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Original Research Article

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

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ABSTRACT

Aims: Revealing anticoccidial potential (that is activity on both causative pathogens, the prokaryotic *Clostridium perfringens)* and the eukaryotic *Eimeria tenella*) of antimicrobial peptides from *Xenorhabdus budapestensis*. **Objectives:** *in vitro* tests of cell-free culture media (CFCM) of *Xenorhabdus budapestensis* DSM 16342 (EMA) and *X. szentirmaii* DSM 16338 (EMC) on 13 *C. perfringens* isolates; and on their cytotoxicity; preparation "**Xenofood**" for future *in vivo* feeding studies aiming at studying the efficacy of EMA and EMC on *C. perfringens* colony-forming units (CFU) in the ileal digests and side-effects.
Study design: Clostridium perfringens samples LH-1-LH24 were collected from chicken and poultry by L. Fodor and L. Makrai. A. Fodor as a visiting scientist were growing EMA and EMC liquid cultures and obtained sterile CFCM and tested in all but 11 LH isolates.
Place and Duration of Study: Department of Microbiology and Infectious Diseases, Faculty of Veterinary Science, Szent István University, Budapest, Hungary between September 2013 and February 2014.
Methodology: Adaptation of previously published i*n vitro* bioassays of EMA and EMC CFCM (with and without proteolytic pepsin and trypsin digestion) on *C. perfringens* isolates. Xenofood is a mixture of an autoclaved, mid-stationary phase culture of *Xenorhabdus budapestensis* and *X. szentirmaii* grown in conventional "starter" and "grower" chicken food.
Results: Antimicrobial (peptides of both EMA and EMC CFCM re heat-stable, trypsin and pepsin resistant. All but one of 13 *C. perfringens* isolates proved sensitive to EMA-CFCM. Previously we found that these antimicrobial products inactivate *E. tenella* cells, but were toxic to permanent chicken liver (LMH) cells. **Conclusion:** We are ready for running an *in vivo* feeding test comparing Xenofood-fed and control broiler cockerels and determine the gastrointestinal (ileal) anti-*Clostridium* and anti-*Eimeria* activity; rate of adsorption; antigenicity; and physiological effects of the heat,- and proteolysis tolerant antimicrobial peptides of *Xenorhabdus budapestensis* and *X. szentirmaii* species.

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

1. INTRODUCTION

Multi-drug resistance (MDR) has gradually been increasing in both Gram-positive [1] (Nawrocki et al., 2014) and Gram-negative [2] (Gruenheid et al., 2012) pathogenic bacterium species. MDR has always been a phenotypic consequence of sequential accumulation of simultaneously appearing mutations, or the up-take of resistance plasmids harboring mobile genetic elements or genomic islands with resistance genes. These encode for either enzymes capable of 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] (Borst et al., 2012); [4] (Miller et al., 2015); [5] (Palmer et al., 2014), in *Clostridium* [6] (Dahms et al., 2014) and in *Salmonella* genera [7] (Lu et al., 2014). The explanation is that the poultry GI is an ideal "market place" for exchange and horizontally transferring resistance gene –carrying plasmids and mobile genetic elements between coexisting bacteria. *Enterococcus cecorum,* for instance, once a simple commensal member of the intestinal microbiota, has become the causative pathogen of arthritis and osteomyelitis worldwide in chickens, such as in Hungary [8] (Makrai et al., 2011) and Poland [9] (Dolka et al., 2016). Evidences of multidrug-resistant plasmid transfer from

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51 Gram positive [10] (Lebreton, 2013) and Gram negative [11] (Szmolka and Nagy, 2013); [12]
52 (Hasman et al., 2015) chicken pathogens via consumed chicken meat to human pathogens,
53 has been accumulating. Apart from the veterinary aspects, this horizontal gene transfer is of
54 clinical importance.

