Microbiology Research Journal International

22(4): 1-17, 2017; Article no.MRJI.38516
ISSN: 2456-7043
(Past name: British Microbiology Research Journal, Past ISSN: 2231-0886, NLM ID: 101608140)

Anti-Coccidiosis Potential of Autoclaveable Antimicrobial Peptides from *Xenorhabdus budapestensis* Resistant to Proteolytic (Pepsin, Trypsin) Digestion Based on *In vitro* Studies

András Fodor[1*], László Makrai[2], László Fodor[2], István Venekei[3], Ferenc Husvéth[4], László Pál[4], Andor Molnár[4], Károly Dublecz[4], Csaba Pintér[5], Sándor Józsa[5] and Michael G. Klein[6]

[1]Department of Genetics, Faculty of Science and Informatics, University of Szeged, Közép fasor 52, Szeged, H-6726, Hungary.
[2]Department of Microbiology and Infectious Diseases, University of Veterinary Medicine, Budapest, Hungary.
[3]Department of Biochemistry, Eötvös Loránd University, Budapest H-1117, Hungary.
[4]Department of Animal Sciences and Animal Husbandry, Georgikon Faculty, University of Pannonia, Széchenyi Street, 11 Keszthely, H-8360 Hungary.
[5]Georgikon Faculty, University of Pannonia, Deák Ferenc utca, 16, Keszthely, H-8360, Hungary.
[6]Department of Entomology, The Ohio State University, 1680 Madison Ave., Wooster, OH-44691, USA.

Authors' contributions

This work was carried out in collaboration between all authors. Author AF designed the study, provided Xenorhabdus cultures, and antimicrobial components of XENOFOOD; he wrote the protocol and wrote the first draft of the manuscript. Author LM designed and organized all the in vitro Clostridium studies and provided the strains. Author LF had been collected and deposited several Clostridium strains used in this study and provided working conditions in his Department. Authors IV and FH tested the endurance of the antimicrobial compounds to enzymatic destructions from trypsin and pepsin, respectively. Author LP made the XENOFOOD ready and he will be in charge of organizing following in vivo feeding experiments and calculating food conversion rates. Author AM carried out majority of the in vitro Clostridium experiments. Author KD provided working conditions for producing Xenorhabdus cultures in large scale; provided expertize for designing XENOFOOD and designed the choreography the appropriate in vivo feeding tests which followed this study Author CP made the photographs. Author SJ performed the statistical analysis. Author MGK managed the analyses of the study, the literature searches and made the final proof-reading. All authors read and approved the final manuscript.

Article Information

DOI: 10.9734/MRJI/2017/38516
Editor(s):
(1) Xing Li, Division of Biomedical Statistics and Informatics, Department of Health Sciences Research, Mayo Clinic College of Medicine, USA.
Reviewers:
(1) Luz del Carmen Camacho Castillo, Instituto Nacional de Pediatría, México.
(2) Nagaraja Suryadevara, MAHSA University, Malaysia.
Complete Peer review History: http://www.sciencedomain.org/review-history/22705

**Corresponding author: E-mail: fodorandras@yahoo.com;*

Original Research Article

Received 25th October 2017
Accepted 5th January 2018
Published 11th January 2018

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 [49].

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 [50,51], 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 this experiment 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. MATERIALS 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 24 h under anaerobic conditions at 40°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°C for 24 h and then scored visually. After 24 h 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 [58] 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°C, and found the best readability between 48 – 72 h. 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.

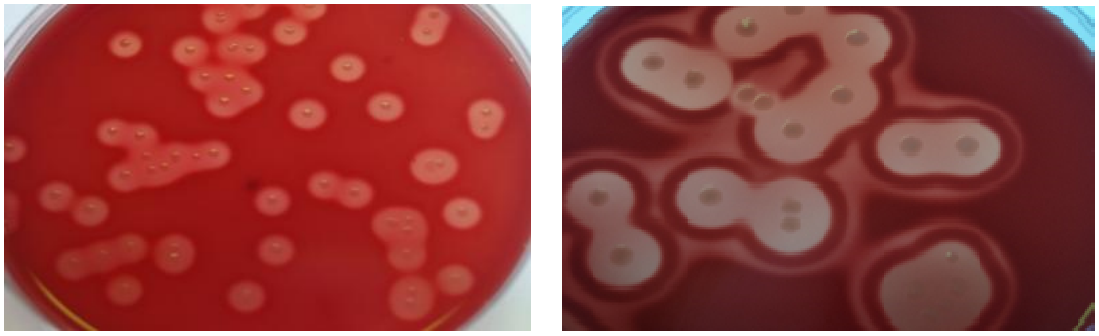


Fig. 1. Shows the *Clostridium* colonies to be counted on a blood agar plate (Photo: Dr. László Makrai, (Department of Microbiology and Infectious Diseases, University of Veterinary Science, Szent István University, Budapest, Hungary)

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

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

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°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°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 [59] 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°C), and then dried by heat (70°C) overnight. The *Xenorhabdus* cells were killed in such a way, while the heat stabile [43] antimicrobial compounds remained active.

