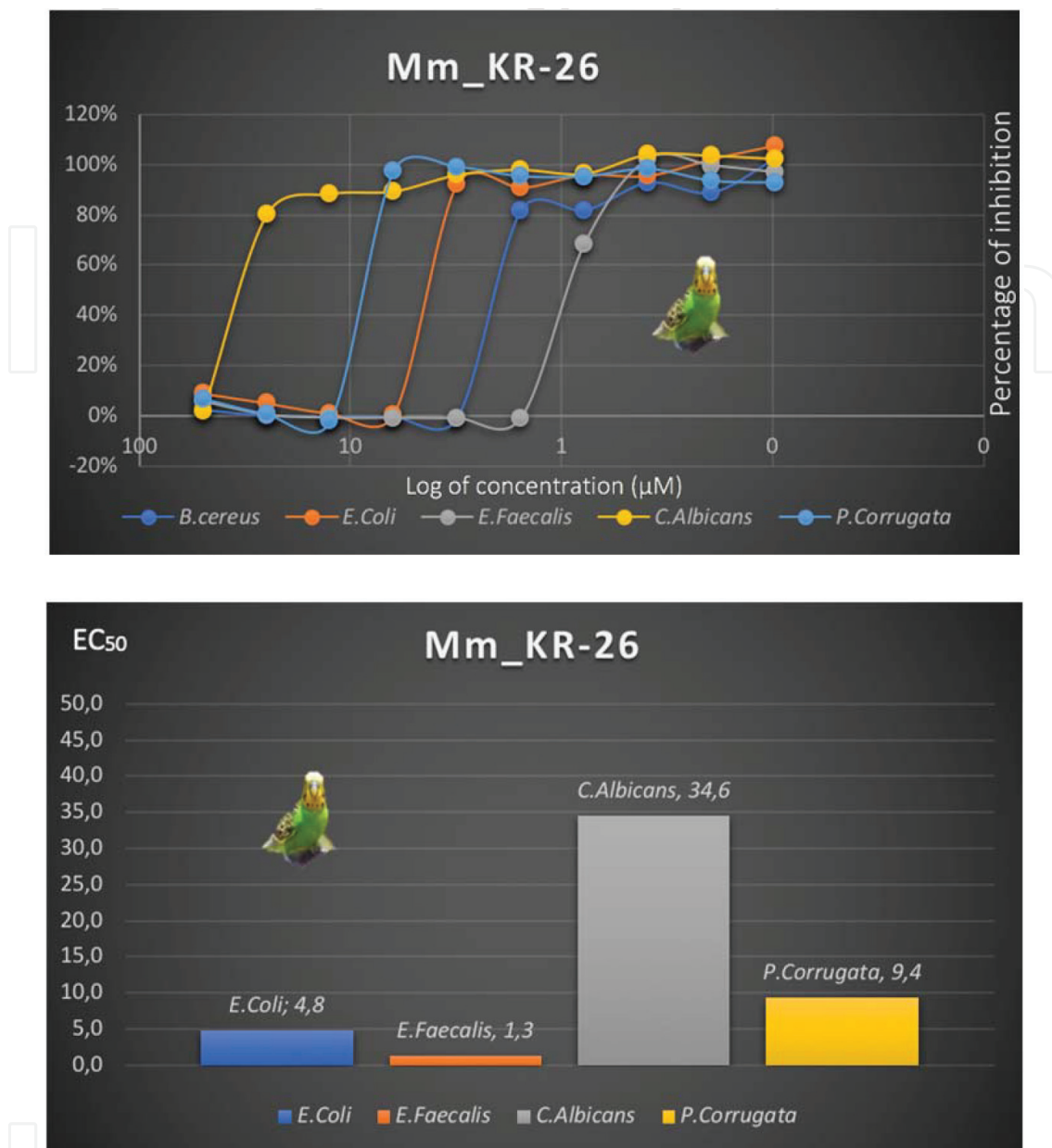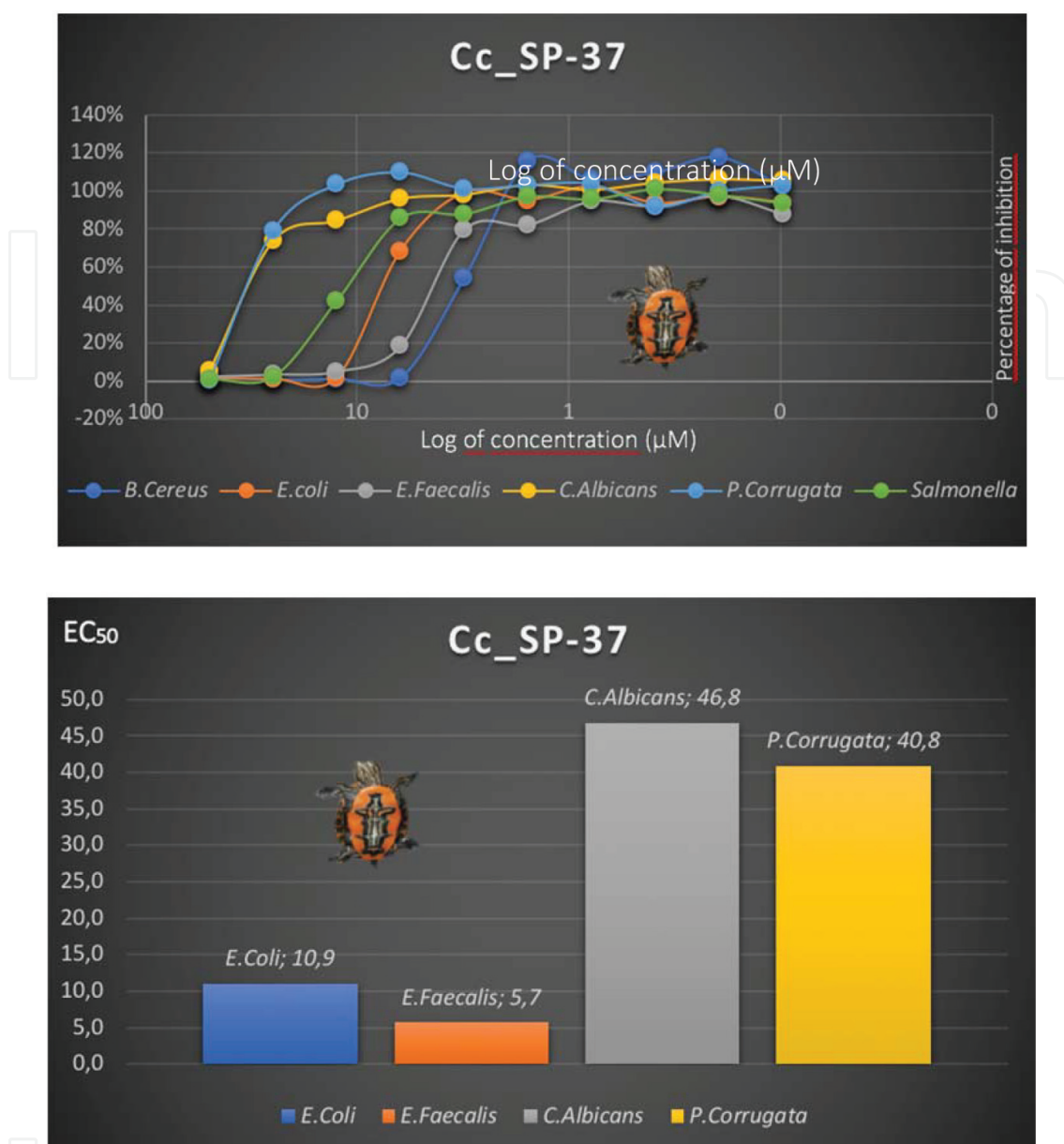
**Figure 4.** Analysis of the in vitro relative antimicrobial activity of the bird peptide Mm_KR-26 against a panel of representative microorganisms, showed as the percentage of inhibition (top) and calculating the effective dose for the inhibition at 50% (EC50), showed bottom.

