Anti-Coccidiosis Potential of Heat Antimicrobial Peptides from *Xenorhabdus budapestensis* Resistant to Proteolytic (Pepsin, Trypsin) Digestion based on *in vitro* studies
ABSTRACT

Aims: Revealing anticoccidial potential (that is activity on both causative pathogens, the prokaryotic Clostridium perfringens) and the eukaryotic Eimeria tenella] of antimicrobial peptides from Xenorhabdus budapestensis. Objectives: in vitro tests of cell-free culture media (CFCM) of Xenorhabdus budapestensis DSM 16342 (EMA) and X. szentirmaii DSM 16338 (EMC) on 13 C. perfringens isolates; and on their cytotoxicity; preparation “Xenofood” for future in vivo feeding studies aiming at studying the efficacy of EMA and EMC on C. perfringens colony-forming units (CFU) in the ileal digests and side-effects.

Study design: Clostridium perfringens samples LH-1-LH24 were collected from chicken and poultry by L. Fodor and L. Makrai. A. Fodor as a visiting scientist were growing EMA and EMC liquid cultures and obtained sterile CFCM and tested in all but 11 LH isolates.

Place and Duration of Study: Department of Microbiology and Infectious Diseases, Faculty of Veterinary Science, Szent István University, Budapest, Hungary between September 2013 and February 2014.

Methodology: Adaptation of previously published in vitro bioassays of EMA and EMC CFCM (with and without proteolytic pepsin and trypsin digestion) on C. perfringens isolates. Xenofood is a mixture of an autoclaved, mid-stationary phase culture of Xenorhabdus budapestensis and X. szentirmaii grown in conventional “starter” and “grower” chicken food.

Results: Antimicrobial (peptides of both EMA and EMC CFCM re heat-stable, trypsin and pepsin resistant. All but one of 13 C. perfringens isolates proved sensitive to EMA-CFCM. Previously we found that these antimicrobial products inactivate E. tenella cells, but were toxic to permanent chicken liver (LMH) cells. Conclusion: We are ready for running an in vivo feeding test comparing Xenofood-fed and control broiler cockerels and determine the gastrointestinal (ileal) anti-Clostridium and anti-Eimeria activity; rate of adsorption; antigenicity; and physiologic effects of the heat- and proteolysis tolerant antimicrobial peptides of Xenorhabdus budapestensis and X. szentirmaii species.

Keywords: Clostridium perfringens, Xenorhabdus Antimicrobial Peptides; in-vitro Bioassay, Xenofood

1. INTRODUCTION

Multi-drug resistance (MDR) has gradually been increasing in both Gram-positive [1] (Nawrocki et al., 2014) and Gram-negative [2] (Gruenheid et al., 2012) pathogenic bacterium species. MDR has always been a phenotypic consequence of sequential accumulation of simultaneously appearing mutations, or the up-take of resistance plasmids harboring mobile genetic elements or genomic islands with resistance genes. These encode for either enzymes capable of destroying the antibiotics, or catalyzing biochemical reactions resulting in inhibition of either binding to, or permeation through, the cellular membrane (CM). The poultry gastro-intestinal (GI) flora is a seed-bed of MDR, as shown by the spectacular ongoing evolution in Enterococcus [3] (Borst et al., 2012); [4] (Miller et al., 2015); [5] (Palmer et al., 2014), in Clostridium [6] (Dahms et al., 2014) and in Salmonella genera [7] (Lu et al., 2014). The explanation is that the poultry GI is an ideal "market place" for exchange and horizontally transferring resistance gene –carrying plasmids and mobile genetic elements between coexisting bacteria. Enterococcus cecorum, for instance, once a simple commensal member of the intestinal microbiota, has become the causative pathogen of arthritis and osteomyelitis worldwide in chickens, such as in Hungary [8] (Makrai et al., 2011) and Poland [9] (Dolka et al., 2016). Evidences of multidrug-resistant plasmid transfer from
Gram positive [10] (Lebreton, 2013) and Gram negative [11] (Szmolka and Nagy, 2013); [12] (Hasman et al., 2015) chicken pathogens via consumed chicken meat to human pathogens, has been accumulating. Apart from the veterinary aspects, this horizontal gene transfer is of clinical importance.

The anaerobic Gram-positive C. perfringens was published first as a globally threatening danger by [13] (Van Immersee et al., 2004), as the causative pathogen of necrotic enteritis. Since then it has become alarming from both veterinary and human clinical aspects. The incidence of C. perfringens-associated necrotic enteritis in poultry has especially been increased in countries that stopped using antibiotic growth promoters. Both the disease and its subclinical forms are caused by C. perfringens type A strains, which produce either the alpha toxin, (to a lesser extent type C), or both alpha and beta toxins, [14] (Timbermont et al., 2009). Some C. perfringens type A isolate also produces an enterotoxin at sporulation, responsible for food-borne disease in humans. A predisposing factor in poultry is the mucosal (gut wall) damage (coccidiosis, caused directly most frequently by the eukaryotic pathogen, Eimeria tenella), preluded by unfavorable changes in the GI biota. The latter could be an indirect consequence of diets with high levels of indigestible, water-soluble, non-starch polysaccharides, which are known to increase the viscosity of the intestinal contents and predispose it to necrotic enteritis, [15] (Van Immersee et al., 2009). This important discovery provides an option for nutrient scientists to contribute to solving Clostridium problems. By other words, the discovery that the gastrointestinal microbiota could significantly be restructured by nutritional factors, provides additional opportunities for nutrition scientists working on the problem coccidiosis [14] (Dahiya et al., 2007), [16] (Teirlynck et al, 2009) or similar problems such as Campylobacter jejuni [18] (Molnár et al., 2015).