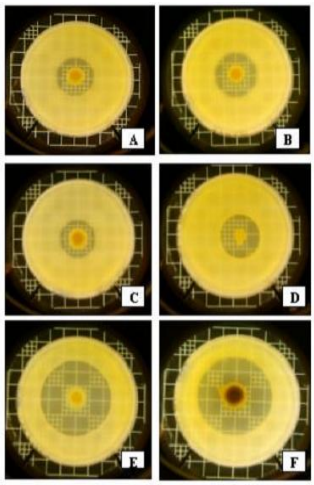
2.7 Statistical Analysis

ANOVA procedures were used following the procedures of the SAS 9.4 Software, mostly due to the unbalanced data set. The significant differences ($\alpha = 0.05$) 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.



A: *X. nematophila* DSM 3370

B: *X. cabanillasii* BP

C: *X. nematophila* ATTC19061

D: *X. bovienii* NYH

E: *X. budapestensis* DSM16342^T

F: *X. szentirmaii* DSM16338^T

Fig. 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).

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.

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).

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.

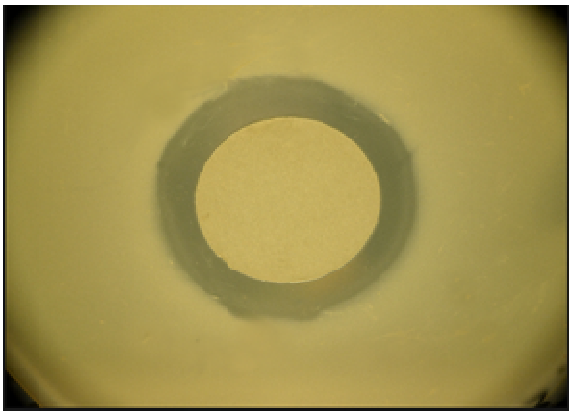


Fig. 3. Experimental evidence that the antimicrobial compounds of *X. budapestensis* cell-free media are resistant to the proteolytic activity of pepsin After 24 h incubation at 37°C a large inactivation zone could be seen, demonstrating a significant antimicrobial activity of the pepsin-treated EMA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

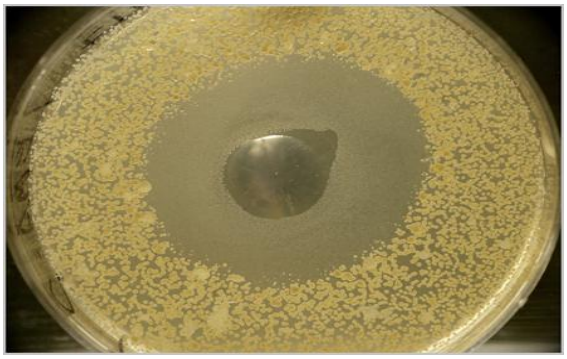
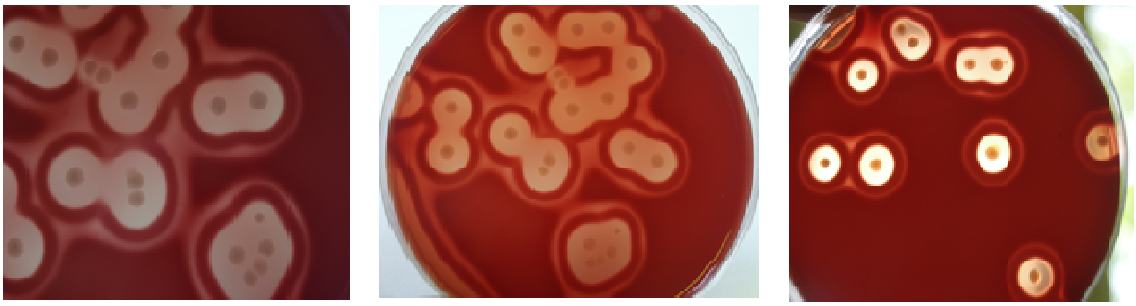


Fig. 4. Anti- *Clostridium* activity of cell-free culture medium of *Xenorhabdus budapestensis* on *Clostridium perfringens* NCAIM 1417 strain in agar diffusion test [46,47]. (Photo: Dr. Csaba Pintér, University of Pannonia, Keszthely, Hungary)



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

Fig. 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

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 24 h, 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]; (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

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

Authors have declared that no competing interests exist.