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

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

77 *C. perfringens* type A cells release several toxins that promote disease development not only
78 in chicken, but also in humans. The necrotic enteritis B-like toxin (NetB) is a β-barrel pore-
79 forming one, which used to be considered as a vaccine candidate [21] (Keyburn et al.,
80 2010). Another toxin called perfringolysin O (PFO, also referred to as θ toxin), is a pore-
81 forming cholesterol-dependent cytolysin (CDC). PFO is secreted as a water-soluble
82 monomer that recognizes and binds membranes via cholesterol. Membrane-bound
83 monomers undergo structural changes that culminate in the formation of an oligomerized
84 pre-pore complex on the membrane surface. The pre-pore then undergoes conversion into
85 the bilayer-spanning pore. Research has demonstrated a role for PFO in gas gangrene
86 progression and in bovine necro-hemorrhagic enteritis, [22] (Verherstraeten et al., 2015). *C.*
87 *perfringens* strains which had been isolated from outbreaks of necrotic enteritis are also
88 capable of secreting factors that inhibit growth of other (competitor) C*. perfringens* strains,
89 including those isolated from the gut of healthy chickens. This feature lends a selective virtue
90 to respective NetB-toxin producing virulent strains, the causative factor of gut lesions. The
91 factor providing this selective virtue to the virulent strain is a novel, chromosomally encoded
92 heat-labile, trypsin - and proteinase-K sensitive protein with bacteriocin activity called perfrin.
93 The gene, which can only be found in *C. perfringens* NetB strains and nowhere else,
94 (despite the fact that the NetB is a plasmid encoded toxin), could be transferred to and

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95 expressed in *E. coli*, in the laboratory, (but theoretically it may happen in the chicken GI at
96 any time) and the recombinant gene product is antibacterial active at a large pH range [23]
97 (Timbermont et al., 2015).

98 Vaccination is an effective but not omnipotent veterinary tool for controlling MDR pathogens
99 and *Clostridia*. The vaccination projects concerning *Enterococcus* seem to be in promising
100 but only at the experimental stage [24] (Romero-Saavedra et al., 2014). None of the seven
101 available publications contain anything on poultry. As for *Clostridia,* the vaccination of
102 chickens against the fatal human pathogen type C (causing botulism*)* were successful [25]
103 (Dohms et al., 1982). The vaccination against *C. perfringens* has seemed to be close to
104 realization for years, but has not been realize yet. The immunization with NetB genetic, or
105 formaldehyde toxoids, seems the most plausible approach [26] (Fernandes Da Costa et al.,
106 2013), but the same team published that only double vaccination (on age 3 and 12 days)
107 with crude supernatant were effective. Immunization not with a single toxin molecule did not
108 give satisfactory protection for chickens against necrotic enteritis lesions, [27] (Mot et al.,
109 2013).

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

122 This approach should invoke a comprehensive strategy, based on Quantitative Structure –
123 Activity Relation (QSAR) analysis and *in silico* modelling [31] (Mojsoska and Jenkinns,
124 2015), and chemical synthesis of modified analogs leading to new antimicrobial agents with
125 novel modes of action. The structural designing AMP candidate molecules has been aiming
126 at improving endurance to proteolytic degradation, binding to, and the penetration through
127 cellular membranes and other biological barriers, which can be achieved by adding modules
128 for passive or active transport, [32] Ötvös and Wade, 2014). Another approach is searching
129 for efficient synergisms, [33] (Lin et al., 2017). Another (ever-green) alternative research line
130 is to search for new antimicrobials of completely novel mode of action in the nature.

131 Our research team has been searching for novel antimicrobials, which are not used in
132 human medicine, are toxic only for chicken pathogens but not toxic for organisms to be
133 protected. We expect to find the best candidates amongst natural antimicrobial peptides,
134 (AMPs) synthetized by the obligate bacterial symbionts (EPB) of entomopathogenic
135 nematodes (EPN) [34] (Forst & Nealson, 1996). These EPB-released AMPs are evolutionary
136 products developed under severe selective pressure and comprise a powerful chemical
137 arsenal against a large scale of prokaryotic and eukaryotic organisms to provide monoxenic

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138 conditions for a given respective EPN / EPB symbiotic complex in polyxenic (insect gut, soil)
139 conditions. There are many EPN-EPB complexes have been existing, many AMP profiles
140 could be determined. Considering that all but one [35] (Nollmann et al., 2015) of the known
141 AMPs can be produced by the bacterium *in vitro*, the EPN/EPB complexes provide a gold
142 mine for researchers interested in new antimicrobials.

143 The majority of EPB-produced AMPs were identified in the last 15 years [36] (Vivas &
144 Goodrich-Blair, 2001); [37] (Park et al., 2009); [38] (Gualtieri et al., 2009); [39] (Bode et al.,
145 2015A). Each of these evolutionarily designed antibiotic arsenals has effectively overcome
146 intruders representing a full scale of antibiotic resistance repertoire in their respective niche.
147 Each EPB-AMP discovered so far is a non-ribosomal peptide (NRP), synthesized by multi-
148 enzyme thiotemplate mechanisms, using non-ribosomal peptide synthetases (NRPS), fatty
149 acid synthases (FAS), and / or related polyketide synthases (PKS), or a hybrid biosynthesis
150 thereof [40] (Reimer & Bode, 2013). The biosynthetic enzymes are encoded by biosynthetic
151 gene clusters [41] (Medema et al., 2015), determining the biosynthetic same word used
152 three times in a sentence pathways.