an $EC_{50}$ value of 5.7 μM) and, in less extent, against *C. albicans* and *P. corrugata* with $EC_{50}$ values of 46.8 and 40.8 μM, respectively (**Figure 5**).

For both cathelicidin peptides, we tested the haemolytic activity at concentrations ranging from 12.5 to 0.2 μM, concentrations at which the peptides were able of inhibiting the growth of pathogenic microorganisms. The haemolytic activity of both peptides was always less than 35%, indicating that the peptides at these concentrations do not show toxicity for human erythrocytes (data not shown).

**Figure 5.** Analysis of the in vitro relative antimicrobial activity of the painted turtle peptide Cc_SP-37 against a panel of representative microorganisms, showed as the percentage of inhibition (top) and calculating the effective dose for the inhibition at 50% (EC50), showed bottom.

Once we tested the antimicrobial activity of the peptides, we next initiated the recombinant production of peptides in the eukaryotic microalga *Chlamydomonas reinhardtii* and in prokaryotic cyanobacteria *Synechococcus elongates*, given the advantages of using these microalga as biofactories [17]. For the eukaryotic microalga, in order to obtain a secreted product, we considered the introduction of the signal peptide of the aryl sulphatase, as previously described [18], and the synthetic DNA with the codon optimisation for this particular microalga. We also added restriction sites for the correct cloning of the synthetic DNA into the vectors pChlamy_4 and pSyn_6. Both expression systems, prokaryotic and eukaryotic microalga have strong promotors and can introduce 6xHis tags for the purification of the recombinant proteins (**Table 3**).

| Vector features | pChlamy_4 | pSyn_6 |
|---|---|---|
| Promoter | HSP70-rbcs2 (strong, endogenous) | psbA1 (strong, endogenous) |
| Selection | Bleomycin/Zeocin | Spectinomycin |
| Epitope tags | TEV-cleavable N-terminal 6His, or C-terminal V5-6His | |
| Gene integration | Stable (random) | Stable (targeted) |
| Growth media | TAP | BG-11 |

**Table 3.** Comparison of the microalga vectors pChlamy_4 and pSyn_6 (Thermofisher) used for the recombinant expression of cathelicidin antimicrobial peptides into the microalga *C. reinhardtii and S. elongatus,* respectively.
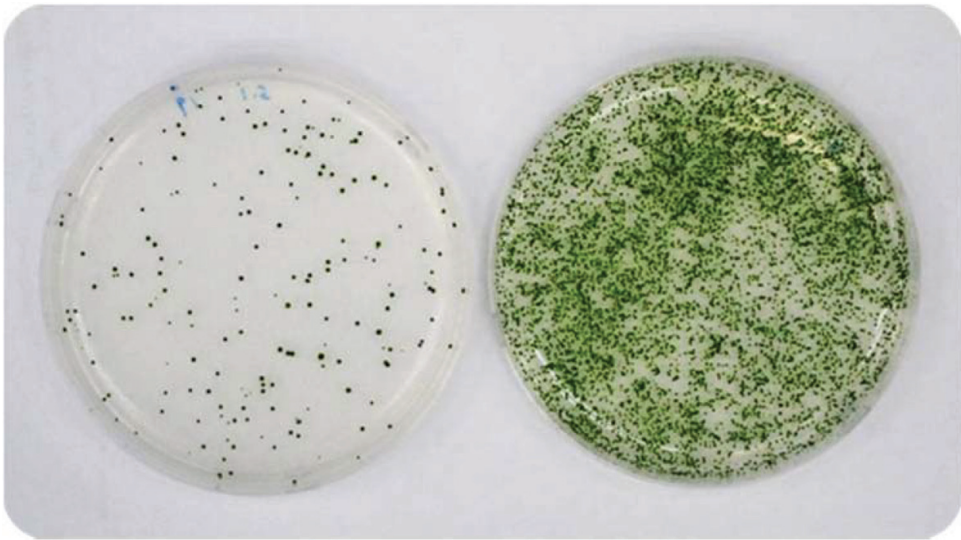


**Figure 6.** Transformed cyanobacteria *S. elongatus* growing in BG-11 agar plates containing spectinomycin (5 μg/ml). Negative control with no insert (left) and containing the bird cathelicidin peptide Mm_KR-26.

Using the commercially available kits for microalga expression, two microalga (*C. reinhardtii and S. elongatus*) were transformed for the recombinant expression of the bird and painted turtle cathelicidins. As an example, **Figure 6** shows the transformation of *S. elongatus* with the DNA coding for the bird cathelicidin peptide Mm_KR-26.

Current experiments are underway in order to optimise the recombinant production of these cathelicidins in a higher culture volume and to study the yield of the peptide production using microalga as biofactories.

## 4. Discussion

Currently, approximately 15 therapeutic agents based on AMP peptides are found in clinical trials of anti-infective or anti-inflammatory indications, generally limited to topical applications

[19]. One of the main questions to bring this type of molecules to the clinical application refers to their toxicity. To date, most clinical trials focus on topical use. Although, many peptides have activity on eukaryotic membranes, this activity is much lower compared to prokaryotes. This may be due to the absence of negatively charged lipids on the surface of eukaryotic membranes, the lower membrane potential in most cells and the presence of cholesterol in eukaryotes. However, despite the large number of peptides described in various organisms (more than 2000) and the advances to take advantage of its clinical potential, there is currently no peptide approved for use in humans by the Food and Drug Administration (FDA) [20].