REFERENCES

1. Nawrocki KL, Crispell EK, McBride SM. Antimicrobial peptide resistance mechanisms of Gram-positive bacteria. Antibiotics (Basel). 201413;3(4):461-92. DOI: 10.3390/antibiotics3040461 PMID: 25419466 Free PMC Article
2. Gruenheid S, Le Moual H. Resistance to antimicrobial peptides in gram-negative bacteria. FEMS Microbiol Lett. 2012;330(2):81-9. DOI: 10.1111/j.1574-6968.2012.02528.x Epub 2012 Mar 12. Review. PMID: 22339775
3. Borst LB, Suyemoto MM, Robbins KM, Lyman RL, Martin MP, Barnes HJ. Molecular epidemiology of Enterococcus cecorum isolates recovered from enterococcal spondylitis outbreaks in the southeastern United States Avian Pathol. 2012;41(5):479-85. DOI: 10.1080/03079457.2012.718070 Epub 2012 Sep 17
4. Miller WR, Munita JM, Arias CA. Mechanisms of antibiotic resistance in enterococci. Expert Rev Anti Infect Ther. 2014;12(10):1221-36. DOI: 10.1586/14787210.2014.956092 Review. PMID: 25199988 Free PMC Article
5. Palmer KL, van Schaik W, Willems RJL, Gilmore MS. Enterococcal Genomics. *In*: Gilmore, M.S., Clewell, D.B., Ike, Y., Shankar, N., (Editors). *Enterococci*: From Commensals to Leading Causes of Drug Resistant Infection [Internet]. Boston, MA, USA: Massachusetts Eye and Ear Infirmary; 2014. PMID: 24649511 Free Books & Documents
6. Dahms C, Hübner NO, Wilke F, Kramer A . Mini-review: Epidemiology and zoonotic potential of multiresistant bacteria and Clostridium difficile in livestock and food GMS Hyg Infect Control. 2014;9(3):Doc21. DOI: 10.3205/dgkh000241. eCollection 2014.
7. Lu Y, Zhao H, Sun J, Liu Y1, Zhou X, Beier RC, Wu G, Hou X. Characterization of multidrug-resistant Salmonella enterica serovars Indiana and Enteritidis from chickens in Eastern China. PLoS One. 2014;9(5):e96050. DOI: 10.1371/journal.pone.0096050. eCollection 2014
8. Makrai L, Nemes C, Simon A, Ivanics E, Dudás Z, Fodor L, Glávits R. Association of Enterococcus cecorum with vertebral osteomyelitis and spondylolisthesis in broiler parent chicks. Acta Vet Hung. 2011;59(1):11-21. DOI: 10.1556/AVet.59.2011.1.2. Crossref, PubMed, Google Scholar
9. Dolka B, Chrobak-Chmiel D, Makrai L, Szeleszczuk P. Phenotypic and genotypic characterization of Enterococcus cecorum strains associated with infections in poultry. BMC Vet Res. 2016;12(1):129. DOI: 10.1186/s12917-016-0761-1 PMID: 27350248.
10. Lebreton F, van Schaik W, McGuire AM, Godfrey P, Griggs A, Mazumdar V, Corander J, Cheng L, Saif S, Young S, Zeng Q, Wortman J, Birren B, Willems RJ, Earl AM, Gilmore MS. Emergence of epidemic multidrug-resistant Enterococcus faecium from animal and commensal strains. MBio. 2013;4(4):pii: e00534-13. DOI: 10.1128/mBio.00534-13
11. Szmolka A, Nagy B. Multidrug resistant commensal *Escherichia coli* in animals and its impact for public health. Front Microbiol. 2013;4:258. DOI: 10.3389/fmicb.2013.00258
12. Hasman H, Hammerum AM, Hansen F, Hendriksen RS, Olesen B, Agersø Y, Zankari E, Leekitcharoenphon P, Stegger M, Kaas RS, Cavaco LM, Hansen DS, Aarestrup FM, Skov RL. Detection of mcr-1 encoding plasmid-mediated colistin-resistant Escherichia coli isolates from human bloodstream infection and imported chicken meat, Denmark 2015. Euro Surveill. 2015;20(49). DOI: 10.2807/1560-7917.ES.2015.20.49.30085. Erratum in: Euro Surveill. 2015;20(50). DOI: 10.2807/1560-7917.ES.2015.20.50.30091
13. Van Immerseel F, De Buck J, Pasmans F, Huyghebaert G, Haesebrouck F, Ducatelle R. Clostridium perfringens in poultry: An emerging threat for animal and public health. Avian Pathol. 2004;33(6):537-49. PMID: 15763720 DOI: 10.1080/03079450400013162 [Indexed for MEDLINE]
14. Timbermont L, Lanckriet A, Gholamiandehkordi AR, Pasmans F, Martel A, Haesebrouck F, Ducatelle R, Van Immerseel F. Origin of clostridium perfringens isolates determines the ability

to induce necrotic enteritis in broilers. Comp Immunol Microbiol Infect Dis. 2009; 32(6):503-12.
DOI: 10.1016/j.cimid.2008.07.001
Epub 2008 Sep 9. Epub 2008 Sep 9.
PMID: 18783830 [Indexed for MEDLINE]

