



Article

Selected Antimicrobial Peptides Inhibit In Vitro Growth of *Campylobacter* spp.

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Abstract: *Campylobacter* is a major cause of acute human diarrheal illness. Broiler chickens constitute a primary reservoir for *C. jejuni* leading to human infection. Consequently, there is a need for developing novel intervention methods. Antimicrobial peptides (AMP) are small proteins which have evolved in most lifeforms to provide defense against microbial infections. To date, over 3000 AMP have been discovered; however, few of them have been analyzed specifically for ability to kill campylobacters. We selected and evaluated a set of 11 unique chemically synthesized AMP for ability to inhibit growth of *C. jejuni*. Six of the AMP we tested produced zones of inhibition on lawns of *C. jejuni*. These AMP included: NRC-13, RL-37, Temporin L, Cecropin–Magainin, Dermaseptin, and C₁₂K-2β₁₂. In addition, MIC were determined for Cecropin–Magainin, RL-37 and C₁₂K-2β₁₂ against 15 isolates of *Campylobacter* representing the three most common pathogenic strains. MIC for campylobacters were approximately 3.1 µg/mL for AMP RL-37 and C₁₂K-2β₁₂. MIC were slightly higher for the Cecropin–Magainin AMP in the range of 12.5 to 100 µg/mL. These AMP are attractive subjects for future study and potential in vivo delivery to poultry to reduce *Campylobacter* spp. populations.

Keywords: antimicrobial peptides (AMP); *Campylobacter*; in vitro inhibition; minimum inhibitory concentration (MIC); synthetic oligo-acyl-lysl (OAK) peptidomimetic



Citation: Line, J.E.; Seal, B.S.; Garrish, J.K. Selected Antimicrobial Peptides Inhibit In Vitro Growth of *Campylobacter* spp.. *Appl. Microbiol.* **2022**, *2*, 688–700. <https://doi.org/10.3390/applmicrobiol2040053>

Academic Editor: Ian Connerton

Received: 1 September 2022

Accepted: 18 September 2022

Published: 21 September 2022

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1. Introduction

Antimicrobial peptides (AMP) are small proteins which have been found in almost every class of living organism where they have evolved as a host-defense mechanism against invading microorganisms [1]. For this reason, they are sometimes referred to as host-defense peptides and are considered key players in innate immunity [2]. AMP represent a unique and diverse group of molecules which can be divided into subgroups on the basis of their amino acid composition and structure [3]. They are generally short, 10–40 amino acids in length, cationic, amphipathic, and can be arbitrarily categorized according to their secondary structure as alpha-helices, beta-sheet, extended helices, and loops [4]. A growing number of AMP are being discovered and synthetic new ones are being designed thanks to tremendous research efforts in response to the dramatic and continued evolution of antibacterial-resistant strains of bacteria and the resulting international crisis in health care [1]. For example, a 2004 database contained 523 known peptides, whereas a 2013 database held 3904 natural and 1643 synthetic ones [5,6]. More recently, a 2021 database contains 22,259 entries which include 5891 general AMP, 16,110 patent AMP, and 77 AMP currently in preclinical or clinical stages of drug development [7]. While it is reasonable to expect that some of the AMP that have been discovered or designed *de novo* to kill a variety of Gram (–) bacteria might also inhibit G (–) *Campylobacter* spp., there have been very few reports in the literature describing effects of AMP on these specific pathogens, perhaps due to the relative difficulty of culturing campylobacters under microaerobic conditions necessary for its growth.

