# XENOFOOD-An Autoclaved Feed Supplement Containing Autoclavable Antimicrobial Peptides-Exerts Anticoccidial GI Activity, and Causes Bursa Enlargement, but Has No Detectable Harmful Effects in Broiler Cockerels despite In Vitro Cytotoxic Ingredients

ANDRÁS FODOR *, Tibor Vellai, Claudia Hess, Petra Ganes, László Makrai *, Károly Dublecz, László Pál, Andor Molnár, László Fodor, Sándor Józsa, Csaba Pintér, Michael Gardner Klein *, Eustachio Tarasco, Michael Hess

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Keywords: multidrug resistance; MDR; EPB (Xenorhabdus; (X. budapestensis; X. szentirmaii; X. innexii); and Photorhabdus) species); CFCM (cell-free conditioned media); NR-AMP (non-ribosomal-templated antimicrobial peptides); anti-microbial; (anti-bacterial; - anti-coccidial; -anti-protist; - activity; cytotoxicity; in vitro; in situ (local) bioavailablity; XENOFOOD; allometry

*Article*

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**András Fodor [1]*, Tibor Vellai [1], Claudia Hess [2], Petra Ganes [2,$], László Makrai [4]*, Károly Dublecz [3], László Pál [3], Andor Molnár [2,3], László Fodor [4], Sándor Józsa [5], Csaba Pintér [5], Michael G. Klein [6]*, Eustachio Tarasco [7] and Michael Hess [2]**

[1] Department of Genetics, Eötvös Loránd University, Hungary, H-1117 Pázmány P. sétány 1C; fodor.andras@ttk.elte.hu; fodorandras@yahoo.com , vellai.tibor@ttk.elte.hu
[2] Clinic for Poultry and Fish Medicine, Department for Farm Animals and Veterinary Public Health, University of Veterinary Medicine (Vetmeduni Vienna), 1210 Vienna, Austria; michael.hess@vetmeduni.ac.at, claudia.hess@vetmeduni.ac.at, [2$]petra.ganas@bfr.bund.de .
[3] Institute of Physiology and Nutrition, Georgikon Campus, Hungarian University of Agriculture and Life Sciences,(MATE), Deák Ferenc utca 16, H-8360, Keszthely, Hungary; dublecz.karoly@uni-mate.hu, pal.laszlo@uni-mate.hu, info.mobildoki@gmail.com, jozsa.sandr@gmail.com,
[4] Department of Microbiology and Infectious Diseases, University of Veterinary Medicine, H-1581 Budapest, P.O.B. 22 , Hungary; makrai.laszlo@univet.hu, fodor.laszlo@univet.hu;
[5] Institute of Plant Pathology, Georgikon Campus, the Hungarian University of Agriculture and Life Sciences, (MATE), Deák Ferenc utca 16, H-8360, Keszthely, Hungary; pintercsaba45@gmail.com
[6] USDA-ARS & Department of Entomology, The Ohio State University, 13416 Claremont Ave, Cleveland, OH 44130, USA; klein.10@osu.edu
[7] Department of Soil, Plant and Food Sciences, University of Bari "Aldo Moro", Via Amendola 165/A, 70126 Bari, Italy; eustachio.tarasco@uniba.it
[8] Division of Infectious Diseases, Department of Internal Medicine, The Ohio State University, Columbus, OH, United States, The Ohio State University, United States
* Correspondence: fodorandras@yahool.com and (cc) makrai.laszlo@univet.hu
$ András Fodor, (cc). László Makrai, and Michael G. Klein are the corresponding authors.

**Abstract:** The ***E***ntomo***p***athogenic ***b***acteria are obligate symbionts of entomopathogenic nematode (EPN) species biosynthesize, and release non-ribosomal-templated hybrid peptides (NR-AMPs), with strong, and large-spectral antimicrobial potential, capable of inactivating pathogens belonging to different prokaryote, and eukaryote taxa. The cell-free conditioned culture media (CFCM) of *Xenorhabdus budapestensis* and *X. szentirmaii* efficiently inactivate poultry pathogens like *Clostridium*, *Histomonas*, and *Eimeria*. To learn whether a bio-preparation containing antimicrobial peptides of *Xenorhabdus* origin with accompanying (*in vitro* detectable) cytotoxic effects could be considered as a potentially applicable preventive feed supplement, we conducted a 42-day feeding experiment on freshly hatched broiler cockerels. XENOFOOD (containing autoclaved *X. budapestensis*, and *X. szentirmaii* cultures developed on chicken feed) were consumed by the birds. The XENOFOOD exerted detectable gastrointestinal (GI) activity reducing the numbers of the colony-forming *Clostridium perfringens* units in the lower jejunum. No animal was lost in the experiment. Neither the body weight, growth rate, feed-conversion ratio, nor the organ-weight data differed between the control (C) and treated (T) groups, indicating that the XENOFOOD diet did not result in any detectable adverse effects. We suppose that the parameters indicating a moderate enlargement of bursas of Fabricius (average weight, size, and individual bursa/spleen weight-ratios) in the XENOFOOD-fed group must be an indication that the humoral immune system neutralized the cytotoxic ingredients in the blood, not allowing to reach their critical cytotoxic concentration in the sensitive tissues.

**Keywords**: multidrug resistance; MDR; EPB (*Xenorhabdus*; (*X. budapestensis*; *X. szentirmaii; X. innexii*); and *Photorhabdus*) species); CFCM (cell-free conditioned media); NR-AMP (non-ribosomal-templated antimicrobial peptides); anti-microbial; (anti-bacterial; - anti-coccidial; -anti-protist; - activity; cytotoxicity; *in vitro*; *in situ* (local) bioavailablity; XENOFOOD; allometry