153 Cabanilasin, from *X. cabanillasii,* exerts of a strong antifungal activity [42] (Houard et al.,
154 2013). In our experiments, the cell-free culture media (CFCM) of *X. cabanillasii* was also
155 extremely toxic to *S. aureus, Escherichia coli* and *Klebsiella pneumoniae* isolated from cows
156 with mastitis syndromes [43] (Furgani et al., 2008). In that experiment, the antibacterial
157 activities of the CFCM of several *Xenorhabdus* species were compared. We found that and
158 those of *X. budapestensis* DSM 16342 (EMA), and *X. szentirmaii* DSM 16338 (EMC) [44]
159 (Lengyel et al., 2005) were by far the best. The CFCM of EMA and EMC also were effective
160 against *Staphylococcus aureus MRSA* (Fodor, McGwire, and Kulkarni, unpublished).
161 Furthermore, the CFCM from EMA and EMC also proved effective against plant pathogens,
162 including both prokaryotic Erwinia *carotavora, Clavibacter michigenense and* several
163 *Xanthomonas* species, [45] (Böszörményi et al., 2009); [46] (Vozik et al., 2015); [47] (Vozik,
164 2017), and all tested eukaryotic Oomycetes (*Phytophthora*) species [42] (Böszörményi et al.,
165 2009); (Muvevi et al., unpublished). Gualtieri confirmed our data declaring that *X.*
166 *szentirmaii* DSM16338 (EMC) was really a source of antimicrobial compounds of great
167 potential, and sequenced this strain [48] (Gualtieri et al., 2014). One of the products
168 (szentiamide) has been chemically synthesized [46] (Nollmann et al., 2012).

169 We suppose that these antimicrobial peptides act in concert. The idea of “Xenofood” is
170 based on the intention to benefit from the joint action of cooperating AMP molecules
171 produced by EMA and EMC cells, not only on a single molecule. We know that the
172 strongest, predominant antibacterial peptide produced by both EMA and MC species is the
173 fabclavine, [51] Fuchs et al. (2014). Many of our experiments with EMA were repeated and
174 confirmed in the laboratory of Professor Helge B Bode (Goethe-Universität, Frankfurt – am -
175 Main Germany), and they confirmed that EMA CFCM exhibited broad-spectrum bioactivity
176 against *Bacillus subtilis, E. coli, Micrococcus luteus, Plasmodium falciparum,*
177 *Saccharomyces cerevisiae, Trypanosoma brucei, and T. cruzi,* [51] (Fuchs et al., 2012).
178 They subjected the CFCM from *X. budapestensis* to MALDI-MS analysis which showed
179 signals of unknown compounds. One compound (1, 1356.96 Da), fabclavine was purified,
180 and its structure was determined. The details of biosynthesis are precisely reconstructed by
181 the authors, but no data about their mode of action has so far been published, [51] Fuchs et

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182 al. (2014). Fabclavines are considered as a novel class of biosynthesized hybrid peptide–
183 polyketide-polyamino natural compounds of extremely high antimicrobial potential in both
184 prokaryotic and eukaryotic pathogen targets, but also with unwanted eukaryotic cell-toxicity,
185 https://www.ncbi.nlm.nih.gov/pubmed/24532262,. They are unambiguously the most
186 effective antimicrobial *Xenorhabdus* peptide-products that have ever been discovered, and
187 they are released by *X. budapestensis* and X*. szentirmaii* [44] (Lengyel et al., 2005).

