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

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ABSTRACT

Aims: To elucidate the anticoccidial potential of antimicrobial peptides from *Xenorhabdus budapestensis* on both causative pathogens (prokaryotic *Clostridium perfringens* and eukaryotic *Eimeria tenella)*. **Objectives:** (1) To establish if the antimicrobial compounds of the cell-free culture media (CFCM) of the entomopathogenic symbiotic bacterium species, *X. budapestensis* DSM 16342 (EMA) and *X. szentirmaii* DSM 16338 (EMC) were active against 13 independent pathogenic isolates of *Clostridium perfringens in vitro*; (2) To create a sterile, autoclaved, bio-preparation called "XENOFOOD", for future *in vivo* feeding studies, aimed at determining the efficacy, and side-effects, of EMA and EMC on *C. perfringens* in chickens.

Study design: *Clostridium perfringens* samples (LH-1-LH24) were collected from chickens and turkeys, and were deposited in the frozen stock collection of Department of Microbiology and Infectious Diseases, Faculty of Veterinary Science, Szent István University, Budapest, Hungary, where the *in vitro* assays were carried out on 13 of these 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 our previously published *in vitro* bioassays for aerobic tests for the anaerobic bacteria *Clostridium perfringens*. When preparing "XENOFOOD" we benefitted from our experimental data about the heat tolerance and endurance to proteolytic enzymatic digestion of the studied antimicrobial peptides.

Results: The studied antimicrobial peptides were heat-stable, trypsin and pepsin resistant. All but one of 13 *C. perfringens* isolates was sensitive to EMA-CFCM. XENOFOOD (made here) is not toxic for chicken, (unpublished).

Conclusion: Since these cell-free cultures killed *E. tenella* cells, but were toxic to permanent chicken liver (LMH) cells, we need to run *in vivo* feeding tests to determine the gastrointestinal (ileac), anti-*Clostridium* and anti-*Eimeria* biological effects of the these heat, - and proteolysis tolerant antimicrobial peptides.

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] and Gram-negative [2] 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 destructing 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 on-going evolution in *Enterococcus* [3] [4] [5], in *Clostridium* [6], as well as in *Salmonella* genera [7]. 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] and Poland [9]. Evidences of multidrug-resistant plasmid transfer from Gram positive [10] and Gram negative [11] [12] chicken pathogens via consumed chicken meat to human pathogens, has been accumulating. Apart from the veterinary aspects, this horizontal gene transfer is of critical clinical importance.

The anaerobic, Gram-positive, *C. perfringens* was first published as a globally threatening danger by Van Immerseel and his associates, [13] 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 also 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]. A few *C. perfringens* type A isolates produce an enterotoxin at sporulation as well, causing disease in humans, [14].

As for the pathogenesis of necrotic enteritis in chickens [15], it is a result of a "joint venture" the eukaryotic *Eimeria* species and *C. perfringens*, [16] [17]. The lesions and damages in the gut wall tissues (mainly in the lamina muscularis mucosae and in the lamina mucosa) provide anaerobic conditions needed for propagation of the toxin-producing *Clostridium*, especially in the ileum. The *Eimeria* (most frequently) *tenella* infection is usually preceded by previous unfavorable changes in the GI biota. The latter might be an indirect consequence of non-appropriate diets which increases the viscosity of the intestinal contents and makes it predisposed to necrotic enteritis [15]. This important discovery provides an opportunity for nutrient scientists to help reduce *Clostridium* infections. In 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 of coccidiosis [18] [19] or similar problems such as *Campylobacter jejuni*, [20]

Clostridium perfringens type A cells release different toxins that causing diseases not only in chickens, but also in humans. One of them, the necrotic enteritis B-like toxin (NetB), is a β-barrel pore-forming one, which used to be a candidate vaccine [21]. Another one, called perfringolysin O (PFO, also called θ toxin), is a pore-forming cholesterol-dependent cytolysin (CDC) [22]. PFO is secreted as a water-soluble monomer that recognizes and binds membranes via cholesterol. Membrane-bound monomer molecules undergo chemical structural changes that culminate in the formation of an oligomerized pre-pore complex on the membrane surface [22]. The pre-pore then undergoes conversion into the bilayer-spanning pore, playing an important role in so-called gas gangrene progression and necro-hemorrhagic enteritis in some mammals [22].