As for the pathogenesis of necrotic enteritis in chicken [15] (Van Immersee at al., 2009), the standardized model describing combined infection with the eukaryotic Eimeria species and C. perfringens, is the most plausible, [19] (Stanley et al., 2014); [20] (Kitessa et al., 2014).

C. perfringens type A cells release several toxins that promote disease development not only in chicken, but also in humans. The necrotic enteritis B-like toxin (NetB) is a β-barrel pore-forming one, which used to be considered as a vaccine candidate [21] (Keyburn et al., 2010). Another toxin called perfringolysin O (PFO, also referred to as θ toxin), is a pore-forming cholesterol-dependent cytolysin (CDC). PFO is secreted as a water-soluble monomer that recognizes and binds membranes via cholesterol. Membrane-bound monomers undergo structural changes that culminate in the formation of an oligomerized pre-pore complex on the membrane surface. The pre-pore then undergoes conversion into the bilayer-spanning pore. Research has demonstrated a role for PFO in gas gangrene progression and in bovine necro-hemorrhagic enteritis. [22] (Verherstraeten et al., 2015). C. perfringens strains which had been isolated from outbreaks of necrotic enteritis are also capable of secreting factors that inhibit growth of other (competitor) C. perfringens strains, including those isolated from the gut of healthy chickens. This feature lends a selective virtue to respective NetB-toxin producing virulent strains, the causative factor of gut lesions. The factor providing this selective virtue to the virulent strain is a novel, chromosomally encoded heat-labile, trypsin - and proteinase-K sensitive protein with bacteriocin activity called perfrin. The gene, which can only be found in C. perfringens NetB strains and nowhere else, (despite the fact that the NetB is a plasmid encoded toxin), could be transferred to and
expressed in *E. coli*, in the laboratory, (but theoretically it may happen in the chicken GI at any time) and the recombinant gene product is antibacterial active at a large pH range [23] (Timbermont et al., 2015).

Vaccination is an effective but not omnipotent veterinary tool for controlling MDR pathogens and *Clostridia*. The vaccination projects concerning *Enterococcus* seem to be in promising but only at the experimental stage [24] (Romero-Saavedra et al., 2014). None of the seven available publications contain anything on poultry. As for *Clostridia*, the vaccination of chickens against the fatal human pathogen type C (causing botulism) were successful [25] (Dohms et al., 1982). The vaccination against *C. perfringens* has seemed to be close to realization for years, but has not been realize yet. The immunization with NetB genetic, or formaldehyde toxoids, seems the most plausible approach [26] (Fernandes Da Costa et al., 2013), but the same team published that only double vaccination (on age 3 and 12 days) with crude supernatant were effective. Immunization not with a single toxin molecule did not give satisfactory protection for chickens against necrotic enteritis lesions, [27] (Mot et al., 2013).

This observation led Professor dr. Van Immerseel (Universiteit Gent, Belgium) and his associates to the conclusion that “immunization with single proteins is not protective against severe challenge and that combinations of different antigens are needed. Most published studies have used multiple dosage vaccination regimens that are not relevant for practical use in the broiler industry” [28] (Mot et al., 2014). Despite some other less pessimistic reports, such as suggesting the use of *C. perfringens* recombinant proteins in combination with Montanide™ ISA 71 VG adjuvant as a vaccine [29] (Jang et al., 2012), or anticoccidial live vaccine [30] (Bangoura et al., 2014), we have to accept the opinion of the #1 expert of that field research field: the vaccination against avian *C. perfringens* type A strains in broiler chicken has not been available yet. Consequently, there is room to work on novel antimicrobials, especially on antimicrobial peptides which might be used to control *C. perfringens* A and also MDR pathogens in the GI system of broiler chicken.

This approach should invoke a comprehensive strategy, based on Quantitative Structure–Activity Relation (QSAR) analysis and *in silico* modelling [31] (Mojoska and Jenkinns, 2015), and chemical synthesis of modified analogs leading to new antimicrobial agents with novel modes of action. The structural designing AMP candidate molecules has been aiming at improving endurance to proteolytic degradation, binding to, and the penetration through cellular membranes and other biological barriers, which can be achieved by adding modules for passive or active transport, [32] Ötvös and Wade, 2014). Another approach is searching for efficient synergisms, [33] (Lin et al., 2017). Another (ever-green) alternative research line is to search for new antimicrobials of completely novel mode of action in the nature.