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

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

207 Prior to the planned in vivo feeding test we carried out in vitro bioassays presented here.
208

209 ## 2. MATERIAL AND METHODS

210

211 ### 2.1 Bacterium Strains

212 *Clostridium perfringens* NCAIM 1417 strain was obtained from the National Collection of
213 Agricultural and Industrial Microorganisms –WIPO
214 (www.wipo.int/budapest/en/idadb/details.jsp?id=5834; of Hungary, Faculty of Food
215 Sciences, Szent István University Somlói út 14-16 1118 Budapest, Hungary) from Dr. Judit
216 Tornai. *C. perfringens* LH1-LH8; LH11-LH16; LH19, LH20 and LH24 used in this study are
217 of chicken origin, from the stock collection of Dr. L.Makrai. *Xenorhabdus* strains, *X.*
218 *budapestensis* DSM 16342 (EMA); *X. szentirmaii,* DSM 16338 (EMC); [44] Lengyel et al,
219 2005) and *X. bovienii* NYH had been isolated by the Fodor laboratory from the
220 entomopathogenic nematodes *Steinernema bicornutum* [Tallósi], [54] (Kaya et al., 2006); *S.*
221 *rarum* and *S. feltiae* HU1 [55], (Tóth, et al, 2005), respectively. EMA and EMC had been
222 deposited by us to the DSMZ, (Leinbniz Institute Deutsche Sammlung von Mikroorganismen
223 und Zellkulturen, Braunchweig, Germany) as DSM 16342 and DSM 16338, respetively. *X.*
224 *nematophila* ATTC 19061 was kndly provided by Professor S. A. Forst (University of
225 Wisconsin – Milwaukee, USA) and *X. nematophila* DSM3370 by Professor E. Stackebrandt,
226 (DSMZ, Braunschweig, Germany). *S. cabanillasii* BP was isolated by ourselves from the

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227 infective dauer juvenile form of the EPN *S. riobrave*, kindly provided by Professor Byron
228 Adams (Bringham Young University, Provo, UT, USA).

229 2.2 Overlay Bioassays for Comparing the Antibacterial Potential of Different 230 *Xenorhabdus* Strains

231 Overlay bioassays for comparing the antibacterial potential of different Xenorhabdus strains
232 (each representing a species) were carried out as previously described [43] Furgani et al.,
233 2008 In order to make sure that we use the proper bacterium, an earlier experiment was
234 repeated in which we compared the antibacterial activities of 5 different *Xenorhabdus* strains
235 on *Klebsiella pneumoniae*.

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

243 2.3 Agar-Diffusion Assay of EMA CFCM on *Clostridium perfringens* NAIM 1417 244 Laboratory Strain

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

251 2.4 Comparison of The Sensitivities (MID Values) of 13 *C. perfringens* Strains 252 Isolated from Poultry in Liquid Cultures to Cell-Free Culture Media (CFCM) of 253 *X. Budapestensis* (EMA)

254 2.4.1 Determination of MID Values

255 In order to quantify the sensitivity of the strains, the maximum inhibiting dilution (MID) values
256 ([43] Furgani et al., 2008); [56] Fodor et al., 2010; [46] (Vozik et al., 2015), [47] (Vozik, 2017)
257 were determined as follows: These studies were carried out in sterile 24-Hole Tissue Culture
258 Plates, with 4 (A-D) rows and 6 (1-6) Columns; in 1 ml final volumes. Each *Clostridium*
259 strains were used in different tissue culture plate. Each hole contained 0.5 ml of 2XRCM
260 (Reinforced Clostridium Media, [57] (Romond et al., 1981) liquid medium and 0.5 ml of
261 sterile, diluted EMA CFCM, in the following distribution: 100, 80, 60, 40, 20 and 0 V/V % in
262 column 1, 2, 3, 4, 5 and 6, respectively. There were 50, 40, 30, 20, 10 and 0 V/V% final
263 concentration of EMA CFCM in columns 1, 2, 3, 4, 5 and 6, such a way. Each cultures in
264 rows A, B and C were inoculated with loopful amount of the respective bacteria obtained
265 from three separate colonies grown on sheep blood agar plates. The holes in row D were not
266 inoculated and served as sterile (negative) controls. Columns 6 served did not contain EMA
267 CFCM and served as positive controls. Each 1-ml culture was overlaid by 0.5 ml sterile
268 (freshly autoclaved) paraffin oil to provide anaerobic conditions. Plates were then incubated
269 at 37°C for 24h and then scored visually. After 24h culturing the growing and inhibited