Clostridium perfringens strains which were isolated from epidemic outbreaks of necrotic enteritis, and were capable of secreting factors that inhibit growth of other (competitor) *C. perfringens* strains, including those isolated from the guts of healthy chickens [23]. 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 strains is a novel, chromosomally encoded, heat-labile, trypsin - and proteinase-K sensitive protein with bacteriocin activity called perfrin [23]. 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*. Theoretically, it may also happen in the chicken GI at any time, and the recombinant gene product is antibacterial active at a large pH range [23].

Several data from the literature seem to support our opinion that although vaccination is an effective, but probably not an omnipotent, veterinary tool for controlling Gram-positive MDR pathogens such as *Clostridia*. The vaccination projects involving *Enterococcus* seem to be in a promising, but only very experimental stage [24]. (None of the seven respective publications have recently been available in PubMed include anything on poultry).

As for *Clostridia,* the vaccination of chickens against the fatal human pathogen type C (causing botulism*),* have fortunately been successful [25].The vaccination against *C. perfringens* however, although seeming to be not too far from realization, but maybe not in the near future. The immunization with NetB genetic, or formaldehyde toxoids, seemed to be the most plausible approach [26], but only the double vaccination (on 3 and 12 days, with crude supernatant), were effective. Immunization with a single toxin molecule did not give satisfactory protection to chickens against necrotic enteritis lesions, which probably is not a realistic option for practical application [27].

This observation led Professor Dr. Van Immersee (Universiteit Gent, Belgium) and his associates to the conclusion that "*immunization with single proteins is not protective against severe challenge. Therefore combinations of different antigens are needed as alternative. In most published studies multiple dosage vaccination regimens were used. It is not a relevant way for practical use in the broiler industry*", [28]. 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] or anticoccidial live vaccine [30] have been noted. Nevertheless, we think that we'd better to accept the opinion of the Expert #1 in that research field: the vaccination against avian *C. perfringens* type A strains in broiler chicken is not yet available [28].

Consequently, there is a room for working on novel antimicrobials, especially on novel antimicrobial peptides which might be used to control *C. perfringens* A and also MDR pathogens in the GI system of broiler chickens. This approach needs a comprehensive strategy, based on Quantitative Structure – Activity Relation (QSAR) analysis and *in silico* modeling [31]. Chemical synthesis of modified analogs leading to new antimicrobial agents with novel modes of action should follow the molecular design to get new antimicrobial peptides, [31]. The structural design of AMP candidate molecules has aimed at improving endurance to proteolytic degradation, binding to, and the penetration through cellular membranes and other biological barriers [32]. This can be achieved by adding modules for passive or active transport [32]. Another approach is searching for efficient synergisms [33].

Another (ever-green) alternative research line is to search for new antimicrobials of completely novel modes of action in 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 the organisms to be protected. We expect to find the best candidates among the natural antimicrobial peptides (AMPs), synthesized by the obligate bacterial symbionts (EPB) of entomopathogenic nematodes (EPN) [34]. 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. They provide monoxenic conditions for a given respective EPN / EPB symbiotic complex in polyxenic (insect gut, soil) conditions. Many EPN-EPB complexes exist, and many AMP profiles could be determined. Considering that all but one [35] 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] [37] [38] [39]. 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]. The biosynthetic enzymes are encoded by gene clusters [41], determining the biosynthetic pathways.