Our research team has been searching for novel antimicrobials, which are not used in human medicine, are toxic only for chicken pathogens but not toxic for organisms to be protected. We expect to find the best candidates amongst natural antimicrobial peptides, (AMPs) synthetized by the obligate bacterial symbionts (EPB) of entomopathogenic nematodes (EPN) [34] (Forst & Nealson, 1996). These EPB-released AMPs are evolutionary products developed under severe selective pressure and comprise a powerful chemical arsenal against a large scale of prokaryotic and eukaryotic organisms to provide monoxenic
conditions for a given respective EPN / EPB symbiotic complex in polyxenic (insect gut, soil) conditions. There are many EPN-EPB complexes have been existing, many AMP profiles could be determined. Considering that all but one [35] (Nollmann et al., 2015) of the known AMPs can be produced by the bacterium in vitro, the EPN/EPB complexes provide a gold mine for researchers interested in new antimicrobials.

The majority of EPB-produced AMPs were identified in the last 15 years [36] (Vivas & Goodrich-Blair, 2001); [37] (Park et al., 2009); [38] (Gualtieri et al., 2009); [39] (Bode et al., 2015A). Each of these evolutionarily designed antibiotic arsenals has effectively overcome intruders representing a full scale of antibiotic resistance repertoire in their respective niche. Each EPB-AMP discovered so far is a non-ribosomal peptide (NRP), synthesized by multi-enzyme thiotemplate mechanisms, using non-ribosomal peptide synthetases (NRPS), fatty acid synthases (FAS), and/or related polyketide synthases (PKS), or a hybrid biosynthesis thereof [40] (Reimer & Bode, 2013). The biosynthetic enzymes are encoded by biosynthetic gene clusters [41] (Medema et al., 2015) determining the biosynthetic pathways.

Cabanilasin, from X. cabanillasii, exerts of a strong antifungal activity [42] (Houard et al., 2013). In our experiments, the cell-free culture media (CFCM) of X. cabanillasii was also extremely toxic to S. aureus, Escherichia coli and Klebsiella pneumoniae isolated from cows with mastitis syndromes [43] (Furgani et al., 2008). In that experiment, the antibacterial activities of the CFCM of several Xenorhabdus species were compared. We found that and those of X. budapestensis DSM 16342 (EMA), and X. szentirmaii DSM 16338 (EMC) [44] (Lengyel et al., 2005) were by far the best. The CFCM of EMA and EMC also were effective against Staphylococcus aureus MRSA (Fodor, McGwire, and Kulkarni, unpublished).

Furthermore, the CFCM from EMA and EMC also proved effective against plant pathogens, including both prokaryotic Erwinia carotovora, Clavibacter michigenense and several Xanthomonas species, [45] (Bőszörményi et al., 2009); [46] (Vozik et al., 2015); [47] (Vozik, 2017), and all tested eukaryotic Oomycetes (Phytophthora) species [42] (Bőszörményi et al., 2009); (Muvevi et al., unpublished). Gualtieri confirmed our data declaring that X. szentirmaii DSM16338 (EMC) was really a source of antimicrobial compounds of great potential, and sequenced this strain [48] (Gualtieri et al., 2014). One of the products (szentiamide) has been chemically synthesized [46] (Nollmann et al., 2012).

We suppose that these antimicrobial peptides act in concert. The idea of “Xenofood” is based on the intention to benefit from the joint action of cooperating AMP molecules produced by EMA and EMC cells, not only on a single molecule. We know that the strongest, predominant antibacterial peptide produced by both EMA and MC species is the fabclavine, [51] Fuchs et al. (2014). Many of our experiments with EMA were repeated and confirmed in the laboratory of Professor Helge B Bode (Goethe-Universität, Frankfurt – am Main Germany), and they confirmed that EMA CFCM exhibited broad-spectrum bioactivity against Bacillus subtilis, E. coli, Micrococcus luteus, Plasmodium falciparum, Saccharomyces cerevisiae, Trypanosoma brucei, and T. cruzi, [51] (Fuchs et al., 2012). They subjected the CFCM from X. budapestensis to MALDI-MS analysis which showed signals of unknown compounds. One compound (1, 1356.96 Da), fabclavine was purified, and its structure was determined. The details of biosynthesis are precisely reconstructed by the authors, but no data about their mode of action has so far been published, [51] Fuchs et
Fabclavines are considered as a novel class of biosynthesized hybrid peptide–polyketide-polyamino natural compounds of extremely high antimicrobial potential in both prokaryotic and eukaryotic pathogen targets, but also with unwanted eukaryotic cell-toxicity, [1] They are unambiguously the most effective antimicrobial Xenorhabdus peptide-products that have ever been discovered, and they are released by X. budapestensis and X. szentirmaii [44] (Lengyel et al., 2005).