Campylobacter is one of the most important human pathogens worldwide, being a major cause of acute human diarrheal illness in the developed world [8–12]. Among foodborne bacterial infections, disease caused by *Campylobacter jejuni* is the most prevalent with approximately 1.5 million cases annually in the United States alone [13]. Commercial broiler chickens serve as a major reservoir for *C. jejuni* with colonization levels as high as 10^{10} CFU per gram of wet feces in the chicken [14–16]. Consequently, commercial broiler chickens constitute the primary reservoir for *C. jejuni* leading to human infections [15,17–19]. Common poultry-associated isolates are present in human clinical cases, providing evidence that poultry is a major contributor to human infection [20]. In addition, an increase in AMR among bacterial pathogens, due in part to the sub-therapeutic use of antibiotics in animal feed, has the potential to compromise public health therapies and remains a concern among scientists and the general public [21–24]. The current consensus of scientific and public opinion is that antibiotic use by humans and in food animals selects for the development of AMR among foodborne bacteria that can complicate public health therapies [25]. A major issue is that antibiotic resistance may not only occur among disease-causing organisms but may also become an issue for other organisms in the host and the environment [26]. Sub-therapeutic use of antibiotics as growth promoters has been discontinued in the European Union [27–29]. These regulations are justified due to the increase in antibiotic resistance among bacterial pathogens [21,30] including bacteria from healthy broilers [31]. Consequently, there is a need for developing novel intervention methods including narrow-spectrum antimicrobials and probiotics that selectively target pathogenic organisms while avoiding the killing of beneficial organisms [30].

Eliminating or dramatically reducing *Campylobacter* spp. contamination during poultry production will reduce foodborne infections [32–34]. Despite various intervention efforts against pathogens including campylobacters and salmonellae by poultry producers, processors, and regulatory agencies, the number of human foodborne diseases caused by these pathogens has not drastically declined [35,36]. There are currently no applicable, on-farm interventions for significantly reducing the colonization of poultry with *C. jejuni*. There is a need for effective interventions that may be practically applied in the poultry industry to reduce the colonization of poultry with *C. jejuni* and, subsequently, reduce consumer exposure to this pathogen [37]. A proposal for on-farm control measures for *Campylobacter* by the EU and implementation of in-plant performance standards for the pathogen by FSIS [38] highlight the urgent need for effective intervention methods. Hence, the objective of this study was to chemically synthesize representative AMP and screen them for ability to kill campylobacters in vitro with the long-term goal of potentially developing novel, practical intervention methods for reduction of these foodborne pathogens in poultry.

2. Materials and Methods

We selected and evaluated a set of 11 unique chemically synthesized and commercially available AMP (Tables 1 and 2) for ability to inhibit growth of *C. jejuni* isolates 11,168 and 81–176 and seven additional pathogenic bacterial isolates including two *Clostridium perfringens* isolates, two *Salmonella* isolates, two *Listeria monocytogenes* isolates, and one *E. coli* O157:H7 in a spot-on-lawn assay. Peptides were synthesized using standard solid-phase (Fmoc) chemistry with a peptide synthesizer (CPC Scientific Inc., Sunnyvale, CA 94089, USA, C₁₂K-2β₁₂; AnaSpec, Fremont, CA 94555, all other AMP).

Table 1. Sources and characteristics of antimicrobial peptides evaluated in this study.

AMP	Source	AA #	Structure and Characteristics	Reference
Apidaecin 1B	Honeybee lymph	18	Cationic, no α -helix formation, high proline content, stable at high temp and low pH, small mol wt 2100	[39]
C ₁₂ K-2 β 12	Synthetic oligo-acyl-lysyl (OAK) hexamer	8	Peptidomimetic, stable at high temp and low pH	[40]
Carnobacteriocin B2	<i>Carnobacterium piscicola</i>	48	Class II bacteriocin, cationic, single alpha-helices involved in coiled-coils or other helix-helix interfaces	[41]
Cecropin A-Magainin 2 hybrid	Cecropia moth/African clawed frog	20	Cationic, short helix-flexible-amphipathic helix, antibacterial as well as antitumor activity	[42]
Dermaseptin	Skin of frog (<i>Phyllomedusa</i>)	34	Cationic, amphipathic α -helix	[43]
Dermcidin DCD	Human sweat glands	48	Forms cation-stabilized oligomeric ion channels in lipid bilayers	[44]
NRC-13 Pleurocidin	American plaice-winter flounder	23	Amphipathic α -helix	[45]
Parasin I	Skin mucus of wounded catfish (<i>Parasilurus asotus</i>)	19	Amphipathic α -helix	[46]
Pyrrhocoricin	European sap-sucking bug (<i>Pyrrhocoris apterus</i>)	20	Cyclic, proline-rich peptide	[47]
RL-37	Bone marrow of Rhesus monkey (<i>Macaca mulatta</i>)	37	Cathelicidin, α -helix	[48]
Temporin L	European red frog skin (<i>Rana temporaria</i>)	13	Stable α -helix, secondary amphipathicity, shortest natural AMP found to date	[49]