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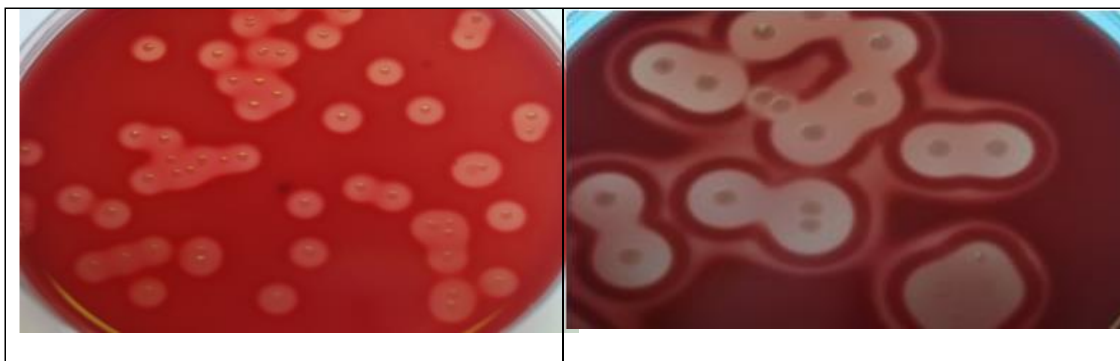
270 cultures could unambiguously be identified. We considered the concentration as MID where
271 neither of the 3 replicates contained visible growth.

272 **2.4.2 Enumeration of *Clostridium perfringens* colony forming units (CFU)**

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

290 2.4.3. **Statistical Analysis**

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



294 .

295 Figure 1 Enumerating *Clostridium* colony forming unites (CFU) on sheep blood agar plates .
296 (Photo: Dr. László Makrai, (Department of Microbiology and Infectious Diseases, University
297 of Veterinary Science, Szent István University, Budapest, Hungary).

298 **2.5 Study of the Endurance of the Antimicrobial Compounds in the Cell-Free**
299 **Culture Media (CFCM) of *X. budapestensis* and *X. szentirmaii* to Proteolytic**
300 **Degradation**

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301 **2.5.1. Trypsin-digested samples** were tested on Gram-positive (*S. aureus*) and Gram
302 negative (*E. coli*) targets in agar diffusion assay, in comparison with untreated CFCM
303 samples. No differences were demonstrated.

304 **2.5.2. Pepsin resistance** was studied as follows**:** in the center of a Luria Broth plate, a 0.22
305 um pore size Millipore filter was laid and infiltrated with HCl and pepsin, and then EMA
306 CFCM was pipetted onto it. The pepsin preparations were prepared by Professor Ferenc
307 Husvéth. After that the plate was overlaid with a *Pseudomonas aeruginosa* suspension
308 diluted with soft agar as described [46] (Vozik et al., 2015); [47] (Vozik et al., 2017). After 24
309 h incubation at 40 ^{o}C, the growth of the test bacterium lawn were checked.

310 **2.6 Preparation of Xenofood**
311
312 Xenofood contained 5% soy-meal, which had been suspended with equal amount (w/w) of
313 EMA and another 5% suspended in equal amount (w/w) of EMC cells obtained from 5 days-
314 old shaken (2000 rpm) liquid cultures, followed by high-speed (Sorwall; for 30 minute)
315 centrifugation. The liquid cultures were in double concentrated (2X) LB (DIFCO) liquid
316 medium, supplemented with meat extract equivalent to the yeast extract. Five days was
317 optimal for antibiotics production at 25 ^{o}C under these conditions, [43] Furgani et al., 2008);
318 [45] Böszörményi et al., 2009). It had previously been discovered that both EMA and EMC
319 grow and produce antibiotics in autoclaved soy-meal containing some water and yeast
320 extract, (Fodor, unpublished). Therefore the original chicken food served as a semi-solid
321 culture media for *Xenorhabdus* cells.
322 Both the separate EMA and EMC culturing semi-solid chicken food that we (Dr. László Pál)
323 prepared daily, were incubated under sterile conditions for another five days. Then the EMA
324 and EMC culture media were combined, autoclaved (20 min, 121 ^{o}C) and then dried by heat
325 (70 ^{o}C) overnight. The *Xenorhabdus* cells were killed such a way, while the heat stabile [43]
326 (Furgani et al., 2008) antimicrobial compounds remained active.

327
328 **3. RESULTS AND DISCUSSION**
329
330 **3.1. Results of Experiments, Aiming at helping to choose the best *Xenorhabdus* strain**
331 **for this study**

332 Results are presented in Fig 2 and qualitative evaluation of the inactivation zones was
333 appropriate to make the right decisions. Based on the results of a repeated experiment we
334 choose *X. budapestensis* DSM16342 (EMA) and *X. szentirmaii* DSM 16338, (EMC) which
335 had been identified in our laboratory [44] (Lengyel et al., 2005) For the exact description and
336 history of these strains, see [43] (Furgani et al., 2008).
337
338 For the exact description and history of these strains, see [43] (Furgani et al., 2008.