We tested CFCM of EMA and EMC were in 2009 in the McGwire laboratory (Ohio State University, Columbus, OH, USA) in different targets and we found that, similarly to several other antimicrobial peptides, ([52] (Kulkarni et al., 2009); [53] (Marr et al., 2012)) they exerted apoptotic effects on eukaryotic cells of Leichmania donovanii. They are also active against Candida sp., and Phytophthora infestans. (A. Fodor et al., unpublished). Considering that there are not only prokaryotic, but eukaryotic pathogens are existing, we decided to continue the “EMA-EMC” project. Coccidiosis is the best example when a prokaryotic and an eukaryotic pathogen act together. Dr. Petra Ganas tested both CFCM in permanent chicken liver cell line at the Vet Med University of Vienna, Austria, and found them toxic to tissue cultures (Ganas, personal communication, for details, see Discussion), even if the cell toxic concentration was 1 order of magnitude higher than the bactericide concentration. These data, and the identification of the most active component (fabclavine), might have discouraged to continue the project.

Considering the presence of multidrug resistance, and even pan-resistance, problems in the GI system of broiler chicken, which may also threaten human health, and the limitation of vaccination, we reconsidered it as a potential tool, on the condition that orally applied compounds were not adsorbed into the meat of broiler chicken. From this aspect we believe that the results of this in vivo experiment are worthwhile, and our conclusions will be taken into consideration by coccidiosis specialists.

Prior to the planned in vivo feeding test we carried out in vitro bioassays presented here.

2. MATERIAL AND METHODS

2.1 Bacterium Strains

Clostridium perfringens NCAIM 1417 strain was obtained from the National Collection of Agricultural and Industrial Microorganisms –WIPO (www.wipo.int/budapest/en/idadb/details.jsp?id=5834; of Hungary, Faculty of Food Sciences, Szent István University Somlói út 14-16 1118 Budapest, Hungary) from Dr. Judit Tornai. C. perfringens LH1-LH8; LH11-LH16; LH19, LH20 and LH24 used in this study are of chicken origin, from the stock collection of Dr. L.Makrai. Xenorhabdus strains, X. budapestensis DSM 16342 (EMA); X. szentirmaii, DSM 16338 (EMC); [44] Lengyel et al, 2005 and X. bovienii NYH had been isolated by the Fodor laboratory from the entomopathogenic nematodes Steinernema bicornutum [Tallósi], [54] (Kaya et al., 2006); S. rarum and S. feltiae HU1 [55], (Tóth, et al, 2005), respectively. EMA and EMC had been deposited by us to the DSMZ, (Leinbniz Institute Deutsche Sammlung von Mikroorganismen und Zellkulturen, Braunchweig, Germany) as DSM 16342 and DSM 16338, respectively. X. nematophila ATCC 19061 was kindly provided by Professor S. A. Forst (University of Wisconsin – Milwaukee, USA) and X. nematophila DSM3370 by Professor E. Stackebrandt, (DSMZ, Braunschweig, Germany). S. cabanillasii BP was isolated by ourselves from the...
infective dauer juvenile form of the EPN S. riobrave, kindly provided by Professor Byron Adams (Bringham Young University, Provo, UT, USA).

2.2 Overlay Bioassays for Comparing the Antibacterial Potential of Different Xenorhabdus Strains

Overlay bioassays for comparing the antibacterial potential of different Xenorhabdus strains (each representing a species) were carried out as previously described [43] Furgani et al., 2008. In order to make sure that we use the proper bacterium, an earlier experiment was repeated in which we compared the antibacterial activities of 5 different Xenorhabdus strains on Klebsiella pneumoniae.

In order to know whether the antimicrobial compounds of EMA were effective against C. perfringens, an overlay experiment [43] Furgani et al., 2008, was carried out (Fig 2). To be sure that the intestinal proteolytic activities would not inactivate our compounds, samples of EMA CFCM were digested with pepsin, following the professional guidance of Professor Ferenc Husvéth (University of Pannonia, Keszthely, Hungary), while another sample was digested with trypsin by István Venekei (Eötvös University, Budapest, Hungary). Both samples preserved their complete antibacterial activity (Fig 3).

2.3 Agar-Diffusion Assay of EMA CFCM on Clostridium perfringens NAIM 1417 Laboratory Strain

Agar Diffusion Tests were similarly carried out, as described by [46] (Vozik et al., 2015), but we applied the method to the anaerobic specimen, C. perfringens. Briefly, the cell-free EMA CFCM exerted strong antimicrobial activity on C. perfringens in an agar diffusion test, as follows: In the hole of the center of the agar plate 100 ul of EMA CFCM were pipetted and overlaid with 3 ml of log phase C. perfringens suspension diluted to 1:250 with soft (0.6 V/V%) agar, and incubated for 24h in anaerobic conditions at 40°C.