Cabanilasin, from *X. cabanillasii,* exerts of a strong antifungal activity [42]. In our experiments, the cell-free culture media (CFCM) of *X. cabanillasii* was also extremely toxic to Staphylococcus *aureus, Escherichia coli* and *Klebsiella pneumoniae*, isolated from cows with mastitis syndromes [43]. 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] proved far the best. The CFCM of EMA and EMC were also effective against *S. aureus* MRSA, (Fodor, McGwire and Kulkarni, unpublished). Furthermore, the CFCM from EMA and EMC also was effective against plant pathogens, including both prokaryotic *Erwinia amylovora, E. carotavora, Clavibacter michigenense and* several *Xanthomonas* species [45] [46] [47] and all tested eukaryotic Oomycetes (*Phytophthora*) species [42] (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 he sequenced this strain [48]. One of the products (szentiamide) has been chemically synthesized [46].

We suppose that these antimicrobial peptides act in concert. The idea of a preparing a bio-product for oral administration to via chicken food, ("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 EMC species is fabclavine [51], but there are also others acting on eukaryotic pathogens as well, especially in EMC [48] [49]. (This is the explanation why we did not use only EMA CFCM alone, but a mixture of EMA and EMC CFCM instead in the experiments reported here).

Many of our experiments with EMA were repeated in the laboratory of Professor Helge B Bode (Goethe-Universität, Frankfurt – am – Main, Germany). 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] as well. They subjected the CFCM from *X. budapestensis* to MALDI-MS analysis and found altogether 4 isomers of fabclavine, one of which was then purified, and its structure was determined. The details of biosynthesis were impressively reconstructed by the authors, but no data about the mode of action has been published so far [51]. Fabclavines are considered a novel class of biosynthesized hybrid peptide–polyketide-polyamino natural compounds with extremely high antimicrobial potential in both prokaryotic and eukaryotic pathogen targets, but also with unwanted eukaryotic cell-toxicity. 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]. (This is a spectacular example of present-day science, when on group of scientists are "sowing" while the other ones are "harvesting").

We tested CFCM of EMA and EMC were in 2009 in the McGwire laboratory (Ohio State University, Columbus, OH, USA) against different targets, and found that, similarly to several other antimicrobial peptides [52] [53] they exerted apoptotic effects on eukaryotic cells of *Leichmania donovanii*. They were also active against *Candida* sp., and *Phytophthora infestans* (A. Fodor et al., unpublished).

Considering that not only prokaryotic, but eukaryotic pathogens also exist, we decided to continue the "EMA-EMC" project. Coccidiosis is the best example of when a prokaryotic and a eukaryotic pathogen act together. Dr. Petra Ganas tested both CFCMs on a permanent chicken liver cell line at the Vet Med University of Vienna, Austria, and found them toxic to the tissue cultures (Ganas, personal communication, for details, see Discussion), even if the toxic cell concentration was 1 order of magnitude higher than the bactericide concentration. These data, and the identification of the most active component (fabclavine), might seem discouraging for the continuation of the project.

However, 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 vaccinations, we reconsidered it as a potential tool, on the prospects that orally applied compounds would not be absorbed into the meat of broiler chickens. Prior to *in vivo* feeding tests we carried out the *in vitro* bioassays presented here, and formulated a chicken food, Xenofood, to test in the in vivo tests. 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.

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 (of Hungary, Faculty of Food Sciences, Szent István University Somlói út 14-16 1118 Budapest, Hungary). *Clostridium perfringens* LH1-LH8; LH11-LH16; LH19, and LH20 are of chicken origin, and LH24 came from a pig; each has been deposited in the (frozen) stock collection of Department of Microbiology and Infectious Diseases, University of Veterinary Medicine Budapest, Hungary.