Table 2. Proposed modes of action, amino acid sequences, and hemolysis reactions of AMP employed in this study.

AMP	AA Sequence	Net Charge	Proposed Modes of Action	Hemolysis
Apidaecin 1B	GNNRP VYIPQ PRPPH PRL	3.1	binding and irreversible combination with a periplasmic receptor/docking molecule, devoid of pore-forming activity	non-hemolytic
C ₁₂ K-2 β 12	C12K-KIK-KIK (C12 represents dodecanoic acid)	4	rapid membrane depolarization and cell permeabilization	non-hemolytic at 1:64 (1.56 mcg/mL)
Carnobacteriocin B2	VNYGN GVSCS KTKCS VNWGQ AFQER YTAGI NSFVS GVASG AGSIG RRP	3.9	cationic membrane-permeabilizing bacteriocin (Class II)	not expected
Cecropin A-Magainin 2 hybrid	KWKLFKKIGIGKFLHSACKF	7.1	trp2 insertion, Lys binding, alpha-helix membrane spanning due to flexible hinge	moderate 1:2 (50 mcg/mL) to 1:32 (3.125 mcg/mL)
Dermaseptin	ALWKT MLKKL GTMAL HAGKA ALGAA ADTIS QGTQ	3.1	forms amphipathic helices when integrated with membrane lipid bilayer	yes
Dermcidin DCD	SSLLE KGLDG AKKAV GGLGK LGKDA VEDLE SVGKG AVHDV KDVLD SVL	-1.9	transmembrane potential formed with nanopore formation upon insertion	not expected
NRC-13 Pleurocidin	GWRTLLKKAEVKTVGKALKHYL	5.1	forms ion channels (probable toroidal pore) in planar lipid bilayers. Inhibits nucleic acid and protein synthesis	moderate 1:2 (50 mcg/mL) to 1:256 (0.39 mcg/mL)
Parasin I	KGRGK QGGKV RAKAK TRSS	8	binds to DnaK, inhibiting its major two functions: ATPase activity and misfolding proteins with inactivation by acting on internal targets	non-hemolytic

Table 2. Cont.

AMP	AA Sequence	Net Charge	Proposed Modes of Action	Hemolysis
Pyrrhocoricin	VDKGS YLPRP TPRP IYNRN	3	binds to 70 kDa heat-shock protein DnaK, inhibiting protein folding	undetermined
RL-37	RLGNFFRKVKKEKIGGGLKKVQKIKDFLGNLVPRTAS	8	amphipathic alpha-helical structure, pore formation	no
Temporin L	FVQWF SKFLG RIL	2	allows Temporin A and B to bypass LPS and access the cytoplasmic membrane by preventing their oligomerization to LPS	yes

Target bacterial cultures, *C. jejuni* 11168 and 81–176 were propagated on Brucella agar with blood (BAB) overnight at 42 °C in a microaerobic gas atmosphere (5% O₂, 10% CO₂, 85% N₂). Cells were aseptically harvested from the plates and suspensions were made in PBS equivalent to No. 1 McFarland standard. Approximately 200 µL of bacterial suspensions were applied to the surface of BAB plates containing 1.5% low electroendosmosis (EEO) agarose [50] (Sigma-A6013) using a swab dipped in the suspension once for the first half the plate, and again for the second half. The inoculated plates were air-dried for approximately 10 min and were then spotted with 10 µL of each antimicrobial peptide or sterile water (control). All AMP solutions were prepared as 1 mg/1 mL; therefore, a 10 µL spot equates to 10 µg. Plates with AMP spots were allowed to dry for approximately 5 min, then were incubated at 37 °C under microaerobic conditions. AMP efficacy was determined as visible zones of inhibition of growth at 24 h.