15. Van Immerseel F, Rood JI, Moore RJ, Titball RW. Rethinking our understanding of the pathogenesis of necrotic enteritis in chickens. Trends Microbiol. 2009;17(1):32-6.
DOI: 10.1016/j.tim.2008.09.005.
Epub 2008 Oct 30.
PMID: 18977143
DOI: 10.1016/j.tim.2008.09.005
[Indexed for MEDLINE]
16. Stanley D, Wu SB, Rodgers N, Swick RA, Moore RJ. Differential responses of cecal microbiota to fishmeal, Eimeria and Clostridium perfringens in a necrotic enteritis challenge model in chickens. PLoS One. 2014;9(8):e104739.
DOI: 10.1371/journal.pone.0104739. eCollection 2014.
PMID: 25167074 Free PMC Article
17. Kitessa SM, Nattrass GS, Forder RE, McGrice HA, Wu SB, Hughes RJ. Mucin gene mRNA levels in broilers challenged with eimeria and/or Clostridium perfringens. Avian Dis. 2014;58(3):408-14.
PMID: 25518436
DOI: 10.1637/10757-122313-Reg.1 [Indexed for MEDLINE].
18. Dahiya JP, Hoehler D, Van Kessel AG, Drew MD. Dietary encapsulated glycine influences Clostridium perfringens and Lactobacilli growth in the gastro-intestinal tract of broiler chickens. J Nutr. 2007; 137(6):1408-14.
PMID: 17513399 Free Article
19. Teirlynck E, Bjerrum L, Eeckhaut V, Huygebaert G, Pasmans F, Haesebrouck F, Dewulf J, Ducatelle R, Van Immerseel F. The cereal type in feed influences gut wall morphology and intestinal immune cell infiltration in broiler chickens. Br J Nutr. 2009;102(10):1453-61.
DOI: 10.1017/S0007114509990407.
Epub 2009 Aug 7.
PMID: 19664304 [Indexed for MEDLINE]
20. Molnár A, Hess C, Pál L, Wágner L, Awad WA, Husvéth F, Hess M, Dublecz K. Composition of diet modifies colonization dynamics of Campylobacter jejuni in broiler chickens. J Appl Microbiol. 2015; 118(1):245-54.
DOI: 10.1111/jam.12679
Epub 2014 Nov 25. PMID: 25358748 [Indexed for MEDLINE]
21. Keyburn AL, Bannam TL, Moore RJ, Rood JI. NetB, a pore-forming toxin from necrotic enteritis strains of Clostridium perfringens. Toxins (Basel). 2010; 2(7):1913-27.
DOI: 10.3390/toxins2071913.
Epub 2010 Jul 23. Review.
PMID: 22069665 Free PMC Article
22. Verherstraeten S, Goossens E, Valgaeren B, Pardon B, Timbermont L, Haesebrouck F, Ducatelle R, Deprez P, Wade KR, Tweten R, Van Immerseel F. Perfringolysin O: The Underrated Clostridium perfringens Toxin? Toxins (Basel). 2015;7(5):1702-21.
DOI: 10.3390/toxins7051702.
Review. PMID: 26008232 Free PMC Article
23. Timbermont L, De Smet L, Van Nieuwerburgh F, Parreira VR, Van Driessche G, Haesebrouck F, Ducatelle R, Prescott J, Deforce D, Devreese B, Van Immerseel F. Perfrin, a novel bacteriocin associated with netB positive *Clostridium perfringens* strains from broilers with necrotic enteritis. Vet Res. 2014;45:40.
DOI: 10.1186/1297-9716-45-40
PMID: 24708344 PMCID: PMC3992141
DOI: 10.1186/1297-9716-45-40 [Indexed for MEDLINE] Free PMC Article
24. Romero-Saavedra F, Laverde D, Wobser D, Michaux C, Budin-Verneuil A, Bernay B, Benachour A, Hartke A, Huebner J. Identification of peptidoglycan-associated proteins as vaccine candidates for enterococcal infections. PLoS One. 2014; 9(11):e111880.
DOI: 10.1371/journal.pone.0111880. eCollection 2014. PMID:25369230 Free PMC Article [Indexed for MEDLINE] Free PMC Article
25. Dohms JE, Allen PH, Cloud SS. The immunization of broiler chickens against type C botulism. Avian Dis. 1982; 26(2):340-5.
PMID:7049149 [Indexed for MEDLINE]
26. Fernandes da Costa SP, Mot D, Bokori-Brown M, Savva CG, Basak AK, Van Immerseel F, Titball RW. Protection against avian necrotic enteritis after immunisation with NetB genetic or formaldehyde toxoids. Vaccine. 2013; 31(37):4003-8.
DOI: 10.1016/j.vaccine. 2013.05.063.
Epub 2013 May 29 PMID:23727000 Free PMC Article