## 1. Introduction

The emergence, and re-emergence of diseases caused by multidrug-resistant (MDR) pathogens, and parasitic organisms in plants, invertebrates, vertebrate animals, and humans provide arguments for the urgent search for new antimicrobial-active drugs with novel modes of action **[1–12].** This study intended to contribute to the research on the drug potential of natural biosynthetic antimicrobial peptides as chemotherapeutic tools against multi-drug resistant pathogens [HM1] [u2] **[13].** The term "antimicrobial peptides" (AMPs) **[14]** includes any polyamide (or even biopolymer with ester, thioester, or otherwise modified backbone) that can be made on a contemporary chemical peptide synthesizer. The limit in size is greater than the arbitrary cutoff of 50 amino acids set up by the US Food and Drug Administration **[15]** for proteins and far exceeds that of biological recognition elements **[14].** Therefore, not only the gene-encoded, ribosomal templated antimicrobial peptides, (RT-AMPs) but other peptides of antimicrobial activity, including enzymatically (bio)-synthesized non-ribosomal templated antimicrobial peptide (NR-AMP) molecules should also be considered. The AMPs are of great perspectives to combat MDR prokaryotes because antibiotic-resistant bacteria perform a high frequency of collateral sensitivity to antimicrobial peptides, **[16].** Furthermore, the mobility patterns of AMP-resistance genes differ from those of the antibiotics-resistance genes **[17]**. RT-[HM2]. AMPs are produced by all but Archea taxa, [**18],** and considered innate components of the innate immune systems of all known eukaryotic organisms, **[19].** The RT-AMPs are usually narrow target spectral drugs, **[20]**, but their target spectrum can be extended *in vivo* **[21] probably** due to their immune-modulatory actions,**[22,23).** The "secrete" of their future drug potential is their molecular versatility, allowing QSAR modeling **[24]**, and computer-aided design of antimicrobial peptide analogs, and peptides **[18],**u1] ] **[25],** and benefiting from the well-suited and powerful tool of proteomics, for better understanding the respective study molecular responses to antimicrobial compounds, **[26]**. The vast majority of MPs are membrane-active **[25]** Others penetrate and bind to intracellular targets like proline-rich PrAMPs, **[27]**.

Several AMPs proved efficient against pathogenic-parasitic *Leishmania* and *Trypanosoma* species.

Unlike those of the RT-AMPs, the target spectrum of most NRP-AMPs is usually large, **[28] [29])**. In eukaryotic targets, some induce apoptotic death of leishmanial protist parasites through calcium-dependent, caspase-independent mitochondrial toxicity mechanism **[30].**

***E***ntomo***p***athogenic ***b***acteria (abbreviation EPBs), the obligate symbionts of entomopathogenic nematode (EPN) species synthesize and release NRP-hybrid [HM1] [u2] [u3] peptides that provide well-balanced pathobiome conditions for the respective EPN/EPB symbiotic complex in polyxenic (insect cadaver in the soil) environments **[31,32]**. These bacteria are considered potential sources of potent natural anti-microbial **[31]**, and anti-protist **[33]** compounds. We have previously found antimicrobial active secreted peptides produce by *Xenorhabdus budapestensis* and *X. szentirmaii* **[34,35]** in both solid, and liquid media that were active against [HM1] [u2] Gram-positive and -negative pathogenic bacteria, **[34,36]**; anti-plant pathogenic bacterial **[37,38],** anti-coccidial **[39],** anti-oomycetes **[37,40]**, as well as antifungal (Ujszegi et al., in preparation) activity.

Recently, it was discovered that the operons encoding the various enzymes of the biosynthetic pathway of each NR-AMP are globally regulated by the gene called *Hfq* in EPB species, providing an option to create EPB strains each of which produces only one single NR AMP molecule. (The method of constructing such a strain in the lab is called by using the „easyPACId" method) **[41]**.

Whether the different NR-AMPs could ever be utilized as drugs not only against prokaryotic (bacteria) pathogens but eukaryotic (fungal pathogens, and parasitic protists) depends on the side

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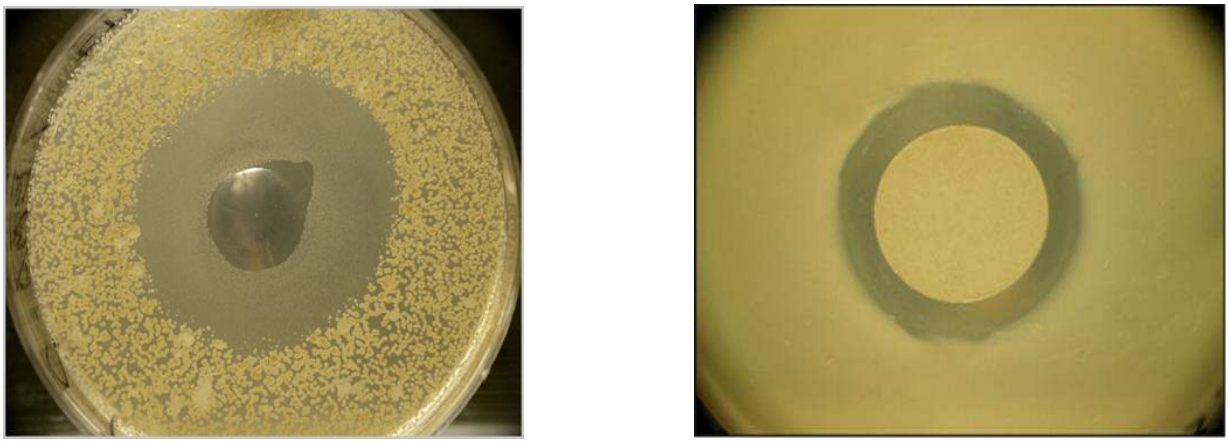
effects. To get experimental experience about the option of applying EPB-produced antimicrobials to pathogens, and parasites of veterinary significance, we present here the results of an *in vitro,* and an accompanying in *vivo* study on chicken. In the *in vitro* study, we tested the cytotoxic potential of the cell-free conditioned culture media (CFCM) of three entomopathogenic bacterium species, - *X. budapestensis,* DSM16342 (EMA); *X. szentirmaii* DSM16338 (EMC); *Photorhabdus luminescens* ssp. *akhurstii* TT01 - on chicken tissue culture cells, namely, on the Leghorn Male Hepatoma (LMH), **[92]** cells, (a permanent confluent hepato-carcinoma cell line). Each CFCM proved rather cytotoxic in this test. In the *in vivo* study, we fed freshly hatched male broiler chickens for 42 days with XENOFOOD **[39]** which contained autoclaved cultures of EMA, and EMC. These bacteria were grown on standard chicken (starter and grower) [HM3] [u4] feed, and the whole culture was used as a "feed supplement". [HM5]. It had been known that these EPB species cannot grow at body temperature (above 33 ˚C).

## 2. Results.

### *2.1. ANticlostridial Activity Of Ema And Emc In VitrO*

Based on the previous results of a repeated experiments, we choose *X. budapestensis* DSM16342 (EMA) and X. *szentirmaii* DSM 16338, (EMC) which had been identified in our laboratory, **[34]**. For the exact description and history of these strains, see Ref. **[35]**. We retested the i*n vitro* efficiency **[36–38]** in overlay bioassays and the pepsin resistance of the active ingredient that we previously published **[39]**, **(Figure 1 A and B**).



(**a**) (**b**)

**Figure 1.** Results of the *in vitro* experiment with EMA CFCM in anaerobic conditions. The active ingredient inhibited the growth of Clostridium *perfringens* cells in agar media before (a) and after (b) pepsin digestion. For details, see Materials, and Method. Photo: Cs. Pintér.