One of the most widely used strategies for the biotechnological production of proteins and recombinant peptides is their expression in microorganisms, such as *E. coli* or yeast. Among the advantages of these expression systems is the well-known physiology of these microorganisms and the existence of specific and well-characterised strains to achieve high levels of expression, the existence of numerous protocols for the transformation of *E. coli* and/or yeasts and the availability of vectors that contain a great diversity of promoters and selection markers, and so on [21]. However among the disadvantages presented by this antimicrobial peptide, production system is, obviously, the potential susceptibility that the producing microbes could have to the harmful antimicrobial peptides, in case these are exposed to. Another disadvantage, in case the peptide is trapped as insoluble form in inclusion bodies, implies the need to further purify these peptides from the extract, and, according to the potential use of the peptides, also the removal of endotoxins. Synthetic biology techniques facilitate the availability of the genetic material that codes for antimicrobial peptides with the codon optimisation of the host and it also allows the incorporation of signal peptides, epitopes for the detection of the final product, or the inclusion of appropriate targets for proteolytic enzymes, tags or tails to facilitate purification [22].

An alternative for an expression system of antimicrobial peptides without contaminating endotoxins could be the transformation of eukaryotic cells from animal or human origin. In this case, it may not be necessary to purify or decontaminate the extracts, since the culture would be free of pathogens. However, the cost of producing recombinant proteins would be considerably higher than using conventional microorganisms. Therefore, the economic yield of these clean expression systems is lower than the microbial ones [23].

We here propose that a production system based on microalga could be an alternative to the conventional fermentation system, which uses bacteria or yeast and/or mammalian cells [24]. First, the antimicrobial peptide is less likely to be toxic to this production system. Second, this production system differs from the others due to the greater volume of scaling that these cultures allow, since it can be spread over large areas, in microalgae production systems. This fact makes these production systems very profitable. Although they can be less efficient, the cost/benefit ratio could be higher for the large-scale production of these peptides. Another advantage of these green production systems is that some microalgae are considered generally regarded as safe (GRAS). Hence, in these cases, depending on the type of application expected for the antimicrobial peptides, purification processes may not be necessary, for example, applications for cosmetics, topical medicines, pesticides for agriculture, food conservation, and so on.

There are already numerous tools for the efficient transformation microalgae. For example, in the case of the microalga *Chlamydomonas reinhardtii*, it is possible to transform the three genomes: nuclear, chloroplastic and mitochondrial. Once transformed, microalgae can be grown in photobioreactors, in controlled conditions and free of pathogens. The microalga used as biofactories of recombinant drugs, on the other hand, could also be grown under controlled conditions to avoid escaping into the environment and to improve the biosecurity of the final product [25].

## 5. Conclusions

Antimicrobial peptides, originating from natural resources, have been recognised as a novel class of antibiotics. We have shown that genome mining is an effective method for finding new antimicrobial peptides, such as cathelicidins, from the genomes of a turtle and a bird. The derived peptides were tested for their *in vitro* antimicrobial activity showing that these antimicrobial peptides were particularly effective against bacterial growth, both Gram+ (*E. faecalis*) and Gram− (*E. coli*). In addition, these peptides had low haemolytic activity in human erythrocytes, which makes them suitable for use in different formulations and/or pharmaceutical, cosmetic and/or phytosanitary compositions, among others. Using synthetic constructions, current experiments are underway to produce these peptides as recombinant chimeric proteins in eukaryotic microalga and cyanobacteria. We hope that our preliminary investigation with these novel peptides could be useful for the design of future strategies that pursue the production of antimicrobial peptides through biotechnology in order to provide novel treatment opportunities based on antimicrobial peptides.

## Acknowledgements

## Conflict of interest

The authors declare no conflict of interest. The funding sponsors had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript, and in the decision to publish the results.

## Author details

José Manuel Pérez de la Lastra*, Cristina Garrido-Orduña, Andrés A. Borges,
David Jiménez-Arias, Francisco Javier García-Machado, Mercedes Hernández,
Concepción González and Alicia Boto

*Address all correspondence to: jm.perezdelalastra@csic.es

Institute of Natural Products and Agrobiology (IPNA), Spanish Research Council (CSIC),
Avda. Astrofísico Fco. Sánchez, San Cristóbal de La Laguna, Santa Cruz de Tenerife, Spain

## References

[1] Kaspar AA, Reichert JM. Future directions for peptide therapeutics development. Drug Discovery Today. 2013;**18**:807-817

[2] Joerger RD. Alternatives to antibiotics: Bacteriocins, antimicrobial peptides and bacterio-phages. Poultry Science. 2003;**82**:640-647