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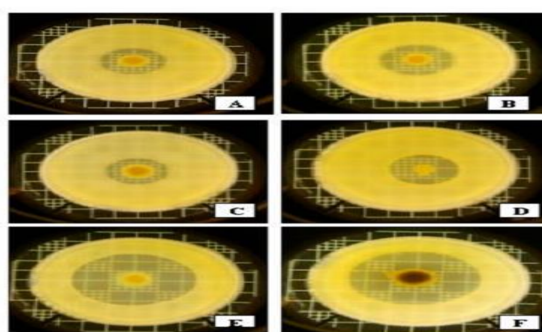

A: *X. nematophila* DSM 3370

B: *X. cabanillasii* BP

C: *X. nematophila* ATTC19061

D: *X. bovienii* NYH

E: *X. budapestensis* DSM16342[T]

F: *X. szentirmaii* DSM16338[T]

339

340 Figure 2 Comparison of the antimicrobial potential of different *Xenorhabdus* strains
341 (representing species) in overlay bioassay, [43] (Furgani et al., 2008). (Photo: Andrea Máthé
342 Fodor. (The Ohio State University, Wooster, OH, USA)

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

348 **3.2. Endurance of the antimicrobial peptides of *X. budapestensis* to pepsin, - and trypsin**
349 **digestion**
350
351 As demonstrated by Fig 3, the overnight pepsin-digested EMA CFCM remained active
352 against *Pseudomonas aeruginosa*. The trypsin-digested samples also preserved their anti-
353 Gram-positive (on *S. aureus*) and anti-Gram-negative (*E. coli*) activities, (not shown).

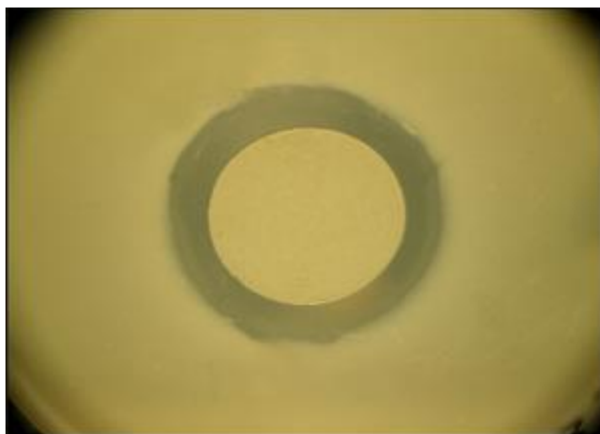

Fig 3 Experimental evidence that the antimicrobial compounds of *X. budapestensis* cell-free media are resistant to the proteolytic activity of pepsin. After 24 h incubation at 37 ^{o}C a large inactivation zone could be seen, demonstrating a significant antimicrobial activity of the pepsin-treated EMA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

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354 **3.3. Efficacy of EMA CFCM on *C. perfringens* Laboratory Strain NCAIM 1471**

355 The cell-free EMA CFCM exerted strong antimicrobial activity on *Clostridium perfringens*
356 laboratory strain NCAIM 1471 in an agar diffusion test. The large inactivation zone of 3.7 cm
357 diameter prove the anti – *Clostridium* activity, see Fig 3. The question arisen whether the
358 pathogen poultry isolates were also sensitive.
359
360

361
362
363
364 Figure 4 Anti- *Clostridium* activity of cell-free culture medium of *X. budapestensis* on
365 *Clostridium perfringens* NCAIM 1417 strain in agar diffusion test [46] (Vozik et al., 2015), [47]
366 (Vozik, 2017). (Photo: Dr. Csaba Pintér, University of Pannonia, Keszthely, Hungary)
367
368 **3.4 Results of the Comparison of the Sensitivities (MID values) of 13 *C. perfringens***
369 **strains isolated from Chicken in Liquid Cultures to Cell-Free Culture Media (CFCM) of**
370 ***X. budapestensis* (EMA)**