Both native and autoclaved CFCM samples of EMA and EMC bacterial strains were bioassayed both in liquid, and agar diffusion tests as described previously **[36-39.** The anticoccidial activities of native and pepsin-treated CFCM samples were compared similarly as described previously **[39]**. All previous data on antimicrobial activity against each of the tested substances on different clinical isolates of Gram-positive *Staphylococcus aureus* **[35–39,43]** was repeatedly confirmed, indicating the antimicrobial activity These data were especially important as confirming all previous findings published in **[38]**. The experiment also confirmed the thermostability and the pepsin durability of the CFCM of EMA, and EMC **[40],** since autoclaving of these CFCMs did not affect the antimicrobial activity against any of the tested organisms.

### *2.2. In Vitro Bioassays: Cytopathogenic Effects Of Cell-Free Condition Media (Cfcm) Of 3 Epb Species On Lmh Chicken Cell Monolayer*

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**Two experiments** were performed on this subject, (see Materials and Methods). In both experiments, permanent, confluent monolayer culture samples (developed in different Falcon flasks) were used as target organisms, and different dilutions of different CFCMs were tested.

(As described in the Materials and Methods, confluent monolayers of LMH cells were parallelly developed in 18 Falcon flasks (for the first experiment), and 36 flasks (for the second experiment). The culture media were Medium RPMI [HM1] [u2] [u3] 1640 supplemented with 10% FBS, penicillin G (200 IU/ml), and streptomycin (200 µg/ml). The incubation time was for 72 hours in both cases, in the controlled atmosphere of 5% CO2 at 37°C and around 85-90% humidity in 25 cm (2) flasks with filtered caps. After the layer formed, the liquid growth media was removed from all but 3 flasks (in the first experiment) and in 2x2 flasks (in the second experiment). Those few (3X1 and 1x2) flasks were referred to as "unchanged" control flasks [HM1] [u2]. As for other flasks, 3x1 (in the first experiment) and 1x2 (in the second experiment) were refilled e fresh culture media (referred to as M199+15% FKS control flasks) while the rest of the flasks were refilled 100 V/V% of different CFCMs (EMA, EMC, TT01 yellow and TT01 red, in the first experiment, and different dilutions of EMA and EMC CFCMs in the second experiments. As for the replicates, in the first experiment we worked with triplicates and in the second experiment with duplicates).

2.2.1. Experiment-1 aimed at comparing the cytopathogenic effects of 4 different undiluted CFCMs obtained from cultures of EMA, EMC, and TT01 yellow, and TT01 red colony color variants, respectively, (see Materials and Methods).

(As described in the Materials and Methods, the EPB cultures had been grown in the same medium and the target (LMH) cells, these cultures were made cell-free by centrifugation and following sterile filtration, and these filtrates were used as CFCMs. The incubation of the LMH cell layers with EPB CFCMs (and respective controls) was carried out exactly in the same conditions as used for developing the confluent cell layers. A prolonged incubation time was characterized by the appearance of holes in the cell monolayer, semi-quantified by using the scoring system **[44]** (Amin et el., 2011) presented in **Supplementary Material, Table 1S**).

It was found that each CFCM exerted destructive effects on the cell layer. The initial lesions on the LMH monolayer were followed by more serious ones consisting of clumps attached to the monolayer, (Score 3). **The results 1** (data are given as empiric score values, [**44**]) **of Experiment** are presented in **Table 1. Table 1.** Results of Experiment-1. Scoring the degree of LMH monolayer destruction caused by CFCM of 3 different EPB strains representing 2 different species.

**Table 1**

| Samples | Replicates | 24h | 48h |
|---|---|---|---|
| **M199 the original (unchanged) culture Media, in which LMH layer had been developed** | **A** | **0** | **0** |
| | **B** | **0** | **0** |
| | **C** | **0** | **0** |
| **Fresh (199 + 15% + PKS) culture media added** | **A** | **0** | **0** |
| | **A** | | |
| | **B** | **0** | **0** |
| | **C** | **0** | **0** |
| **EMA CFCM (EMA had been cultured inM199)** | **A** | **3** | **4** |
| | **B** | **3** | **4** |
| | **C** | **3** | **4** |
| **EMC CFCM (EMC had been cultured inM199)** | **A** | **4** | **4** |
| | **B** | **4** | **4** |
| | **C** | **4** | **4** |
| **TT01 YELLOW CFCM** | **A** | **4** | **4** |
| | **B** | **4** | **4** |

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| | | | |
|---|---|---|---|
| **(TT01 had been cultured inM199)** | **C** | **4** | **4** |
| **TT01 RED CFCM** | **A** | **4** | **4** |
| **TT01 had been cultured** | **B** | **4** | **4** |
| **inM199)** | **C** | **4** | **4** |

[1]Table 1 Footnotes. The numbers in the table are the score values (see Table 1). Abbreviations: M199 Unchanged C growth control media (M199, unchanged); M199 supplemented with + 15% + PKS C growth control media (fresh M199, supplemented with 15% fibroblast growth media); EMA CFCM: cell-free conditioned culture media of *X. budapestensis* DSM16442 (EMA); EMC CFCM: cell-free conditioned culture media of *X. szentirmaii* DSM16338 (EMC); TT01 YELLOW CFCM: cell-free conditioned culture media of *Photorhabdus luminescens ssp. akhurstii* TT01, yellow colony-color variant (from McConkey agar media); TT01 RED CFCM: cell-free conditioned culture media of *P. luminescens* ssp. *akhurstii* TT01 RED colony-color variant (from McConkey agar media).

2.2.2. Experiment-2 aimed at comparing the cytopathogenic effects of serial dilutions of the CFCM filtrates of EMA, and EMC cultures, considering that both the both EMA and EMC CFCMs showed much stronger antimicrobial activity than the two TT01 CFCM. The stock solutions, (and serial dilutions of them) used in this experiment were: EMA 60%, and EMC 80%, respectively. Confluent monolayer from LMH cells was developed again in Medium RPMI 1640 supplemented with 10% FBS, penicillin G (200 IU/ml), and streptomycin (200 µg/ml) for 72 hours in a controlled atmosphere of 5% $CO_2$ at 37°C and around 85-90% humidity in 25 $cm^2$ Falcon flasks with filtered caps (see also in Materials, and Methods). The same score system [44] (Amin, 2012, see Table S1) was used for evaluating the results.