2.4 Comparison of The Sensitivities (MID Values) of 13 C. perfringens Strains Isolated from Poultry in Liquid Cultures to Cell-Free Culture Media (CFCM) of X. Budapestensis (EMA)

2.4.1 Determination of MID Values

In order to quantify the sensitivity of the strains, the maximum inhibiting dilution (MID) values ([43] Furgani et al., 2008); [56] Fodor et al., 2010; [46] (Vozik et al., 2015), [47] (Vozik, 2017) were determined as follows: These studies were carried out in sterile 24-Hole Tissue Culture Plates, with 4 (A-D) rows and 6 (1-6) Columns; in 1 ml final volumes. Each Clostridium strains were used in different tissue culture plate. Each hole contained 0.5 ml of 2XRCM (Reinforced Clostridium Media, [57] (Romond et al., 1981) liquid medium and 0.5 ml of sterile, diluted EMA CFCM, in the following distribution: 100, 80, 60, 40, 20 and 0 V/V % in column 1, 2, 3, 4, 5 and 6, respectively. There were 50, 40, 30, 20, 10 and 0 V/V% final concentration of EMA CFCM in columns 1, 2, 3, 4, 5 and 6, such a way. Each cultures in rows A, B and C were inoculated with loopful amount of the respective bacteria obtained from three separate colonies grown on sheep blood agar plates. The holes in row D were not inoculated and served as sterile (negative) controls. Columns 6 served did not contain EMA CFCM and served as positive controls. Each 1-ml culture was overlaid by 0.5 ml sterile (freshly autoclaved) paraffin oil to provide anaerobic conditions. Plates were then incubated at 37°C for 24h and then scored visually. After 24h culturing the growing and inhibited
cultures could unambiguously be identified. We considered the concentration as MID where neither of the 3 replicates contained visible growth.

2.4.2 Enumeration of Clostridium perfringens colony forming units (CFU)

Sampled taken from the first hole in which bacterial proliferation could not visually been detected, 0.5 ml of culture were sucked out cautiously from below the paraffin oil and serial dilutions were prepared up to $10^{-5}$ and 100 μl volumes were simultaneously spread onto the surface of sheep blood agar (by D. László Makrai, see Fig 1) and Tryptose-Sulfite-Cycloserine (TSC) agar [59] (Harmon et al., 1971), plates. The latter was designed as a highly selective solid medium for growing and enumerating C. perfringens colony forming units. The TSC allows virtually complete recovery C. perfringens, while inhibits practically all facultative anaerobes tested and known as more selective than SFP Agar). Three replicates were used for each dilutions. (In preliminary experiments (carried out by András Fodor and Andor Molnár, at that time both of us were affiliated at the Department of Animal Sciences and Animal Husbandry, Georgikon Faculty, University of Pannonia, Keszthely, Hungary) TSC plates were incubated in anaerobic conditions at 40 °C and found the best readability between 48 – 72h. The C. perfringens colonies were unambiguously recognized by colony color and the black reduced sulfides granules around them, but the color of the agar also gave a kind of qualitative information. The colonies used in these preliminary experiments were obtained from chicken ileal digests were reproducibly counted and also from the stock collection of Dr. L. Makrai).

2.4.3. Statistical Analysis

ANOVA procedure was used following the fundamentals of the SAS 9.4 Software mostly due to the unbalanced data set. The significant differences ($\alpha = 0.05$) between treatment means were assessed using the Least Significant Difference (LSD).

Figure 1 Enumerating Clostridium colony forming unites (CFU) on sheep blood agar plates.

(Photo: Dr. László Makrai, (Department of Microbiology and Infectious Diseases, University of Veterinary Science, Szent István University, Budapest, Hungary).

2.5 Study of the Endurance of the Antimicrobial Compounds in the Cell-Free Culture Media (CFCM) of X. budapestensis and X. szentirmaii to Proteolytic Degradation
2.5.1. Trypsin-digested samples were tested on Gram-positive (S. aureus) and Gram negative (E. coli) targets in agar diffusion assay, in comparison with untreated CFCM samples. No differences were demonstrated.

2.5.2. Pepsin resistance was studied as follows: in the center of a Luria Broth plate, a 0.22 um pore size Millipore filter was laid and infiltrated with HCl and pepsin, and then EMA CFCM was pipetted onto it. The pepsin preparations were prepared by Professor Ferenc Husvéninth. After that the plate was overlaid with a Pseudomonas aeruginosa suspension diluted with soft agar as described [46] (Vozik et al., 2015); [47] (Vozik et al., 2017). After 24 h incubation at 40 °C, the growth of the test bacterium lawn were checked.

2.6 Preparation of Xenofood

Xenofood contained 5% soy-meal, which had been suspended with equal amount (w/w) of EMA and another 5% suspended in equal amount (w/w) of EMC cells obtained from 5 days-old shaken (2000 rpm) liquid cultures, followed by high-speed (Sorwall; for 30 minute) centrifugation. The liquid cultures were in double concentrated (2X) LB (DIFCO) liquid medium, supplemented with meat extract equivalent to the yeast extract. Five days was optimal for antibiotics production at 25 °C under these conditions. [43] Furgani et al., 2008; [45] Bőszörményi et al., 2009. It had previously been discovered that both EMA and EMC grow and produce antibiotics in autoclaved soy-meal containing some water and yeast extract, (Fodor, unpublished). Therefore the original chicken food served as a semi-solid culture media for Xenorhabdus cells. Both the separate EMA and EMC culturing semi-solid chicken food that we (Dr. László Pál) prepared daily, were incubated under sterile conditions for another five days. Then the EMA and EMC culture media were combined, autoclaved (20 min, 121 °C) and then dried by heat (70 °C) overnight. The Xenorhabdus cells were killed such a way, while the heat stable antimicrobial compounds remained active.