Xenorhabdus strains, *X. budapestensis* DSM 16342 (EMA), *X. szentirmaii* DSM 16338 (EMC) [44] and *X. bovienii* NYH which had been isolated from the entomopathogenic nematodes *Steinernema bicornutum* [Tallósi] [54], *S. rarum* and *S. feltiae* HU1 [55], are originated from the Fodor laboratory, Eötvös University, Budapest, Hungary. EMA and EMC had also been deposited by us in the DSMZ, (Leinbniz Institute Deutsche Sammlung von Mikroorganismen und Zellkulturen, Braunchweig, Germany) as DSM 16342 and DSM 16338, respectively. *Xenorhabdus nematophila* ATTC 19061, was from Forst Laboratory at the University of Wisconsin – Milwaukee, USA) and *X. nematophila* DSM 3370 DSMZ, Braunschweig, Germany). *Steinernema cabanillasii* BP was isolated by us from infective dauer juveniles from the EPN *S. riobrave*.

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].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 *K. pneumoniae*.

To determine if the antimicrobial compounds from EMA were effective against *C. perfringens*, an overlay experiment was carried out [43].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 our coauthor Professor Ferenc Husvéth (University of Pannonia, Keszthely, Hungary), while another sample was digested with trypsin by István Venekei (Eötvös University, Budapest, Hungary)

2.3 Agar-Diffusion Assay of EMA CFCM against *Clostridium perfringens* NCAIM 1417 Laboratory Strain

Agar Diffusion Tests were similarly carried out, as described by [46], but we converted the method for the anaerobic specimen, *C. perfringens*. An agar diffusion test was conducted as follows: In a hole at the center of the agar plate, 100 ul of EMA CFCM were pipetted and overlaid with 3 ml of a log phase *C. perfringens* suspension diluted to 1:250 with soft (0.6 V/V%) agar. They were incubated for 24h under anaerobic conditions at 40 ^{o}C.

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

2.4.1 Determination of MID Values

To quantify the sensitivity of the strains, the maximum inhibiting dilution (MID) values [43] [56] [46] [47] were determined as below. 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* strain was used in a different tissue culture plate. Each hole contained 0.5 ml of 2XRCM Reinforced Clostridium Media [57] liquid medium, and 0.5 ml of sterile, diluted EMA CFCM, with the following distribution: 100, 80, 60, 40, 20 and 0 volume / volume (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. Each culture in rows A, B and C were inoculated with loopful 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^{o}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 none of the 3 replicates contained visible growth.

2.4.2 Enumeration of *Clostridium perfringens* colony forming units (CFU)

Samples were taken from the first hole in which bacterial proliferation was not visually 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] 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 it inhibits practically all facultative anaerobes tested, and is known as being more selective than SFP Agar. Three replicates were used for each dilution. In preliminary experiments, carried out by András Fodor and Andor Molnár, both then at the Department of Animal Sciences and Animal Husbandry (Georgikon Faculty, University of Pannonia, Keszthely, Hungary), TSC plates were incubated under anaerobic conditions at 40^{o}C, and found the best readability between 48 – 72h. The *C. perfringens* colonies were 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 (Fig. 1). The colonies used in these preliminary experiments were obtained from chicken ileal digests, and from the stock collection of Dr. L. Makrai, were reproducibly counted.

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Figure 1 shows the *Clostridium* colonies to be counted on a blood agar plate (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 (*Staph. aureus*) and Gram negative (*E.coli*) targets in agar diffusion assay, and compared 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 Millipore filter of 0.22 um pore size was laid and infiltrated with HCl and pepsin. Then EMA CFCM was pipetted onto it. The pepsin preparations were prepared by Professor Ferenc Husvéth. After that the plate was overlaid with a *Pseudomonas aeruginosa* suspension diluted with soft agar as described [46] [47]. After 24 h incubation at 40 ^{o}C, the growth of the test bacterium lawn was checked.

2.6 Preparation of XENOFOOD

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 2XLB (DIFCO), supplemented with meat extract equivalent to the yeast extract. Five days was optimal for antibiotic production at 25^{o}C under these conditions [43] [45]. It had previously been discovered that both EMA and EMC grow and produce antibiotics in autoclaved soy-meal containing some water and yeast extract, or in autoclaved 0.5% w/w yeast (Fodor, unpublished). Therefore the original chicken food served as a semi-solid culture media for the *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 ^{o}C), and then dried by heat (70^{o}C) overnight. The *Xenorhabdus* cells were killed in such a way, while the heat stabile [43] antimicrobial compounds remained active.