27. Mot D, Timbermont L, Delezie E, Haesebrouck F, Ducatelle R, Van Immerseel F. Day-of-hatch vaccination is not protective against necrotic enteritis in broiler chickens. Avian Pathol. 2013 Apr;42(2):179-84.
DOI: 10.1080/03079457.2013.778955
PMID: 23581446 [Indexed for MEDLINE]
28. Mot D, Timbermont L, Haesebrouck F, Ducatelle R, Van Immerseel F. Progress and problems in vaccination against necrotic enteritis in broiler chickens. Avian Pathol. 2014;43(4):290-300.
DOI: 10.1080/03079457.2014.939942
PMID: 24980518
DOI: 10.1080/03079457.2014.939942
[Indexed for MEDLINE]
29. Jang SI, Lillehoj HS, Lee SH, Lee KW, Lillehoj EP, Hong YH, An DJ, Jeong W, Chun JE, Bertrand F, Dupuis L, Deville S, Arous JB. Vaccination with clostridium perfringens recombinant proteins in combination with Montanide™ ISA 71 VG adjuvant increases protection against experimental necrotic enteritis in commercial broiler chickens. Vaccine. 2012;30(36):5401-6.
DOI: 10.1016/j.vaccine.2012.06.007
Epub 2012 Jun 17.
PMID: 22713719.
30. Bangoura B, Alnassan AA, Lendner M, Shehata AA, Krüger M, Daugschies A. Efficacy of an anticoccidial live vaccine in prevention of necrotic enteritis in chickens. Exp Parasitol. 2014;145:125-34.
DOI: 10.1016/j.exppara.2014.08.004
Epub 2014 Aug 14. PMID: 25131774. [Indexed for MEDLINE]
31. Mojsoska B, Jenssen H. Peptides and Peptidomimetics for Antimicrobial Drug Design. Pharmaceuticals (Basel). 2015;8(3):366-415.
DOI: 10.3390/ph8030366. Review.
PMID: 26184232 Free PMC Article
32. Ötvös L Jr, Wade JD. Current challenges in peptide-based drug discovery. A Specialty Grand Challenge Article. Front Chem. 2014;2:62.
DOI: 0.3389/fchem.2014.00062. eCollection 2014.
PMID: 25152873 PMCID: PMC4126357. Free PMC Article
33. Lin L, Nonejuie P, Munguia J, Hollands A, Olson J, Dam Q, Kumaraswamy M, Rivera H Jr, Corriden R, Rohde M, Hensler ME1, Burkart MD, Pogliano J, Sakoulas G, Nizet V. Azithromycin synergizes with cationic antimicrobial peptides to exert bactericidal and therapeutic activity against highly multidrug-resistant gram-negative bacterial pathogens. EBioMedicine. 2015 2(7):690-8.
DOI: 10.1016/j.ebiom.2015.05.021
eCollection 2015 Jul.
PMID: 26288841 PMCID: PMC4534682
DOI: 10.1016/j.ebiom.2015.05.021
[Indexed for MEDLINE] Free PMC Article. Comment in Rethinking the Antibiotic Discovery Paradigm. [EBioMedicine. 2015]
34. Forst S, Nealson K. Molecular biology of the symbiotic-pathogenic bacteria *Xenorhabdus* spp. and *Photorhabdus* spp. Microbiol Rev. 1996;60(1):21-43. Review.
PMID: 8852894 PMCID: PMC239416
[Indexed for MEDLINE] Free PMC Article
35. Nollmann FI, Dauth C, Mulley G, Kegler C, Kaiser M, Waterfield NR, Bode HB. Insect-specific production of new GameXPeptides in *Photorhabdus luminescens* TTO1, widespread natural products in entomopathogenic bacteria. Chembiochem. 2015;16(2):205-8.
DOI: 10.1002/cbic.201402603.
Epub 2014 Nov 25.
PMID: 25425189
DOI: 10.1002/cbic.201402603 [Indexed for MEDLINE]
36. Vivas EI, Goodrich-Blair H. *Xenorhabdus nematophilus* as a model for host-bacterium interactions: rpoS is necessary for mutualism with nematodes. J Bacteriol. 2001;183(16):4687-93.
PMID: 11466270 PMCID: PMC99521
DOI: 10.1128/JB.183.16.4687-4693.2001
[Indexed for MEDLINE] Free PMC Article
37. Park D, Ciezki K, van der Hoeven R, Singh S, Reimer D, Bode HB, Forst S. Genetic analysis of xenocoumacin antibiotic production in the mutualistic bacterium *Xenorhabdus nematophila*. Mol Microbiol. 2009;73(5):938-49.
DOI: 10.1111/j.1365-2958.2009.06817.x.
Epub 2009 Aug 4.
PMID: 19682255 [Indexed for MEDLINE]
38. Gualtieri M, Aumelas A, Thaler JO. Identification of a new antimicrobial lysine-rich cyclolipopeptide family from Xenorhabdus nematophila. J Antibiot (Tokyo). 2009;62(6):295-302.
DOI: 10.1038/ja.2009.31
Epub 2009 Apr 17.
PMID: 19373275
DOI: 10.1038/ja.2009.31[Indexed for MEDLINE]