3. RESULTS AND DISCUSSION

3.1. Results of Experiments, Aiming at helping to choose the best Xenorhabdus strain for this study

Results are presented in Fig 2 and qualitative evaluation of the inactivation zones was appropriate to make the right decisions. Based on the results of a repeated experiment we choose X. budapestensis DSM16342 (EMA) and X. szentirmaii DSM 16338, (EMC) which had been identified in our laboratory [44] (Lengyel et al., 2005) For the exact description and history of these strains, see [43] (Furgani et al., 2008).

For the exact description and history of these strains, see [43] (Furgani et al., 2008).
Figure 2 Comparison of the antimicrobial potential of different *Xenorhabdus* strains (representing species) in overlay bioassay, ([43](Furgani et al., 2008)); (Photo: Andrea Máthé Fodor. (The Ohio State University, Wooster, OH, USA)

As expected, *X. budapestensis* (EMA) and *X. szentirmaii* proved to be the best, ([Fig 2](#)). Results of the overlay bioassay experiment with different *Xenorhabdus* strains on *Klebsiella pneumoniae* helped to make the right decision when choosing antimicrobial producing strain. The repeated experiment convinced us that *X. budapestensis* and *X. szentirmaii* should be used for this experiment.

### 3.2. Endurance of the antimicrobial peptides of *X. budapestensis* to pepsin, - and trypsin digestion

As demonstrated by Fig 3, the overnight pepsin-digested EMA CFCM remained active against *Pseudomonas aeruginosa*. The trypsin-digested samples also preserved their anti-Gram-positive (on *S. aureus*) and anti-Gram-negative (*E. coli*) activities, (not shown).

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Fig 3 Experimental evidence that the antimicrobial compounds of *X. budapestensis* cell-free media are resistant to the proteolytic activity of pepsin. After 24 h incubation at 37 °C a large inactivation zone could be seen, demonstrating a significant antimicrobial activity of the pepsin-treated EMA CFCM.
3.3. Efficacy of EMA CFCM on *C. perfringens* Laboratory Strain NCAIM 1471

The cell-free EMA CFCM exerted strong antimicrobial activity on *Clostridium perfringens* laboratory strain NCAIM 1471 in an agar diffusion test. The large inactivation zone of 3.7 cm diameter prove the anti-*Clostridium* activity, see Fig 3. The question arisen whether the pathogen poultry isolates were also sensitive.

![Image of agar diffusion test](https://example.com/image.png)

Figure 4 Anti-*Clostridium* activity of cell-free culture medium of *X. budapestensis* on *Clostridium perfringens* NCAIM 1417 strain in agar diffusion test [46] (Vozik et al., 2015), [47] (Vozik, 2017). (Photo: Dr. Csaba Pintér, University of Pannonia, Keszthely, Hungary)

3.4 Results of the Comparison of the Sensitivities (MID values) of 13 *C. perfringens* strains isolated from Chicken in Liquid Cultures to Cell-Free Culture Media (CFCM) of *X. budapestensis* (EMA)

Table 1 show the MID values as a qualitative parameter of the sensitivity of each poultry isolate to the antibacterial compounds of *X. budapestensis*. Majority of the examined strains are sensitive but one of the 13 was resistant (LM24). No direct interrelation between the degree of EMA sensitivity and other behavior cannot be demonstrated. The results provide a good message: the majority of *C. perfringens* isolates are sensitive. But the results also provide a bad message: there are EMA-resistant resistant *C. perfringens* isolates, even if they are rare.
Table 1 MID values of *C. perfringens* isolates from chicken differing in colony morphology and hemolytic behavior

<table>
<thead>
<tr>
<th>Clostridium perfringens isolates from poultry (L. Makrai, unpublished)</th>
<th>Minimum Inhibiting Dilutions (MID) Values (V/V%) of the cell-free culture medium (CFCM) of Xenorhabdus budapestensis (EMA) Inhibiting Bacterial Proliferation</th>
<th>Conclusion</th>
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<tbody>
<tr>
<td>LM 1</td>
<td>&lt; 10</td>
<td>Extremely sensitive</td>
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<tr>
<td>LM 2</td>
<td>&lt; 30</td>
<td>Sensitive</td>
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<tr>
<td>LM 3</td>
<td>&lt; 10</td>
<td>Extremely sensitive</td>
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<td>LM 4</td>
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<td>LM 5</td>
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<td>LM 8</td>
<td>&lt; 30</td>
<td>Sensitive</td>
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<td>LM 11</td>
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<td>LM 14</td>
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<td>LM 20</td>
<td>&lt; 30</td>
<td>Sensitive</td>
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<tr>
<td>LM 24</td>
<td>&gt; 50</td>
<td>Resistant</td>
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</tbody>
</table>

None of the samples taken from cultures of no visible proliferation contained any CFU, indicating that the toxicity was complete. Whether the differences in the sensitivities could related with the cellular phenotype was not revealed by this experiment, although the *C. perfringens* isolates were rather different concerning colony morphology and hemolytic behavior, see Fig 5.