2.7. Statistical Analysis

ANOVA procedures were used following the procedures of the SAS 9.4 Software, mostly due to the unbalanced data set. The significant differences (α = 0.05) between treatment means were assessed using the Least Significant Difference (LSD).

3. RESULTS

3.1. Results of Experiments, Aimed at Helping to Choose the Best *Xenorhabdus* Strains for This Study

Results shown in Fig. 2, and a qualitative evaluation of the inactivation zones, indicated the appropriate bacteria to use. As expected, *X. budapestensis* (EMA) and *X. szentirmaii* were the best. Results of the overlay bioassay experiment with different *Xenorhabdus* strains on *K. pneumoniae* helped to make the right decision when choosing antimicrobial producing strains.

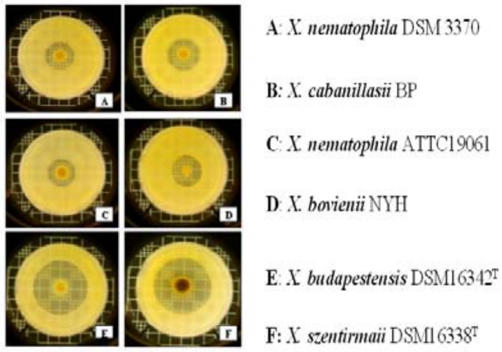


Figure 2 Comparison of the antimicrobial potential of different *Xenorhabdus* strains (representing species) in overlay bioassays [43]. (Photo: Andrea Máthé Fodor. The Ohio State University, Wooster, OH, USA)

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).

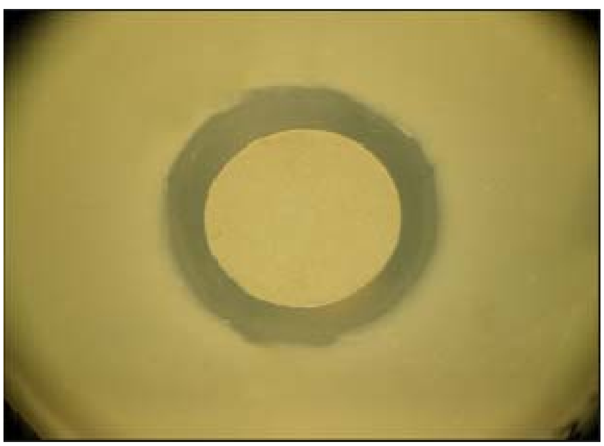


Fig. 3. Experimental evidence that the antimicrobial compounds of *X. budapestensis* cell-free media are resistant to the proteolytic activity of pepsin. After 24 h incubation at 37 ^{o}C a large inactivation zone could be seen, demonstrating a significant antimicrobial activity of the pepsin-treated EMA CFCM.

3.3. Efficacy of EMA CFCM on *C. perfringens* Laboratory Strain NCAIM 1471

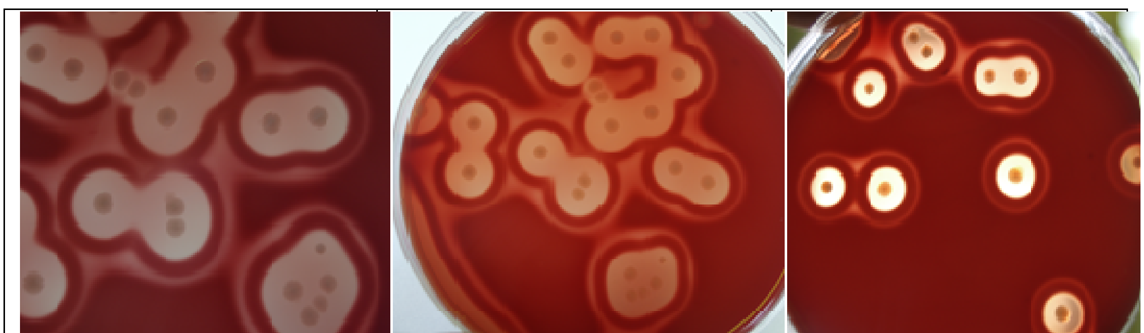
The cell-free EMA CFCM exerted strong antimicrobial activity on *C. perfringens* laboratory strain NCAIM 1471 in an agar diffusion test. The large inactivation zone of 3.7 cm diameter shows the anti – *Clostridium* activity (Fig 3). The question arises as to whether the pathogenic poultry isolates were also sensitive.