The results **of Experiment 2** are presented in **Figure s 2 & 3**, and the data are given in Supplementary material **(Table 2S & 3S).**

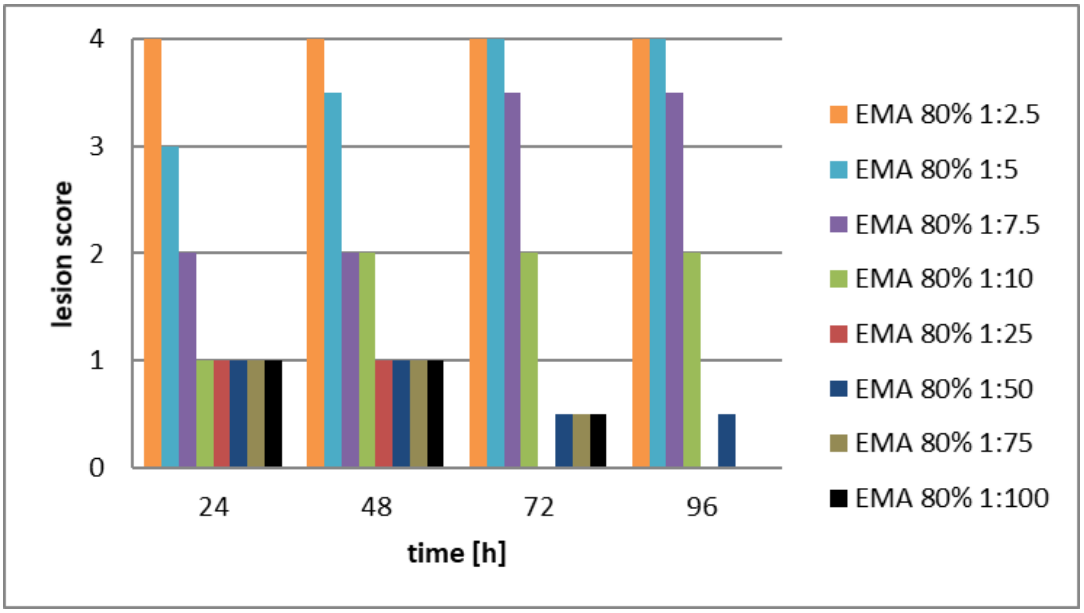


**Figure 2.** Destruction of LMH [42] cell monolayer caused by serially diluted EMA CFCM samples. [HM1] The scoring system is **[44]** shown in **Table S1. (**Abbreviations: EMA= *Xenorhabdus budapestensis* DSM16342(T) (Lengyel). CFCM= cell-free conditioned culture media). Data are given in Supplementary **Material TableS2 and TableS3.**

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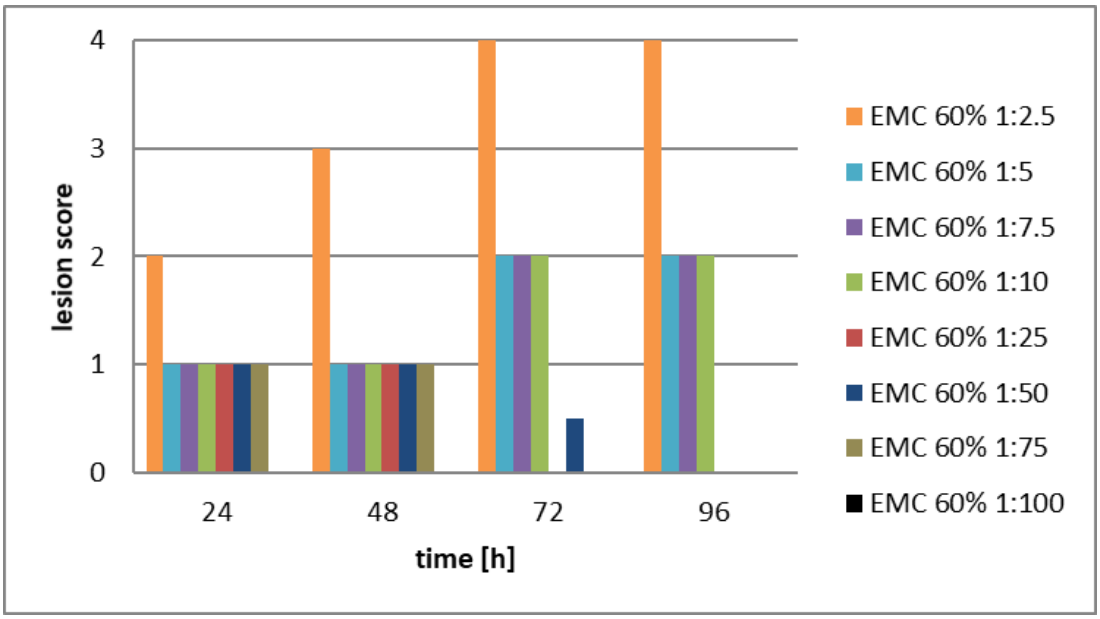


**Figure 3.** Destruction of LMH [42] cell monolayer caused by serially diluted EMC CFCM samples. [HM1] The scoring system is **[44]** shown in **Table S1. (**Abbreviations: EMC= *Xenorhabdus budapestensis* DSM16338(T) (Lengyel). CFCM= cell-free conditioned culture media). Data are given in Supplementary **Material TableS2 and TableS3.**

The data may allow us to hypothesize that the *in vivo* application of EMA NR_AMP peptides did cause fatal or even detectable cytopathogenic in sensitive organs (like the liver) and tissues where they are present in less than 3.2 V/V % (1:25 dilution) EMA-CFCM or 2.4V/V% EMC.

*2.3. Results Of The In Vivo Xenofood-Feeding Experiment*

2.3.1 Gastrointestinal activity of XENOFOOD

To examine whether the gastrointestinal potential of the XENOFOOD was considerable we counted *Clostridium* CFUs in the lower ileum (see **Figure 4**) of XENOFOOD-fed and untreated animals

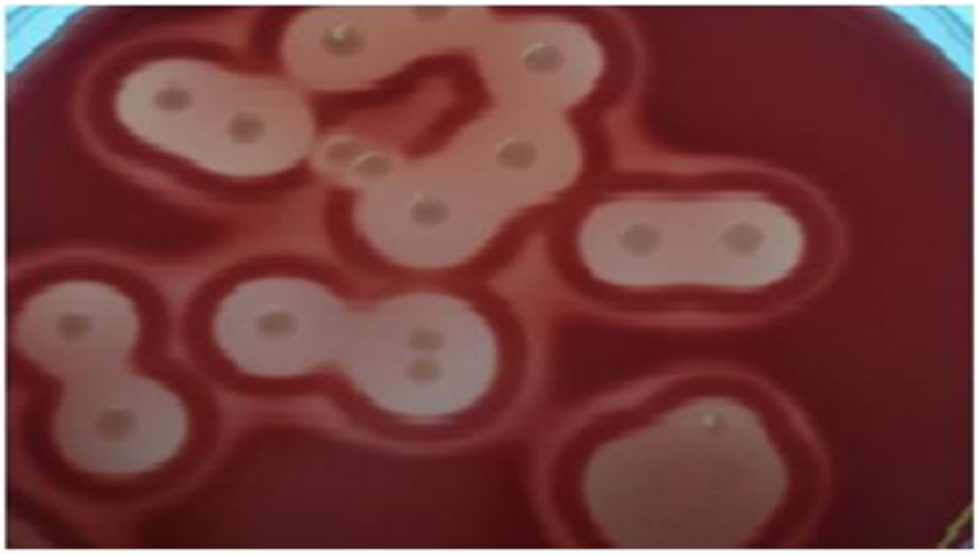

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**Figure 4.** Determination of viable cell count using plate counting method (*Clostridium perfringens*, blood agar). .