[3] Cars O, Hedin A, Heddini A. The global need for effective antibiotics—Moving towards concerted action. Drug Resistance Updates. 2011;**14**:68-69

[4] Zasloff M. Antimicrobial peptides of multicellular organisms. Nature. 2002;**415**:389-395

[5] Peschel A, Sahl HG. The co-evolution of host cationic antimicrobial peptides and microbial resistance. Nature Reviews Microbiology. 2006;**4**:529

[6] Zasloff M. Antimicrobial Peptides: Do they have a Future as Therapeutics? In: Harder J, Schröder JM, editors. Antimicrobial Peptides. Birkhäuser Advances in Infectious Diseases. Cham: Springer International Publishing; 2016. pp. 147-154

[7] Barra D, Simmaco M. Amphibian skin: A promising resource for antimicrobial peptides. Trends in Biotechnology. 1995;**13**:205-209

[8] Yoo WG, Lee S, Lee MR, Yun MR, Kwon T, Kim DW. Genome-wide identification of antimicrobial peptides in the liver fluke, Clonorchis sinensis. Bioinformation. 2015;**11**:17

[9] Wang P, Hu L, Liu G, Jiang N, Chen X, Xu J, et al. Prediction of antimicrobial peptides based on sequence alignment and feature selection methods. PLoS One. 2011;**6**:e18476

[10] Bals R, Wilson JM. Cathelicidins-A family of multifunctional antimicrobial peptides. Cellular and Molecular Life Sciences. 2003;**60**:711-720

[11] Zanetti M. Cathelicidins, multifunctional peptides of the innate immunity. Journal of Leukocyte Biology. 2004;**75**:39-48

[12] Lehrer RI, Ganz T. Cathelicidins: A family of endogenous antimicrobial peptides. Current Opinion in Hematology. 2002;**9**:18-22

[13] Pérez de la Lastra JP, Garrido-Orduña C, Borges CL, Borges AA, Boto A. Antimicrobial activity of cathelicidins of mammals from avian, aquatic and terrestrial environments. In: Mendez-Vilas, editor. Antimicrobial research: Novel bioknowledge and educational programs. Formatex; 2017. pp. 9-18

[14] Torrent M, Di Tommaso P, Pulido D, Nogués M, Notredame C, Boix E, Andreu D. AMPA: An automated web server for prediction of protein antimicrobial regions. Bioinformatics. 2012;**28**:130-131

[15] Wang G, Li X, Wang Z. APD3: The antimicrobial peptide database as a tool for research and education. Nucleic Acids Research. 2016;**44**:D1087-D1093

[16] Wiegand I, Hilpert K, Hancock RE. Agar and broth dilution methods to determine the minimal inhibitory concentration (MIC) of antimicrobial substances. Nature Protocols. 2008;**3**:163-175

[17] Wijffels RH, Kruse O, Hellingwerf KJ. Potential of industrial biotechnology with cyanobacteria and eukaryotic microalgae. Current Opinion in Biotechnology. 2013;**24**(3): 405-413

[18] Rasala BA, Lee PA, Shen Z, Briggs SP, Mendez M, Mayfield SP. Robust expression and secretion of Xylanase1 in Chlamydomonas reinhardtii by fusion to a selection gene and processing with the FMDV 2A peptide. PLoS One. 2012;**7**(8):e43349

[19] Wimley WC, Hristova K. Antimicrobial peptides: Successes, challenges and unanswered questions. The Journal of Membrane Biology. 2011;**239**:27-34

[20] Kang SJ, Park SJ, Mishig-Ochir T, Lee BJ. Antimicrobial peptides: Therapeutic potentials. Expert Review of Anti-Infective Therapy. 2014;**12**(12):1477-1486

[21] Terpe K. Overview of bacterial expression systems for heterologous protein production: From molecular and biochemical fundamentals to commercial systems. Applied Microbiology and Biotechnology. 2006;**72**(2):211

[22] Welch M, Villalobos A, Gustafsson C, Minshull J. You're one in a googol: Optimizing genes for protein expression. Journal of the Royal Society Interface. 2009;**6**(Suppl 4):S467-S476

[23] Wurm FM. Production of recombinant protein therapeutics in cultivated mammalian cells. Nature Biotechnology. 2004;**22**(11):1393-1398

[24] Potvin G, Zhang Z. Strategies for high-level recombinant protein expression in transgenic microalgae: A review. Biotechnology Advances. 2010;**28**(6):910-918

[25] Boto A, Pérez de la Lastra JM, González CC. The road from host-defense peptides to a new generation of antimicrobial drugs. Molecules. 2018;**23**:311