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

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

Table 1 lists the MID values as a qualitative parameter of the sensitivity of each of the poultry isolates to the antibacterial compounds of *X. budapestensis*. A 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 could be demonstrated. The results provide a good message: The majority of *C. perfringens* isolates are sensitive. However, they also provide a bad message: There are EMA-resistant resistant *C. perfringens* isolates, even if they are rare.

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



LM1(from chicken)	LM2 (from turkey)	LM24 (from pig)

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

Table 1 MID values of *Clostridium perfringens* isolates from chicken differing in colony morphology and hemolytic behavior

C. perfringens isolates from poultry (L. Makrai, unpublished)	Minimum Inhibiting Dilutions (MID) Values (V/V%) of the cell-free culture medium (CFCM) of *Xenorhabdus budapestensis* (EMA) Inhibiting Bacterial Proliferation	Conclusion
LM 1	< 10	Extremely sensitive
LM 2	< 30	Sensitive
LM 3	< 10	Extremely sensitive
LM 4	< 10	Extremely sensitive
LM 5	< 10	Extremely sensitive
LM 8	< 30	Sensitive
LM 11	< 10	Extremely sensitive
LM 14	< 10	Extremely sensitive
LM 15	< 10	Extremely sensitive
LM 16	< 10	Extremely sensitive
LM19	< 10	Extremely sensitive
LM20	< 30	Sensitive
LM 24	> 50	Resistant

4. Discussion

The *in vitro* experiments demonstrated that antimicrobial peptides of *X. budapestensis* (EMA) were highly toxic for all but one (LM 24) *C. perfringens* isolates. Dr. Klaus Teichmann (Biomin, Tulln, Austria), as a courtesy, tested EMA and EMC CFCM preparations, obtained from us. He declared that the CFCM of EMA exerted an extremely strong anticoccidial activity on both *Clostridium* and *Eimeria* cells. He declared that he had not ever worked with such an efficient anticoccidial preparation before as EMA CFCM. Dr. Teichmann found a lower concentration range within which *E. tenella* cells died, while the cells of the chicken tissue culture were not affected, (Klaus Teichmann, personal communication). These facts are arguments for taking the potential use of EMA and EMC antimicrobial peptides, as potential anticoccidial agents administered *per os*, into consideration.

But there are arguments against using XENOFOOD as well, and they are those data which showed in vitro cytotoxicity on the permanent chicken liver cell line LMH [60]. Dr. Ganas and her associates (Aziza Amin, Irina Profjeva, and Micheal Hess) tested the cytopathogenic effect of different dilutions of the same samples of sterile cell-free media (CFCM) of EMA and EMC on permanent chicken liver LMH cells, as Dr. Teichmann. They demonstrated that EMA CFCM at a dose of < 5% V/V concentration was harmless, but at >5%V/V 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 resulted in about a 50% damage within the first 24h, and this damage 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 also resulted in ~ 50% permanent damage, calculated on the base of the score scale of Amin et al. [60] (2012); (Petra Ganes et al., personal communication).