Six of the AMP we tested produced zones of inhibition on lawns of *C. jejuni*. Three AMP were chosen for further investigation on the basis of anti-*Campylobacter* activity, water solubility, and reported reduced cytotoxicity to mammalian cells. Cecropin-Magainin, RL-37, and C₁₂K-2β₁₂ were tested for ability to produce zones of inhibition in spot-on-lawn assays against 24 different bacteria including *C. jejuni*, *C. coli*, and *C. lari* isolates as well as two strains of *Salmonella*, *Clostridium perfringens*, *Listeria monocytogenes*, *Lactobacillus*, and *E. coli* O157:H7. The spot-on-lawn assay was conducted as described earlier with the addition of Brain Heart Infusion agar with 1.5% EEO agarose for salmonellas, *E. coli*, clostridia, and *Listeria*. *Lactobacillus* MRS agar with 1.5% EEO agarose was utilized for lactobacilli. *Campylobacter* and lactobacilli were incubated in a microaerobic atmosphere (10% CO₂, 5% O₂, 85% N₂) at 37 °C, and ambient air cultivation at 37 °C was utilized for *Salmonella*, *E. coli*, and *Listeria*. An anaerobic environment (5% CO₂, 5% H₂, 90% N₂) at 37 °C was used for incubation of *C. perfringens*.

In addition, a modification of the CLSI M26A [51] and Wu and Hancock [52] assays were utilized to determine minimum inhibitory concentrations (MIC) for the AMP Cecropin-Magainin, RL-37, and the oligo-acyl-lysyl (OAK) C₁₂K-2β₁₂ against 15 isolates of campylobacter in microtiter plates. Briefly, target campylobacter cultures were grown in Brucella broth for 18 h in 25 cm² vented tissue culture flasks in a microaerobic gas environment (5% O₂, 10% CO₂, 85% N₂) at 37 °C. Visual adjustment of suspensions equivalent to a 0.5 McFarland standard for 1.5 × 10⁸ cfu/mL baseline were made using fresh Brucella broth. The standardized suspensions were further diluted in fresh Brucella broth 1:1000 for an inoculum of 1.5 × 10⁵. Enumeration of inoculum levels was assessed on BAB. Cecropin-Magainin, RL-37, and C₁₂K-2β₁₂ antimicrobial peptides were serially diluted in polypropylene strip tubes using 0.2% bovine serum albumin and 0.1% acetic acid diluent. An amount of 10 µL of each peptide dilution was added to 90 µL of bacterial inoculum culture in Greiner 96-well V-bottom polystyrene tissue culture-treated microtiter plates in triplicate for targets. One well was inoculated for each target with 10 µL 0.2% bovine serum albumin and 0.1% acetic acid diluent with 90 µL of bacterial inoculum as positive control. One well was inoculated with 10 µL 0.2% bovine serum albumin and 0.1% acetic acid diluent with 90 µL of Brucella broth as negative control. Plates were placed in gallon-sized zip-lock freezer bags (SC Johnson, Racine WI 53403, USA), flush-filled with the microaerobic gas mixture, and incubated for 40 h with gas replacement at 24 h. MIC was defined

as the minimum concentration that prevented growth based on visual observation using mirrored white light.