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Using ANOVA statistical analysis, we compared the average of the lower ileal Clostridium CFUs (see **Figure 2**) of the XENOFOOD-fed animals (**48.1)** with those of the controls (**149.9**). We found that despite the 3X difference, only a slightly significant could be demonstrated, probably due to the large standard deviation in the control values **(Table 4).**

**Table 4.** Gastro-enteral antimicrobial potential (on *Clostridium* (CFU) in the lower ileum CFU). The absolute, and relative weights of the immune organs (bursa of Fabricius, and spleens of XENOFOOD[HM1] .

| TREATMENTS | | SPLEEN | bursa of Fabricius | | | CLOSTRIDIUM CFU |
|---|---|---|---|---|---|---|
| | | weight | weight | size | individual bursa/spleen | (lower ileum) |
| | N | mg | mg | mm | ratio | |
| C (Control) | 10 | 2266.6 | 2761.0 | 25.2 | 1.39 | 149.9 |
| T (Xenofood-fed) | 10 | 1618.0 | 3618.2 | 26.9 | 2.38 | 48.1 |
| t | | +2.09 | +3.16 | 3.02 | 3.7 | -2.128733 |
| P | | 0.056 | 0.006 | 0.009 | 0.006 | 0.028 |
| Significance | | NS | ** | ** | * | * |

[1] Table 4: The average measurements (sizes and (weights) of the spleen, as well as those of the bursas of Fabricius.

The data show that the average weights of the spleens were smaller, while the average measurements of the sizes of the bursas were larger in the XENOFOOD-fed group, and the differences were significant. The same can be stated about the *Clostridium* colony-forming units, (CFUs): there were fewer in the XENOFOOD-fed group (on average). The XENOFOOD exerted unambiguously detectable gastrointestinal antimicrobial activity. But the original infestation of the population from which the experimental animals were selected rate was very low. These results allow concluding that XENOFOOD feeding was harmless. We suppose that the bursa-dependent humoral immune system must have neutralized the cytotoxic peptide-like molecules (including the GI-active NR-APMPs) of the XENOFOOD in the blood, protecting the sensitive tissues and organs in such a way**.**

2.3.2. Growth rate, feed consumption and the post-mortal data

During the 42-day-long experiment, no animal was lost. We did not detect any significant differences either in the body weight, (**Table 5),** in the feed conversion rates between the control and XENOFOOD-fed groups, or the growth rates of the two groups **(Table 6).**

**Table 5.** Body Weights.

| TREATMENTS | N | DAYS OF THE EXPERIMENT | | |
|---|---|---|---|---|
| | | 10 | 14 | 42 |
| C (Control) | 34 | 252.9 +/- 33.4 | 1196.8 +/- 123 | 2842.5 +/- 184 |
| T (XENOFOOD-fed) | 34 | 271.3 +/- 39.4 | 1132.7+/- 162 | 2984.2+/- 207 |
| T-value | | -177 | 187 | - 1.02 |
| P-value | | 0.08 | 0.07 | 0.32 |
| Significance | | ns | ns | ns |

[1] Table 5 footer: During the 42-day experiment, no animal was lost. We did not detect any significant differences either in the body weights.

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**Table 6.** Feed Conversion Rates of XENOFOOD – Fed Chicken and Control Chicken.

| TREATMENTS | N | GROWTH PERIODS (DAYS) | | |
|---|---|---|---|---|
| | | 1-10 | 11-24 | 1-24 |
| C (Control) | 34 | 1.06 | 1.68 | 1.53 |
| T (XENOFOOD-fed) | 34 | 0.97 | 1.71 | 1.52 |
| Significance | | ns | ns | ns |

Table 6 footer: As data in Table 6 prove, there was not any detectable difference between the food conversions of the two groups.

As for the organ weight, they followed the allometric rules, according to which the regular and systematic patterns of growth such as the mass or size of any organ or part of a body can be expressed about the total mass or size of the entire organism according to the allometric equation: $Y = bx^{\alpha}$, where Y = mass of the organ, x = mass of the organism, $\alpha$ = growth coefficient of the organ, and b = a constant (only bursa- and spleen data are given). This fact itself excludes any organ-development abnormality of the treated (XENOFOOD-fed) animals. But the larger bursa weight and the smaller spleen weights, as well as larger bursa/spleen ratios in the XENOFOOD-fed groups may indicate indirectly an intensified humoral immune activity, leading to the neutralization of the cytotoxic AMP-components in the blood (**Table 6**).

**3. Discussion.**

AMPs comprise a potentially promising toolkit combatting MDR because antibiotic-resistant bacteria perform a high frequency of collateral sensitivity to antimicrobial peptides, **[13,16,17].** *X. budapestensis* (EMA) and *X. szentirmaii* (EMC) are abundant sources of NR-AMP molecules of large target spectra and strong anti-pathogen potential, **[35].** Each NR-AMP molecule in the *Xenorhabdus* CFCM is the end-product of a biosynthetic pathway. Each biosynthetic pathway is encoded by genes clustered in the respective operon (biosynthetic gene complex, BCG). In EMC 71 BCGs have been identified, **[70]**. Their large spectral NR-AMP products of overlapping activities and cooperating potential are coexisting as ingredients of the CFCM. Theoretically, there are two alternative ways of research philosophy for the geneticist of agricultural or veterinary commitments. One option is to focus on searching for individual molecules as new potential drugs, benefitting the global (co)regulation of the biosynthesis of different NR_AMPs, searching for or constructing EPB strains synthesizing only single NR-AMP molecules. As for this alternative, the recent discovery, according to which the different NRPS-encoding operons are under the global control of the post-transcriptional regulator, *Hfq*, provides an option for constructing *Xenorhabdus* strains producing single NR-AMP only, **[41]**. It is a great challenge! Another strategy is to try to benefit from the co-existence of naturally cooperating NR_AMP molecules in the CFCMs by producing harmless but effective preventive biopreparations like food supplements either as food supplements for veterinary, or soil additives for plant protection goals against pathogens and parasites. This latter option may be discussed here in light of the experiment's results where chickens were fed by XENOFOOD, a food supplement, in which *in vitro* active ut heat-sterlized NR-AMPs were present as ingredients. We know about these compounds that they were of peptides, **[28],** in vitro anti-coccidial active, [**39**] which did not lose their antimicrobial potential either when heat-sterilized **[36].** or when subjected to pepsin digestion, **[39]**. But in the *in vitro* experiment we presented here we found that each EPB CCM we tested exerted strong cytopathogenic effects on LMH permanent confluent chicken cells. We do not know whether one single (most probably the fabclavine) **[48–51]** molecule, or more than one molecule can be taken as responsible for the cyto-pathogenecity. There are several candidates (see Table 7).