39. Bode HB, Brachmann AO, Jadhav KB, Seyfarth L, Dauth C, Fuchs SW, Kaiser M, Waterfield NR, Sack H, Heinemann SH, Arndt HD. Structure elucidation and activity of kolossin A, the D-/L-pentadecapeptide product of a giant nonribosomal peptide synthetase. Angew Chem Int Ed Engl. 2015;54(35):10352-5.
DOI: 10.1002/anie.201502835
Epub 2015 Jun 26.
PMID: 26118790
DOI: 10.1002/anie.201502835 [Indexed for MEDLINE]
40. Reimer D, Bode HB. A natural prodrug activation mechanism in the biosynthesis of non-ribosomal peptides. *Nat. Prod. Rep*. 2013;**31**(2):154-159.
DOI: 0.1039/c3np70081j. Review.
PMID: 24356302
41. Medema MH, Kottmann R, Yilmaz P, 1, Cummings M, Biggins JB, Blin K, Claesen J, et al. Minimum Information about a Biosynthetic Gene cluster. Nat Chem Biol. 2015;11(9):625-31.
DOI: 10.1038/nchembio.1890
DOI: 10.1038/nchembio.1890.
PMID: 26284661 PMCID: PMC5714517
DOI: 10.1038/nchembio.1890 [Indexed for MEDLINE] Free PMC Article
42. Houard J, Aumelas A, Noël T, Pages S, Givaudan A, Fitton-Ouhabi V, Villain-Guillot P, Gualtieri M. Cabanillasin, a new antifungal metabolite, produced by entomopathogenic *Xenorhabdus cabanillasii* JM26. J Antibiot (Tokyo). 2013;66(10):617-20.
DOI: 10.1038/ja.2013.58. Epub 2013 Jun 12. PMID: 23756685
DOI: 10.1038/ja.2013.58 [Indexed for MEDLINE]
43. Furgani G, Böszörményi E, Fodor A, Máthé-Fodor A, Forst S, Hogan JS, Katona Z, Klein MG, Stackebrandt E, Szentirmai A, Sztaricskai F, Wolf SL. Xenorhabdus antibiotics: a comparative analysis and potential utility for controlling mastitis caused by bacteria. *J* Appl Microbiol. 2008;104(3):745-58.
Epub 2007 Nov 1. PMID: 17976177
DOI: 10.1111/j.1365-2672.2007.03613.x [Indexed for MEDLINE]
44. Lengyel K1, Lang E, Fodor A, Szállás E, Schumann P, Stackebrandt E. Description of four novel species of *Xenorhabdus*, family Enterobacteriaceae: *Xenorhabdus budapestensis* sp. nov., *Xenorhabdus ehlersii* sp. nov., *Xenorhabdus innexi* sp. nov., and *Xenorhabdus szentirmaii* sp. nov. Syst Appl Microbiol. 2005;28(2):115-22. Erratum in: Syst Appl Microbiol. 30 (1): 83. Also in March/April 2014 Volume 2 Issue 2 e00190-14 Genome Announcements genomea.asm.org ehlersii sp. nov., *Xenorhabdus innexi* sp. nov., and *Xenorhabdus szentirmaii* sp. nov. Syst. Appl. Microbiol. 28:115.
http://dx.doi.org/10.1
016/j.syapm.2004.10.004.227
PMID: 15830803
DOI: 10.1016/j.syapm.2004.10.004
[Indexed for MEDLINE]
45. Böszörményi E, Érsek T, Fodor A, Fodor AM, Földes LS, Hevesi M, Hogan JS, Katona Z, Klein MG, Kormány A, Pekár S, Szentirmai A, Sztaricskai F, Taylor RA. Isolation and activity of Xenorhabdus antimicrobial compounds against the plant pathogens Erwinia amylovora and Phytophthora nicotianae. J Appl Microbiol. 2009;107(3):746-59.
DOI: 10.1111/j.1365-2672.2009.04249.x. Epub 2009 Mar 23. PMID: 19320949
DOI: 10.1111/j.1365-2672.2009.04249.x
[Indexed for MEDLINE]
46. Vozik D, Bélafi-Bakó K, Hevesi M, Böszörményi E, Fodor A. Effectiveness of a peptide-rich fraction from *Xenorhabdus budapestensis* culture against fire blight disease on apple blossoms. Not. Bot. Horti. Agrobo. 2015;43(2):547-553.
DOI: 10.15835/nbha4329997
Available:www.notulaebotanicae.ro
47. Vozik D. Agricultural utilization of antimicrobial compounds produced by entomopathogenic bacteria, PhD Thesis in Hungarian] Entomopatogén Baktériumok Antimikrobiális Hatású Anyagcseretermékeinek Felhasználhatósága a Növényvédelemben Ph. D. Thesis, University of Pannonia, Veszprém, Hungary; 2017.
48. Gualtieri M, Ogier JC, Pagès S, Givaudan A, Gaudriault S. Draft Genome Sequence and Annotation of the Entomopathogenic Bacterium *Xenorhabdus szentirmaii* Strain DSM16338. Genome Announc. 2014;2(2). pii:e00190-14.
DOI: 10.1128/genomeA.00190-14.
PMID: 24625876 PMCID: PMC3953197
DOI: 10.1128/genomeA.00190-14
Free PMC Article
49. Nollmann FI, Dowling A, Kaiser M, Deckmann K, Grösch S, Ffrench-Constant