3. Results

A set of 11 unique AMP were chemically synthesized and evaluated for ability to lyse *C. jejuni* in a spot-on-lawn assay (Figure 1). Table 3 shows the initial screening results for the 11 AMP against two isolates of *C. jejuni* as well as additional human foodborne pathogens including isolates of *Clostridium perfringens*, *Salmonella*, *Listeria*, and enterotoxigenic *E. coli*. Six of the selected AMP produced obvious zones of inhibition against growth of *C. jejuni* isolates. These antimicrobial peptides included: NRC-13 Pleurocidin, RL-37, Temporin L, Cecropin–Magainin, Dermaseptin, and C₁₂K-2β₁₂. Interestingly, these same six AMP were also able to lyse most of the other pathogenic bacteria tested.

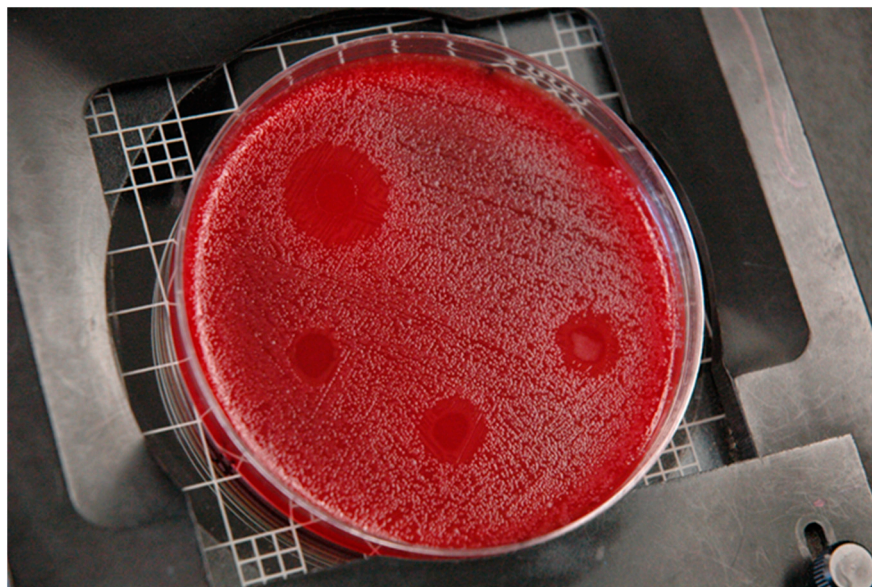


Figure 1. “Spot-on-lawn” zone assay demonstrating inhibition of *C. jejuni* by selected antimicrobial peptides: (Counter-clockwise from top) RL-37, Cecropin A–Magainin 2 hybrid, the synthetic OAK C₁₂K-2β₁₂, ampicillin (positive control), and sterile water (negative control, no zone of inhibition).

Cecropin–Magainin, RL-37, and C₁₂K-2β₁₂ were chosen for further investigation on the basis of anti-*Campylobacter* activity, water solubility, and limited cytotoxicity to mammalian cells and were tested for ability to produce zones of inhibition in spot-on-lawn assays against 24 different bacteria including 15 unique *C. jejuni*, *C. coli*, and *C. lari* isolates as well as two strains of *Salmonella*, *Clostridium perfringens*, *Listeria monocytogenes*, *Lactobacillus*, and *E. coli* O157:H7. These three active AMP produced obvious zones of inhibition on all 15 of the campylobacter isolates tested, regardless of the strain (Table 4). They also inhibited the other pathogens tested with the only exceptions being the lack of zone formation by the Cecropin–Magainin and RL-37 AMP on *Lactobacillus helveticus* and *Clostridium perfringens* 39.

MIC of C₁₂K-2β₁₂, Cecropin–Magainin, and RL-37 against 15 different *C. jejuni*, *C. coli*, and *C. lari* isolates are presented in Table 5. MIC for C₁₂K-2β₁₂ ranged from 1.6 to 3.1 µg/mL with no discernable differences between strains of campylobacter. MIC for RL-37 varied from 1.6 to 6.3 µg/mL also with no obvious differentiation between campylobacter strains. The MIC for the Cecropin–Magainin hybrid were significantly higher, ranging from 12.5 to 100 µg/mL. No significant differences between campylobacter stains were observed in response to the Cecropin–Magainin AMP.