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

378

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

Clostridium 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

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

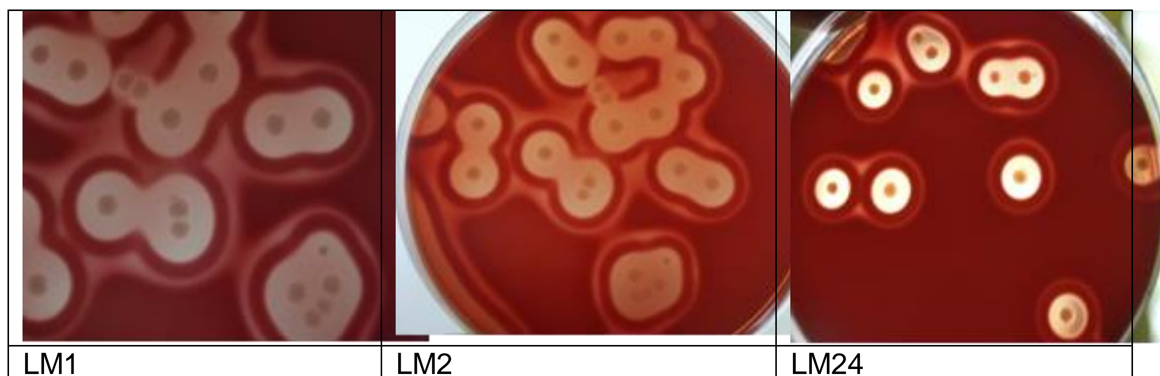

Fig 5 *Clostridium perfringens* isolates LM1, LM2 and LM24 differing in colony morphology, sporulation willingness and hemolytic behavior. (Photo: Dr. László Makrai, (Department of Microbiology and Infectious Diseases, University of Veterinary Science, Hungary).

4. Discussion

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396 The *in vitro* experiment demonstrated that antimicrobial peptides of *X. budapestensis* (EMA)
397 were highly toxic for all but one *C. perfringens* isolates. Dr. Klaus Teichmann (Biomin, Tulln,
398 Austria), also for courtessy, was working with the same EMA and EMC preparation as
399 Ganes et al., and declared that the CFCM of EMA exerted an extremely strong anticoccidial
400 activity on both *Clostidium* and *Eimeria* cells. (He told he has never worked with such an
401 efficient anticoccidium peparation before). He found a concentration range whithin which
402 *Eimeria tenella* cells died, while the cells of the chicken tissue culture were not affected,
403 (Klaus Teichmann personal communication). These facts are arguments for taking EMA
404 antimicrobial peptides as a potential anticoccidial agent into consideration.

405 But there are arguments against *Xenofood* as well, and they are those data which prove *in*
406 *vitro* cytotoxicity on the permanent chicken liver cell line LMH, [60] Amin et al. (2012), as
407 found by Dr. Petra **Ganas** (Clinic for Avian, Reptile and Fish Medicine, Vetmeduni, Vienna,
408 Austria). These fellow - scientists tested the cytopathogenic effect of sterile cell-free media
409 (CFCM) of EMA and EMC on eukaryotic cells *for courtesy*. Dr. Ganas and her associates
410 (Aziza Amin, Irina Profjeva, and Micheal Hess) treated the permanent chicken liver LMH
411 cells with different dilutions of our EMA and EMC preparations, and for evaluation, used the
412 score-scale published by [60] Amin et al. (2012). They demonstrated that EMA CFCM at a
413 dose of V/V < 5% concentration was harmless, but at V/V >5% concentrations they
414 seriously damaged the cell layer. Doses >10 V/V% caused total destruction of the cell layer,
415 while that of 5 – 10 V/V dose range resulted in about a 50% demage within the first 24h,
416 and this demage was not repaired in the next 72 hrs. As for EMC, only the dose of 32%
417 resulted in complete cell layer destruction, but the lower doses of 1-20 V/V% range also
418 resulted in a permanent ~ 50% demage, calculate on the base of the score scale of [60]
419 Amin et al. (2012), (Petra Ganes et al., personal communication).

420 Fabclavines are the predominant antimicrobial compound poduced by both EMA and EMC
421 and were isolated and purified by [51] (Fuchs et al, 2014),who did not suggest it as a drug of
422 the future because of its extreme large target size and toxicity in eukaryotic targets.

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

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

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

430 Second, there has no practically applicable vaccination technique available against *C.*
431 *perfringens*, [28] (Mot et al., 2014).