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**Table 7.** A list of the non-ribosomal templates antimicrobial peptides discovered in different *Xenorhabdus species.*

**R**

| **Natural NR-AMPs** | ***Xenorhabus specoes*** | **refeence** |
|---|---|---|
| Xenofuranone A and B | *X. szetirmaii* (EMC) DSM(16338)T | [45] Brachmann, et al., 2006 |
| Nemaucin | *X. cabanillasii* | [46] Gualtieri, et al., 2009)a,[47] Gualtieri, et al., 2012 |
| Fabclavine | *X. budapestensis* DSM16342)T (EMA) | [48] (Fuchs et al., 2012 |
| Fabclavine, A, B | *X. szentirmaii* DSM(16338)T (EMC) | [49] (Fuchs et al., 2014) |
| Fabclavine, biosynthetic intermediers, derivatives, and analogs | *X. szentirmaii* DSM(16338)T (EMC)<br>All but e few *Xenorhabdus* | [50] Wenski et al., 2019.<br>[51] Wenski et al., 2020 |
| Cabanillasin | *X. cabanillasii*, *X. khoisanae*, SB10 | [52] Houard et al., 2013, |
| PAX peptides | *X. nematophila* | [54] Gualtieri et al, 2009b |
| | | [55] Fuchs et al, 2011), |
| | *X. khoisanae*, SB10 | [53] Dreyer, et al., 2019 |
| Odilorhabdins | *X. riobrave* | [56] Isaacson and Webster, 2013 |
| | | [57] Pantel, et al, 2018 |
| | | [58] Sarciaux et al., 2018 |
| | | [69] Racine, and Gualtieri 2019 |
| | | [60] Lanois-Nouri, et al., 2022. |
| Anti-oomycete peptides | *X. budapestensis* NMC-10 | [61] (Xiao et al., 2012) |
| Xenortide | *X. nematophila* | [62] (Reimer, 2014 |
| Xenortide A-D | *X. nematophila* | [63] Esmati, et al., 2018 |
| Rhabdopeptide | *X. nematophila* | [64] Reimer at al., 2013 |
| Rhabdopeptide (with nematicide activity) | *X. budapestensis SN84* | [65] (Bi et l., 2018) |
| Rhabdoopeptide/xenortide-like peptides | *Xenorhabdus innexi* | [66] Zhao, 2018 |
| New cyclic depsipeptide xenematide F, and G, (anti-oomycete activity) | *X. budapestensis* SN84 | [67] (Xi et al, 2019 |
| Szentiamide | *X. szentirmaii* DSM16338$^T$ (EMC) | [68] Ohlendorf, et al., 2011) |
| | | [69] (Nollmann, et al, 2012) |
| Genomic information: 71 NR-AMP operons in. | *X. szetirmaii* (EMC)$^T$ DSM16338 | [70] Gualtieri et al.,2014 |

It is not known whether the mechanism of the antimicrobial action of a given NR-AMP molecule is the same as those causing cytopathogenicity in the organisms. But the XENOFOOD food supplement proved in this experiment harmless and GI active against a bacterium pathogen, Clostridium *perfringens*. But in our *in vivo* feeding experiment the lower number of *Clostridia* does not automatically indicate a "protection" exerted by the XENOFOOD, since the natural infestation rate of the experimental animals was very low, but we do not see any other plausible explanation. The moderate but detectable anti-Clostridial effect without any detectable harmful side effects may be explained in more ways than one. An explanation may come from the previous experiments on *Eimeria* (unpublished) that the effective cytotoxic concentration is much higher than the effective anti-clostridial dose. Another possible explanation is that the actual local NP-AMR concentration

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(bioavailability) was much lower in the blood and the other organs and tissues than in the gut. We suppose that the second option is more probable because the immune system of the birds is probably capable of neutralizing these peptide-like compounds. The higher average weights of the bursas in the T-groups suggest that humoral immunity is mainly responsible for that.

As for the perspetives, see Conclusons.

**4. Material, and Method**

- ***Anti-clostridial potential of EPB CFCM:*** The respective methods have been published in [39]. Briefly, *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 the Department of Microbiology and Infectious Diseases, University of Veterinary Medicine Budapest, Hungary. *Xenorhabdus* strains, *X. budapestensis* DSM 16342(T) (Lengyel) (EMA), and X. *szentirmaii DSM* 16338 (T) (Lengyel)(EMC) [34,35]. of EMA and EMC CFCM bioassays were tested on different Gram-positive strains including *Clostridium perfringens* strains were carried out as described before**, [35–39].**
- ***.In vitro* experiments:** Cell-free conditioned culture media (CFCM), of antibiotic-producing bacteria, were tested, and LHM (tissue culture) cells were in two different experiments.
- ***Experiment 1:*** Testing cytotoxicity of different EPB CFCM on confluent LMH (leghorn male hepatoma, LMH) cell line [42]: *Developing Confluent layers of LMH (leghorn male hepatoma, LMH) cell line* [42]: (LMH; ATCC Number: CRL-2117™) were developed culture Falcon flasks in Medium RPMI 1640 (Invitrogen/GIBCO), supplemented with 10% heat-inactivated fetal bovine serum FBS (Invitrogen/GIBCO), penicillin G (200 IU/ml) and streptomycin (200 µg/ml), respectively**.**
- In detail, cells were inoculated into 25 cm (2) flasks with filtered caps (Sarstedt) containing an end volume of 7 ml culture and incubated in a controlled atmosphere of 5% CO2 at 37 °C and around 85-90% humidity. After 72 hours of incubation, a confluent monolayer of LMH cells was obtained per flask**.**
- Altogether 18 Falcon tubes, - each containing 9 ml Medium 199 with Earle's Salts, L-glutamine, 25 mM HEPES and L-amino acids (Invitrogen/GIBCO) and supplemented with 15% heat-inactivated fetal bovine serum FBS (Invitrogen/GIBCO) and 0.22% rice starch were used, 2x3 controls and 4X3 experimental tubes.
- ***Preparation of CFCMs***: Tubes with thw same media (except fo for streptomicine, were inoculated with 4 different bacterial strains, EMA, EMC, TT01 yellow or TT01 red, representing 2 *Xenorhabdus*, (*X. budapestensis* nov. DSM16342(T), Lengyel) (EMA), [34,35], and *X. szentirmaii* nov. DSM16338 (T) (Lengyel) (EMC, **[34,35],** and 1 *Photorhabdus* (*P. luminescens ssp. akhurstii* TT01, **[71]** species. The latter is obtained from the Boemare laboratory (Montpellier, France). TT01 yellow and TT01 red names were used for two colony-color variants segregating spontaneously in McConkey agar plates, (P. Ganas, unpublished). (*Xenorabdus*, and *Photorhabdus* are penicillin resistant species).
- These antibiotic-producing bacteria were freshly taken from frozen cultures and grown on the bacterial species grown on MacConkey agar plates before they were transferred to the liquid