Fabclavines are the predominant antimicrobial compound produced by both EMA and EMC and were isolated and purified [51], and was not suggested as a future drug because of its extremely large target size and toxicity to eukaryotic targets. This kind of “certification” is usually quite enough to place a candidate drug molecule into the wastebasket, 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 also exist. Coccidiosis is the best example where a prokaryotic *C. perfringens* and a eukaryotic *E. tenella* cooperate in causing the disease, and both should be controlled.

Second, there is practically no vaccination technique against *C. perfringens* [28]. So the introduction of new antimicrobial compounds should be taken into consideration.

We are not the only team walking on this road. Recently, there have been several research directions attempting to solve the coccidiosis problem. A project includes a search for novel antibiotic-delivery systems, such as using ovotransferrin as a targeting molecule [61]. Another approach is to improve the usefulness of commonly used anticoccidials and antibiotics, which have recently been tested on a subclinical necrotic enteritis model [62]. Recently proline-rich antimicrobial peptides are considered as potential therapeutics against antibiotic-resistant bacteria [63]. The designer proline-rich antibacterial peptide A3-APO prevents the Gram-positive *Bacillus anthracis* mortality by deactivating bacterial toxins [64]. Even more recently two (NZ2114 and MP1102) novel plectasin-derived peptides have been designed for targeting Gram-positive bacteria, and the tests on gas gangrene-associated *C. perfringens* provided encouraging results [65].

The hopes of applying probiotics have been also emerging [66] [67] [68]. The use of vegetative *Bacillus amyloliquefaciens* cells did not justify the hopes: they did not confer protection against necrotic enteritis in broilers, despite the high antibacterial activity of its supernatant against *C. perfringens* in vitro [69].

5. Conclusions

There are two alternative approaches to control coccidiosis in broiler chicken: the vaccination and the “chemotherapy”, (that is, a search for gastro-intestinally active, autoclaveable antimicrobial peptides active against both *C. perfringens* and *E. tenella*).

Considering that there are publications about antibiotic resistant and multiresistant pathogen *C. perfringens* [70] [71], and that the coccidiosis problem has not yet seem to be solved by using vaccination, the search for new efficient antimicrobials to control coccidiosis have probably been justified.

On the basis of *in vitro* studies, fabclavine alone (and / or as a component of interacting antimicrobial active peptide complexes present in the CFCM of EMA and EMC) fulfil the criteria of a promising chemotherapeutic agent *in vitro,* that is, acting as strong antibacterial on *C. perfringens* and as strong apoptotic cytotoxic compounds on the unicellular eukaryotic pathogen, *E. tenella*.

However, the cytotoxicity may pose a serious problem of practical use. Indeed, we found that the CFCM of both EMA and EMC were cytotoxic *in vitro* in permanent chicken liver cells. But the *in vitro* and the *in vivo* situation are completely different.

If it happened that the orally administered fabclavine (and/or the whole AMP complex), due to their proteolytic endurance), might act *in vivo* as strong anti-Clostridia and anti-Eimeria agents in the GI, without causing any harm of the organism to be protected, it would have a chance to be register and use Xenofood as an anticoccidial bio-preparation. This option cannot be ruled out if the adsorption from the gut, were similarly low as that of the orally administred vancomycin [72].

We believe that an *in vivo* XENOFOOD feeding experiment would be necessary to learn whether the orally administrated antimicrobial peptides produced by *X. budapestensis* (EMA) and *X. szentirmaii* (EMC), *in vitro* against both the prokaryotic (*C. perfringens*) and the eukaryotic (E. tenella) pathogens causing coccidiosis in chicken, could be used in broiler cockerels.

We are ready for *in vivo* bioassay and looking for cooperative partners.

ACKNOWLEDGEMENTS

A The authors express their sincere thanks and appreciations to our coauthor Professor **Károly Dublecz**, the previous Dean of the Georgikon Faculty, for generously providing full (moral and even manual) help needed for preparing and accomplishing the in vivo experiments and following *Clostridium* bioassays in his Unit.