Fig 5 *Clostridium perfringens* isolates LM1, LM2 and LM24 differing in colony morphology, sporulation willingness and hemolytic behavior. (Photo: Dr. László Makrai, (Department of Microbiology and Infectious Diseases, University of Veterinary Science, Hungary).

4. Discussion
The \textit{in vitro} experiment demonstrated that antimicrobial peptides of \textit{X. budapestensis} (EMA) were highly toxic for all but one \textit{C. perfringens} isolates. Dr. Klaus Teichmann (Biomin, Tulln, Austria), also for courtesy, was working with the same EMA and EMC preparation as Ganes et al., and declared that the CFCM of EMA exerted an extremely strong anticoccidial activity on both \textit{Clostridium} and \textit{Eimeria} cells. (He told he has never worked with such an efficient anticoccidial preparation before). He found a concentration range within which \textit{Eimeria tenella} cells died, while the cells of the chicken tissue culture were not affected, (Klaus Teichmann personal communication). These facts are arguments for taking EMA antimicrobial peptides as a potential anticoccidial agent into consideration.

But there are arguments against \textit{Xenofood} as well, and they are those data which prove \textit{in vitro} cytotoxicity on the permanent chicken liver cell line LMH, \cite{Amin2012}, as found by Dr. Petra Ganas (Clinic for Avian, Reptile and Fish Medicine, Vetmeduni, Vienna, Austria). These fellow - scientists tested the cytopathogenic effect of sterile cell-free media (CFCM) of EMA and EMC on eukaryotic cells for courtesy. Dr. Ganas and her associates (Aziza Amin, Irina Profjeva, and Micheal Hess) treated the permanent chicken liver LMH cells with different dilutions of our EMA and EMC preparations, and for evaluation, used the score-scale published by \cite{Amin2012}. They demonstrated that EMA CFCM at a dose of V/V < 5% concentration was harmless, but at V/V >5% concentrations they seriously damaged the cell layer. Doses >10 V/V% caused total destruction of the cell layer, while that of 5 – 10 V/V dose range resulted in about a 50% demage within the first 24h, and this demage was not repaired in the next 72 hrs. As for EMC, only the dose of 32% resulted in complete cell layer destruction, but the lower doses of 1-20 V/V% range also resulted in a permanent ~ 50% demage, calculate on the base of the score scale of \cite{Amin2012}, (Petra Ganas et al., personal communication).

Fabclavines are the predominant antimicrobial compound poduced by both EMA and EMC and were isolated and purified by \cite{Fuchs2014}, who did not suggest it as a drug of the future because of its extreme large target size and toxicity in eukaryotic targets.

This kind of „certificatation” is usually quite enough to place a candidate drug molecule into the waste basket, despite its super strong antimicrobial effects.

However, an exception with fabclavine may be considered because of the following arguments:

First, there are not only prokaryotic, but eukaryotic pathogens are also existing. The coccidiosis is the best example where the prokaryotic \textit{C. perfringens} and the eukaryotic \textit{Ei. tenella} cooperate in causing the disease and both should be controlled.

Second, there has no practically applicable vaccination technique available against \textit{C. perfringens}, \cite{Mot2014}.

Recently there are several research directions trying to solve the coccidiosis problem. The projects, among others, include search for novel antibiotic-delivery systems, (such as that uses ovotransferrin as targeting molecule), \cite{Ibrahim2015} (Ibrahim et al., 2015). There are also works toward improving the usefulness of commonly used anticoccidials and antibiotics (which have recently been tested on a subclinical necrotic enteritis model), \cite{Lanckriet2016} (Lanckriet et al,
Quite recently the hopes toward applying probiotics have been emerging. However, the coccidiosis problem has not seem to be solved yet. Until the coccidiosis problem has not been solved, to search for new efficient antimicrobials is probably justified.

There are two questions which could be answered by an appropriate in vivo feeding test:

1. Whether the orally administered fabclavine has a useful anti-\textit{Clostridium} and anti-\textit{Eimeria} potential were strong enough for curing of preventive applications;

2. Whether the orally administrated fabclavine would be adsorbed or inactivated by the immune system and cannot effect any adverse effects.

This is why we prepared Xenofood and suggest to use it in a feeding test.

For decades, antibiotics have been used extensively in animal production worldwide, as growth promoting agents. Added in low doses to the feed of farm animals, they have been shown to increase daily weight gain and conversion of feed into body mass, leading to some economic advantages for farmers. However, there are serious concerns that the use of antibiotics in the feed an increasing multidrug resistance and the trend is to reduce antibiotics in feedstuff. Since 1 January 2006, legislation has been in place in Europe to prohibit the use of antibiotics as growth promoters, and in other continents, the use of antimicrobial growth promoters in feedstuffs is under debate.

The negative consequences of the previous practice is unambiguously demonstrated by the alarming phenomenon of multidrug - and pan-drug resistance especially spectacular in Enterococci, \cite{vanHoorebeke2011}, \cite{Lebreton2013}; \cite{Miller2014}; \cite{Palmer2014}; and, although in a lesser scale, in \textit{C. perfringens} \cite{Gholamiandehkordi2009}; \cite{Dahms2014}; \cite{Ngamwongsatit2016} as well.