R, Bode HB. Synthesis of szentiamide, a depsipeptide from entomopathogenic *Xenorhabdus szentirmaii* with activity against *Plasmodium falciparum*. Beilstein J Org Chem. 2012;8:528-33.
DOI: 10.3762/bjoc.8.60
Epub 2012 Apr 11. PMID: 22563351
PMCID: PMC3343279 Free PMC Article

50. Fuchs SW, Sachs CC, Kegler C, Nollmann FI, Karas M, Bode HB. Neutral loss fragmentation pattern based screening for arginine-rich natural products in and *Photorhabdus*. Anal Chem. 2012; 84(16):6948-55.
DOI: 10.1021/ac300372p.
Epub 2012 Aug 6. PMID: 22873683 [Indexed for MEDLINE]
51. Fuchs SW, Grundmann F, Kurz M, Kaiser M, Bode HB. Fabclavines: Bioactive peptide-polyketide-polyamino hybrids from *Xenorhabdus*. Chembiochem. 2014;15(4):512-6.
DOI: 10.1002/cbic.201300802.
Epub 2014 Feb 13 PMID: 24532262
DOI: 10.1002/cbic.201300802 [Indexed for MEDLINE]
52. Kulkarni MM, McMaster WR, Kamysz W, McGwire BS. Antimicrobial peptide-induced apoptotic death of leishmania results from calcium-de pend ent, caspase-independent mitochondrial toxicity. J Biol Chem. 2009;284(23):15496-504.
DOI: 10.1074/jbc.M809079200
Epub 2009 Apr 8. PMID: 19357081
PMCID: PMC2708846
DOI: 10.1074/jbc.M809079200 [Indexed for MEDLINE] Free PMC Article
53. Marr AK, McGwire BS, McMaster WR. Modes of action of Leishmanicidal antimicrobial peptides. Future Microbiol. 2012;7(9):1047-59.
DOI: 10.2217/fmb.12.85. Review.PMID: 22953706. [Indexed for MEDLINE]
54. Kaya H Aguillera MM, Alumai A, Choo HY, de la Torre M, Fodor A. et al. Status of entomopathogenic nematodes and their symbiotic bacteria from selected countries or regions of the world. Biol Cont. 2006;38: 134–155.
PMID: 15782660
DOI: 10.1556/AVet.53.2005.1.7 [Indexed for MEDLINE]
55. Tóth EM, Márialigeti K, Fodor A, Lucskai A, Farkas R. Evaluation of efficacy of entomopathogenic nematodes against larvae of Lucilia sericata (Meigen, 1826) (Diptera: Calliphoridae). Acta Vet Hung. 2005;53(1):65-71.
PMID: 15782660
DOI: 10.1556/AVet.53.2005.1.7 [Indexed for MEDLINE]
56. Fodor A Fodor AM Forst S Hogan JS Klein MG Lehoczky É. Comparative analysis of antibacterial activities of *Xenorhabdu*s species on related and non-related bacteria in vivo. J Microbiol Antimicrobials. 2010;2(3):30-35.
57. Romond C, Beerens H, Criquelion J, Lepage C. De´nombrement en milieu liquide de *C. perfringens* dana leswas analysed five times, after decimal dilution of the meat.aliments. Annales des Falsifications de l'Expertise Chimique et deToxicologie. 1981;74:181–184.
58. Harmon SM, Kautter DA, Peeler JT. Improved medium for enumeration of *Clostridium perfringens*. *Appl Microbiol*. 1971;22(4):688–692. PMCID: PMC376387
59. Current Trends in Consumption of Animal Products. Chapter 1 In: Designing Foods: Animal Product Options in the Marketplace. National Research Council (US) Committee on Technological Options to Improve the Nutritional Attributes of Animal Products. Washington (DC): National Academies Press (US); 1988.
60. Amin A1, Bilic I, Berger E, Hess M. *Trichomonas gallinae*, in comparison to *Tetratrichomonas gallinarum*, induces distinctive cytopathogenic effects in tissue cultures. Vet Parasitol. 2012;186(3-4):196-206.
DOI: 10.1016/j.vetpar.2011.11.037.
Epub 2011 Nov 20. PMID: 22172581
DOI: 10.1016/j.vetpar.2011.11.037 [Indexed for MEDLINE]
61. Ibrahim HR, Tatsumoto S, Ono H, Van Immerseel F, Raspoet R, Miyata T. A novel antibiotic-delivery system by using ovotransferrin as targeting molecule Eur J Pharm Sci. 2015;66:59-69.
DOI: 10.1016/j.ejps.2014.10.005. Epub 2014 Oct 12. PMID:25315410 [Indexed for MEDLINE]
62. Lanckriet A, Timbermont L, De Gussem M, Marien M, Vancraeynest D, Haesebrouck F, Ducatelle R, Van Immerseel F. The effect of commonly used anticoccidials and antibiotics in a subclinical necrotic enteritis model. Avian Pathol. 2010;39(1):63-8.
DOI: 10.1080/03079450903505771
PMID: 20390538. [Indexed for MEDLINE]