We thank **Dr. Petra Ganas** for unselfishly accomplishing those experiments which clarified the cytotoxicity of the CFCM by EMA and EMC, and kindly provided all data to us with the generous permission of his boss, Professor Michael Hess (University of Veterinary, Vienna, Austria). We thank **Dr. Klaus Teichmann** (Biomin, Tulln, Austria) who tested the same samples (that Petra Ganas worked with) on *Eimeria* cells and shared his conclusions with us unselfishly.

We would like to express thanks and appreciations the professional technical help to the lab technicians in the Vet School for their invaluable technical help, especially **Miss Éva Kolozsvári** and **Miss Teréz Halasi**.

We are extremely grateful to **Mrs. Swam Short** (Head of the Library in Ohio State University, Wooster, OH) for providing shelter and professional support to accomplish the MS properly. Many thanks for that.

This MS would have never been materialized in the absence of the strong and unselfish help and support of **Mrs. Andrea Máthé-Fodor**, (Molecular and Cellular Imaging Center, Ohio State University, Wooster, OH, USA) throughout in every stage of the work.

The *in vitro Clostridium* bioassays preceding Xenofood experiments were supported by **TÁMOP-4.2.2/B-10/1-2010-0025** project based on the financial support the State of Hungary and the EU.

COMPETING INTERESTS

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.
Authors have declared that no competing interests exist.

AUTHORS' CONTRIBUTIONS

Dr. Habil. András Fodor was the project initiator and put the MS together. Furthermore, he provided the bacterium cultures and inoculated and fermented Xenofood with *X. budapestensis* and *X. szentirmaii*.

Professors László Makrai and **László Fodor** (University of Veterinary Sciences, Budapest, Hungary) were the intellectual leader and governing the *Clostridium* tests. We worked on the *Clostridium perfringens* isolates (LM1 - LM24), previously collected, identified and deposited by them in the Department's stock collection. All but a few experiments were carrying on in their Laboratory at Department of Microbiology and Infectious Diseases, Faculty of Veterinary Science, Szent István University, Budapest, Hungary.

Associate Professor Dr. István *Venekei* carried out the trypsin digestions and bio-assayed the antimicrobial activities of the digested preparations with A. Fodor at the Department of Biochemistry at Eötvös University in Budapest, Hungary.

Professor Emeritus Ferenc Husvéth, (University of Pannonia, Keszthely, Hungary biochemist, independently from the others, guided our experiments on testing the pepsin sensitivity of the antimicrobial active compounds EMA and EMC CFCM in Keszthely, many thanks for it. This work was essential and Dr. Husvéth should definitely be our coauthor.

Associate Professor Dr. László Pál (University of Pannonia, Keszthely, Hungary) prepared the media by mixing up the food components before A. Fodor inoculated it with X. budapestensis and X. szentirmaii. He weighted the animals daily and calculated the food conversion. His work was essential and Dr. Pál should definitely be one of the coauthors.

Research Associate Dr. Andor Molnár (University of Pannonia, Keszthely, Hungary) and A. Fodor has applied previously published overlay,- and agar diffusion technique to C. perfringens and he was the pioneer of the *Clostridium* experiments at the Department of Animal Sciences and Animal Husbandry, Georgikon Faculty, University of Pannonia, (Keszthely, H-8360 Hungary) working with chicken ileal digestions.

The statistical analyses were carried out or guided by **Adjunct Emeritus Professor Dr. Sándor Józsa** (University of Pannonia, Keszthely, Hungary).

Adjunct Emeritus Professor Dr. Csaba Pintér (University of Pannonia, Keszthely, Hungary) made all but one (made by Andrea ***Máthé – Fodor***, Ohio State University, Wooster, OH, USA) photos published here

Adjunct Professor Michael G. *Klein* (The Ohio State University) made the final shaping and proofreading of the MS and was the spiritual rector of putting the data and ideas in the present form, which has been an invaluable contribution.

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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

GI = gastro-intestinal system

MDR = multi drug resistance (multiple antibiotic resistance)