We believe that keeping \textit{C. perfringens} in the cross hairs of fellow-scientists working on novel AMPs is important, because of the recent, but hopefully just temporary, problems concerning vaccinations \cite{Mot2014}. In the efforts to overcome coccidiosis, we should neutralize a prokaryotic (\textit{C. perfringens}) and the collaborating eukaryotic (\textit{Eimeria tenella}) pathogens. Powerful antibiotics with large scale spectra and of novel mode of action, like fabclavines, are needed to slow the evolutionary process of multidrug resistance in the chicken gastrointestinal biota, against which vaccination is not an effective tool at present. If a novel antimicrobial is effective, has a novel mode of action, does not evoke immediate resistance and its application does not mean biohazard, it should be taken into consideration as potential drug.

The antimicrobially active compounds of EMA and EMC (present in the Xenofood) \textit{in vitro} act as strong antimicrobials and cytotoxic compounds, while \textit{in vivo} act as strong
antimicrobial in the GI but we forecast that they would not act as cytotoxic compound neither in the blood nor in any organs. Supposedly, only an insignificant amount of orally administered fabclavine would enter the circulation, similarly to orally administered vancomycin, \cite{71}.

Working on developing a new antimicrobial peptide is justified only if the candidate molecule is efficient, has a novel mood of action and exerts no cytotoxic, or any other adverse effects in the protected organisms. Antimicrobial peptoids are a group of potential candidates for consideration to be tested. Recently a library of 22 cationic amphipathic peptoids designed to target bacteria have been examined, \cite{73} (Mojsoska and Jenssen, 2015). All these peptoids share an overall net charge of +4 and are 8 to 9 residues long. However, the hydrophobicity and charge distribution along the abiotic backbone varied, thus allowing an examination of the structure-activity relationship within the library. In addition, the toxicity profiles of all the peptoids were assessed in human red blood cells (hRBCs) and HeLa cells, revealing the low toxicity of the majority of the peptoids. The structural optimization also identified two peptoid candidates, 3 and 4, with high selectivity ratios of 4 to 32 and 8 to 64, respectively, and a concentration-dependent bactericidal mode of action against Gram-negative \textit{E. coli} \cite{74} (Mojsoska et al., 2015). Another group of candidate AMPs are the oligomers of the proline-rich antimicrobial peptide (PrAMP), Chex1-Arg20, which has been designed (by Professor L. Ötvös Jr., (Temple University, Philadelphia Department of Biology) and his associates at The Semmelweis Medical School, Budapest, Hungary) in order to improve antibacterial selectivity and potency, \cite{75} (Li et al., 2017).

Recently two (NZ2114 and MP1102) novel plectasin-derived peptides have been designed for targeting Gram-positive bacteria \cite{76} (Zheng et al., 2017). The antibacterial characteristics and mechanism of NZ2114 and MP1102 against gas gangrene-associated \textit{C. perfringens} were studied for the first time. The minimal inhibitory concentration and minimal bactericidal concentration of both against resistant \textit{C. perfringens type A} strain CVCC 46 were impressively low. As for the mechanisms, they induced serious membrane damage; and bound to the genomic DNA leading to change its conformation. The cell cycle analysis showed that \textit{C. perfringens} CVCC 46 cells exposed to these drugs were arrested at Phase I. Both are suggested as new antimicrobial agents for gas gangrene infection resulting from resistant \textit{C. perfringens} \cite{76} (Zheng et al., 2017).

As for perspective, we would eagerly like to cooperate with organic preperative chemists to „domesticate” fabclavine to a derivative with reduced cell toxicity. We believe that the main targets of the modified fabclavine would be the Enterocci.

5. Conclusions

We believe that a Xenofood feeding experiment would be essential to reveal whether the orally administrated antimicrobial peptides produced by \textit{Xenorhabdus budapestensis} (EMA), and \textit{X. szentirmaii} (EMC) - which had previously proven active \textit{in vitro} against both the prokaryotic (\textit{Clostridium perfringens}) and the eukaryotic (\textit{Eimeria tenella}) pathogens causing coccidiosis in chicken, but also proven cytotoxic \textit{in vitro} in permanent chicken liver cells, were antibiotic active \textit{in vivo} without causing any harm in the animals to be protected.
REFERENCES


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ABBREVIATIONS

List of Abbreviations

AMP = Antimicrobial Peptides

CFCM = cell-free culture media

EMA = *Xenorhabdus budapestensis*, (obligate bacterium symbiont of the nematode *Steinernema bicornutum* but can easily be grown in vitro, even in supplemented chicken food)

EMC = *Xenorhabdus szentirmaii*, (obligate bacterium symbiont of the nematode *Steinernema rarum* but can easily be grown in vitro, even in supplemented chicken food)

EPB = entomopathogenic (nematode-symbiotic) bacterium

EPN = entomopathogenic nematode

FCR = feed conversion ratio: kg of consumed food/ kg body weight

GI = gastro-intestinal system

MDR = multi drug resistance (multiple antibiotic resistance)

PBS = Physiological buffered (NaCl) Solution