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medium as described before, **[35–40]**. The bacterial species were grown on MacConkey agar plates before they were transferred to the liquid medium, and unexpectedly two different types of colonies for TT01 were observed on the agar plates: red-brown colored colonies which adsorbed the neutral red from the MacConkey agar and yellow-colored colonies which did not so. Both types of colonies were tested for the effect of cell-free filtrates on LMH monolayers. In this particular experiment, the antibiotic-producing bacteria were cultured at 28 °C. The bacteria were incubated for 65 hours at 30 °C in a shaker (225 rpm. Bacterial cultures were then centrifuged at 3,300xg for 5 min and then the supernatants from the cultures were filtered through 0.22 μm cellulose acetate filters (Millipore).

- ***Experimental design*:** From all but 3 of the Falcon flasks (with the 72-hrs old LMH-layers), the culture medium of the LMH monolayers was removed from the flasks and replaced by the 4 CFCMs. Each of the CFCMs was tested in triplicates. There were two sets of controls. There were also 3 flasks with fresh medium (fresh Medium 199 supplemented with 15% FBS) without CFCM, and another 3 with the original, ("unchanged", that is 72h-old) culture media. All these 4X3 experimental and 2X3 control flasks were incubated for another 72hrs. The cultures were incubated in a controlled atmosphere of 5% CO2 at 37 °C and around 85-90% humidity.
- ***In vitro Experiment* 2:** permanent chicken liver cells (LMH; ATCC Number: CRL-2117™) [42] were grown in Medium RPMI 1640 (Invitrogen/GIBCO) supplemented with 10% heat-inactivated fetal bovine serum FBS (Invitrogen/GIBCO), penicillin G (200 IU/ml) and streptomycin (200 μg/ml).
- Cells were inoculated into 25 $cm^2$ flasks with filtered caps (Sarstedt) containing an end volume of 7 ml culture and incubated in a controlled atmosphere of 5% $CO_2$ at 37 °C and around 85-90% humidity**.**
- After 72 hours of incubation, a confluent monolayer of LMH cells was obtained per flask.
- **All but the so-called "Unchanged culture flasks" LMH monolayers,** the culture medium of the LMH monolayers was removed from the flasks and replaced by *something*.
- **The control flasks** were refilled with fresh media **(**Medium RPMI 1640 (Invitrogen/GIBCO) supplemented with 10% heat-inactivated fetal bovine serum FBS (Invitrogen/GIBCO), penicillin G (200 IU/ml) and streptomycin (200 μg/ml).
- **The experimental flasks** were refilled with cell-free (centrifuged and filtered serially diluted stock solutions (EMA: stock solution: 60V/V; EMC stock solution 80V/V%). The dilutions were 1:2.5; 1:5; 1:10 1:.25; 1:.50; 1:75, and 1:100, respectively.
- **The EPB cells had been also cultured in Medium RPMI 1640** supplemented with 10% FBS, penicillin G, without streptomycin, (*Xenorhabdus* are pecicillin resistant).
- Each of the different filtrate analyses and the controls was performed in duplicate.
- The cultures were incubated in a controlled atmosphere of 5% $CO_2$ at 37 °C and around 85-90% humidity.
- **Each monolayer was investigated visually** by an inverted light microscope to detect the effect of the cell-free filtrates on LMH monolayers. According to the degree of monolayer destruction, the following scoring system was established and also published, [44], (See **Supplementary Material, Table S1**).
- ***XENOFOOD preparation:*** XENOFOOD contained 5% soy-meal, which had been suspended with an equal amount (w/w) of EMA and another 5% suspended in an equal amount (w/w) of

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EMC cells obtained from 5 days-old shaken (2000 rpm) liquid cultures by high-speed (Sorwall; for 30 minutes) centrifugation.

- The liquid cultures were in 2XLB; (DIFCO); supplemented with meat extract equivalent amount to the yeast extract. The 5 days had been proven optimal for antimicrobial substance production at 25 °C in these conditions).
- It had previously been discovered that both EMA and EMC grow and produce antimicrobial substances in autoclaved soy meal containing some water and yeast extract or autoclaved yeast, (in 0.5 w/w %).
- Therefore, the original chicken food served as a semi-solid culture media of *Xenorhabdus* cells. Both EMA and EMC culturing semi-solid chicken food, which was prepared daily, and have been incubated in sterile conditions for another five days; then the EMA and EMC culturing media were united; autoclaved (20 min, 121 °C), and then dried by heat overnight. The *Xenorhabdus* cells were killed in such a way, while the heat stabile [36] antimicrobial compounds remained active
- ***XENOFOOD in vivo feeding experiment*:** *Experimental animals*: One-day-old male broiler chickens (N=2x34 = 68) were equally distributed into two groups: Control (C) and Treated (T) groups. The latter was fed with XENOFOOD. The C group was kept on a normal starter (1-10-d) and grower (11-42d) diet according to the standard international protocol.
- *Food, feeding, evaluations:* The T Group T was kept on "starter (1-10-d) XENOFOOD", and "grower XENOFOOD" (11-42d). Body weights were measured daily between 1-42 days. Growth and FCR were monitored for 24-d. In the *in vivo* feeding experiment, we fed 39 birds with XENOFOOD, and there were 39 control birds.
- *Body weights* were measured daily between 1-42 days. *Growth, - and food-conversion rates (GR and FCR respectively,* were monitored for every 24-d.
- *Dissection, Post mortam data:* Not all but a sample (N=2x10=20) of 42-day-old birds were dissected on the 42nd day are presented here. We dissected a sample of 10 birds from the XENOFOOD-fed and a sample of 10 birds from the Control groups. [HM1] [u2] That is, not all but samples (N=2x10) of the 2x34) from 42-day-old birds were dissected to get post-mortam data about a few body organs as well as about the number of *Clostridium* germs in their ilea. The body weights of these selected animals did not differ from the average of their respective (C, or T) experimental groups. After dissection, the weights of the different organs were measured. The absolute and relative weights of the spleens and the bursae of Fabricii are presented in the Results section.
- *CFU determination*:The content of the lower ileum was washed taken out, diluted, and equilibrated and the colony-forming units were determined on BAM Media M75: Lactose-Gelatin Medium.
- ***Statistical Analysis*** ANOVA procedure was carried out by using the respective propositions of the SAS 9.4, see Acknowledgment section Software mostly due to an unbalanced data set. The significant differences ($\alpha$ = 0.05) between treatment means were assessed using the Least Significant Difference (LSD**)**.