63. Li W, Tailhades J, O'Brien-Simpson NM, Separovic F, Ötvös L. Jr, Hossain MA, Wade JD. Proline-rich antimicrobial peptides: Potential therapeutics against antibiotic-resistant bacteria. Amino Acids. 2014;46(10):2287-94.
DOI: 10.1007/s00726-014-1820-1
Epub 2014 Aug 21. Review. PMID: 25141976

64. Ötvös L. Jr, Flick-Smith H, Fox M, Ostorházi E, Dawson RM, Wade JD. The designer proline-rich antibacterial peptide A3-APO prevents *Bacillus anthracis* mortality by deactivating bacterial toxins. Protein Pept Lett. 2014;21(4):374-81. PMID: 24164262

65. Zheng X, Wang X, Teng, Mao R, Hao Y, Yang N, Zong L, Wang J. Mode of action of plectasin-derived peptides against gas gangrene-associated Clostridium perfringens type A. PLoS One. 2017; 12(9):e0185215.
DOI: 10.1371/journal.pone.0185215. eCollection 2017. PMID: 28934314 Free PMC Article

66. Ducatelle R, Eeckhaut V, Haesebrouck F, Van Immerseel F. A review on prebiotics and probiotics for the control of dysbiosis: present status and future perspectives. Animal. 2015;9(1):43-8.
DOI: 10.1017/S1751731114002584
Epub 2014 Oct 22. Review. PMID:25336177 [Indexed for MEDLINE]

67. Skrivanova E Van Immerseel F Hovorkova P Kokoska L. In Vitro Selective Growth-Inhibitory Effect of 8-Hydroxyquinoline on *Clostridium perfringens* versus Bifidobacteria in a Medium Containing Chicken Ileal Digesta. PLoS One. 2016; 11(12):e0167638.
DOI: 10.1371/journal.pone.0167638
eCollection 2016. PMID: 27936245 Free PMC Article

68. Eeckhaut V, Wang J, Van Parys A, Haesebrouck F, Joossens M, Falony G, Raes J, Ducatelle R, Van Immerseel F. The probiotic butyricicoccus pullicaecorum reduces feed conversion and protects from potentially harmful intestinal microorganisms and necrotic enteritis in broilers. Front Microbiol. 2016;7:1416. eCollection 2016. PMID: 27708624 Free PMC Article

69. Geeraerts S, Delezie E, Ducatelle R, Haesebrouck F, Devreese B, Van Immerseel F. Vegetative *Bacillus amyloliquefaciens* cells do not confer protection against necrotic enteritis in broilers despite high antibacterial activity of its supernatant against Clostridium perfringens in vitro. Br Poult Sci. 2016; 57(3):324-9.
DOI: 10.1080/00071668.2016.1169246
PMID:27122203

70. Gholamiandehkordi A, Eeckhaut V, Lanckriet A, Timbermont L, Bjerrum L, Ducatelle R, Haesebrouck F, Van Immerseel F. Antimicrobial resistance in Clostridium perfringens isolates from broilers in Belgium. Vet Res Commun. 2009;33(8):1031-7.
DOI: 10.1007/s11259-009-9306-4
PMID:19597952

71. Ngamwongsatit B, Tanomsridachchai W, Suthienkul O, Urairong S, Navasakuljinda W, Janvilisri T. Multidrug resistance in Clostridium perfringens isolated from diarrheal neonatal piglets in Thailand. Anaerobe. 2016;38:88-93.

72. Aradhyula S, Manian FA, Hafidh SA, Bhutto SS, Alpert MA. Significant absorption of oral vancomycin in a patient with *Clostridium difficile* colitis and normal renal function. South Med J. 2006; 99(5):518-20.
PMID: 16711316
DOI:10.1097/01.smj.0000216477.06918.a3 [Indexed for MEDLINE]

Peer-review history:
The peer review history for this paper can be accessed here:
http://www.sciencedomain.org/review-history/22705