432 Recently there are several research directions trying to solve the coccidiosis problem. The
433 projects, among others, include search for novel antibiotic-delivery systems, (such as that
434 uses ovotransferrin as targeting molecule), [61] (Ibrahim et al., 2015). There are also works
435 toward improving the usefulness of commonly used anticoccidials and antibiotics (which
436 have recently been tested on a subclinical necrotic enteritis model), [62] (Lanckriet et al,

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437 2010). Quite recently the hopes toward applying probiotics, [63] (Ducatelle et al., 2015); [64]
438 (Skrivanova et al., 2016); [65] (Eeckhaut et al., 2016); [66] (Geeraerts et al., 2016) have
439 been emerging. However, the coccidiosis problem has not seem to be solved yet. Until the
440 coccidiosis problem has not been solved, to search for new efficient antimicrobials is
441 probably justified.

442 A question is whether in the age of the boom of multidrug resistance an attempt to introduce
443 a new antibiotic were scientifically justified.

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

445 1. Whether the orally administered fabclavine has a useful anti-*Clostridium* and anti-
446 *Eimeria* potential were strong enough for curing of preventive applications;

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

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

450 For decades, antibiotics have been used extensively in animal production worldwide, [15]
451 (Van Immerseel et al., 2009) as growth promoting agents. Added in low doses to the feed of
452 farm animals, they have been shown to increase daily weight gain and conversion of feed
453 into body mass, leading to some economic advantages for farmers. However, there are
454 serious concerns that the use of antibiotics in the feed an increasing multidrug resistance
455 and the trend is to reduce antibiotics in feedstuff. Since 1 January 2006, legislation has been
456 in place in Europe to prohibit the use of antibiotics as growth promoters, and in other
457 continents, the use of antimicrobial growth promoters in feedstuffs is under debate, [15] (Van
458 Immerseel et al., 2009); [67] Huyghebaert et al., 2011).

459 The negative consequences of the previous practice is unambiguously demonstrated by the
460 alarming phenomenon of multidrug - and pan-drug resistance especially spectacular in
461 Enterococci, [68] (van Hoorebeke, 2011), [10} (Lebreton et al., 2013); [4] (Miller et al., 2014);
462 [5] (Palmer et al., 2014); and, although in a lesser scale, in *C. perfringens* [69]
463 (Gholamiandehkordi et al., 2009); [6] (Dahms et al., 2014); [70] (Ngamwongsatit et al., 2016)
464 as well.

465 We believe that keeping *C. perfringens* in the cross hairs of fellow-scientists working on
466 novel AMPs is important, because of the recent, but hopefully just temporary, problems
467 concerning vaccinations [25] (Mot el al., 2014). In the efforts to overcome coccidiosis, we
468 should neutralize a prokaryotic (*C. perfringens)* and the collaborating eukaryotic (*Eimeria*
469 *tenella*) pathogens. Powerful antibiotics with large scale spectra and of novel mode of action,
470 like fabclavines, are needed to slow the evolutionary process of multidrug resistance in the
471 chicken gastrointestinal biota, against which vaccination is not an effective tool at present. If
472 a novel antimicrobial is effective, has a novel mode of action, does not evoke immediate
473 resistance and its application does not mean biohazard, it should be taken into consideration
474 as potential drug.

475 The antimicrobially active compounds of EMA and EMC (present in the Xenofood) *in vitro*
476 act as strong antimicrobials and cytotoxic compounds, while *in vivo* act as strong

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477 antimicrobial in the GI but we forecast that they would not act as cytotoxic compound neither
478 in the blood nor in any organs. Supposedly, only an inignificant amount of orally adminitered
479 fabclavine would enter the circulation, similarly to orally administred vancomycin, [71]
480 (Aradhyula et al., 2006).

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

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

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

511 5. Conclusions

512 We believe that a Xenofood feeding experiment would be essential to reveal whether the
513 orally administrated antimicrobial peptides produced by *Xenorhabdus budapestensis* (EMA),
514 and *X. szentirmaii* (EMC) - which had previously proven active *in vitro* against both the
515 prokaryotic (*Clostridium perfringens*) and the eukaryotic (*Eimeria tenella*) pathogens causing
516 coccidiosis in chicken, but also proven cytotoxic *in vitro* in permanent chicken liver cells,
517 were antibiotic active *in vivo* without causing any harm in the animals to be protected.
518

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876 **ABBREVIATIONS**

877 **List of Abbreviations**

878 **AMP** = Antimicrobial Peptides

879 **CFCM** = cell-free culture media

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

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

885 **EPB** = entomopathogenic (nematode-symbiotic) bacterium

886 **EPN** = entomopathogenic nematode

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

888 **GI** = gastro-intestinal system

889 **MDR** = multi drug resistance (multiple antibiotic resistance)

890 **PBS** = Physiological buffered (NaCl) Solution