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## 5. Conclusions

The work presented here can be considered as a contribution to the field of application of biosynthetic non-ribosomal encoded antimicrobial peptides (NR-AMPs) controlling multi-drug resistance (MDR). Regarding the future potential application of natural compounds, several aspects have to be taken into consideration: (A) the antimicrobial potential; (B) the durability, thermotolerance, and bioavailability; (C) cytotoxicity, and another, unwanted side effects, especially if one group of target organisms are of eukaryotic (protist) pathogens. We have not found any publication concerning the comparisons of the *in vitro* and the *in vivo* effects of antimicrobial active NR-AMP molecules of EPB origin on the same eukaryote (host) organism to be protected from pathogens or parasites.

Herein we demonstrated that both the heat-inactivated and the native cell-free conditioned culture media (CFCM) of Xenorhabdus *budapestensis* DSM16342 (EMA), and that of *Xenorhabdus szentirmaii* DSM16338 (EMC) which has broad-spectrum antimicrobial activity against several prokaryotic and eukaryotic microbial pathogens, also exerted a robust cytopathogenic effect on confluent (cultured) chicken cell layer, LHM).

The results of the *in vivo* experiment presented in **Tables 4-6** may allow concluding that XENOFOOD feeding was harmless. It means that the NR-AMPs, present in the CFCM of *X. budapestensis* and *X. szentirmaii* - at least when applied as autoclaved food ingredients - did not cause any detectable side effects *in vivo* in broilers.

Their metabolic incorporation of undecomposed NR-AMPs into proteins or other tissue components is biochemical nonsense. However, their deposition into the egg yolk cannot theoretically be ruled out, therefore, we may recommend XENOFOOD as protecting food supplement only for cockerels but not for pullets.

The XENOFOOD exerted gastrointestinal antimicrobial activity in the GI has been reflected in the reduced average value of the *Clostridium* CFUs in the XENOFOOD-fed group. We suppose that the bursa-dependent humoral immune system neutralized the cytotoxic side components (of peptide nature) of the XENOFOOD, (that is, cytotoxic NR-AMPs of CFCMs in the blood (but, naturally, not in the gut).

The presence of the active antimicrobial compounds in the GI are proved by the significantly reduced number of colony-forming germs in the lower jejunum of the XENOFOOD-fed (T-group of) animals compared to that of the controls, (C-group). Based on those facts, we may recommend XENOFOOD as a useful food supplement to protect broiler cockerels against coccidiosis.

However, before drawing that conclusion, we recommend another experiment on experimentally *Clostridium*-pre-infested chickens, (also on cockerels, not pullet) to reproducibly determine the *in vivo* anti-Clostridial effects of the XENOFOOD diet quantitatively. (As mentioned above, we do not recommend the use of pullets, because the deposition of the XENOFOOD ingredients is unpredictable). We have been looking for a cooperating partner for carrying on this final experiment, and this is one of our strongest arguments for publishing these data in Pathogens

## 6. Patents

This work has not resulted in any patented result, we offer **XENOFOOD recipe** for the Scientific Community for free**. Conflict of Interest Statement**: The authors declare that the research was conducted without any commercial or financial relationships that could be construed as a potential conflict of interest.

Supplementary Material: Scores of HML Monolayer Destruction caused by by EPB CFCMs.

**Author Contributions:** Conceptualization, ANDRÁS FODOR, László Makrai, Károly Dublecz and Michael Hess; Data curation, Sándor Józsa; Formal analysis, Claudia Hess, László Pál and Michael Klein; Funding acquisition, Tibor Vellai; Investigation, Claudia Hess, Petra Ganes and Andor Molnár; Methodology, Petra Ganes, László Makrai, László Pál, Andor Molnár, Sándor Józsa and Csaba Pintér; Project administration, László Makrai; Resources, Tibor Vellai and Károly Dublecz; Validation, Claudia Hess, László Fodor and Sándor Józsa; Writing – original draft, ANDRÁS FODOR; Writing – review & editing, Tibor Vellai, Claudia Hess, Michael Klein, Eustachio Tarascoi and Michael Hess. All authors will be informed about each step of manuscript processing including submission, revision, revision reminder, etc. via emails from our system or assigned Assistant Editor. The final order of the authors is as follows: ANDRÁS **FODOR***; Tibor **Vellai**; Claudia **Hess**; Petra **Ganes**; László **MAKRAI***; Károly **DUBLECZ***; László **Pál**; Andor **Molnár**; László **Fodor**; Sándor **Józsa**; Csaba **Pintér**; Michael Gardner **KLEIN***; Eustachio **Tarasco**; Michael **Hess. Author Credit Statement**. The authors confirm their proportional but e important, equally essential contribution to the paper. The authors demonstrate that they were sharing work contributed to the report as follows: **Conceptualization**: AF, LM, MH, KD; **Data curation**: SJ; **Formal analysis**: MGK, LF; **Funding acquisition**: collective, TV, LM **Methodology**: LM, (*Clostridium* in vitro, colony counting), CH, PG, (*in vitro* bioassays in LMH), LP (feeding experiments, food conversion), LM (post mortem examinations); AF (CFCM preparations); CSP (photo), SJ (statistics). The first draft was written by AF, corrected, and improved by TV, MGK, CH, and MH; **Supplementary Material:** PG

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**Institutional Review Board Statement:** The *in vivo* feeding experiments were actually carried out according to the protocol that was asked for at the University of Veterinary Medicine, Budapest, Hungary; (Attached). We are aware that the Editorial Office might ask you for further information and are ready to provide it. We are aware that the Editorial Office might ask you for further information and are ready to provide it. A copy of the official permission is available.

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