Table 3. Formation of zones of inhibition by selected AMP against bacterial isolates.

AMP	Cj1	Cj2	Cp1	Cp2	S1	S2	Lm1	Lm2	Ec
Apidaecin 1B	-	-	-	-	+	+	-	-	+
C ₁₂ K-2β ₁₂	+	+	+	+	+	+	+	+	+
Carnobacteriocin B2	-	-	-	-	-	-	-	-	-
Cecropin A–Magainin 2 hybrid	+	+	-	+	+	+	+	+	+
Dermaseptin	+	-	+	+	-	+	+	+	+
Dermcidin DCD	-	-	-	-	-	-	-	±	-
NRC-13 Pleurocidin	+	+	-	+	±	+	+	+	+
Parasin I	-	-	-	-	-	-	-	-	-
Pyrrhocorin	-	-	-	-	-	-	-	-	-
RL-37	++	++	-	+	+	+	+	+	+
Temporin L	+	+	+	++	+	+	+	+	+
Ampicillin (control)	-	-	++	++	+	+	+	++	++
Acetic acid (control)	-	-	+	+	+	+	+	+	+

Key to bacterial target isolates: Cj1 = *Campylobacter jejuni* 11168; Cj2 = *Campylobacter jejuni* 81-176; Cp1 = *Clostridium perfringens* CP39; Cp2 = *Clostridium perfringens* CP509; S1 = *Salmonella enteritidis* serovar Typhimurium 14028; S2 = *Salmonella enteritidis* serovar Heidelberg 130NR; Lm1 = *Listeria monocytogenes* A49594 (4b); Lm2 = *Listeria monocytogenes* 311-WT; Ec = *Escherichia coli* O157:H7 (233_RC1-WT). Key to table symbols: - no inhibition; ± presence of satellite colonies within zone of inhibition; + 5 mm mean zone diameter; ++ 13.5 mm mean zone diameter.

Table 4. Formation of zones of inhibition by selected AMP against various target bacteria.

Target Bacteria	C ₁₂ K-2β ₁₂	Cecropin A–Magainin 2	RL-37
<i>Campylobacter jejuni</i> 14118	++	+	+++
<i>Campylobacter jejuni</i> 81-116	++	+	++
<i>Campylobacter jejuni</i> 81-176 ^a	++	+	++
<i>Campylobacter jejuni</i> 11168 ^{^a}	++	+	++
<i>Campylobacter jejuni</i> RM1221 ^a	+++	+	+++
<i>Campylobacter jejuni</i> A74C	+++	+	++
<i>Campylobacter jejuni</i> A49943 *	++	+	++
<i>Campylobacter jejuni</i> A33250 *	++	+	++
<i>Campylobacter jejuni</i> A29428 *	++	+	++
<i>Campylobacter coli</i> Epi 33-WT	+++	+	+++
<i>Campylobacter coli</i> A49941 *	+++	+	+++
<i>Campylobacter coli</i> A33559 *	++	+	+++
<i>Campylobacter lari</i> RM2100	+++	+	+++
<i>Campylobacter lari</i> A35221 *	++	+	++
<i>Campylobacter lari</i> "slaughter beach"	++	+	++
<i>Salmonella enterica</i> serovar Typhimurium Epi 3	++	+	+
<i>Salmonella enterica</i> serovar Heidelberg Epi 42	++	+	+
<i>Lactobacillus acidophilus</i> -WT	++	+	+
<i>Lactobacillus helveticus</i> -WT	++	-	-
<i>Clostridium perfringens</i> 39	+	-	-
<i>Clostridium perfringens</i> 509	+	+	+
<i>Listeria monocytogenes</i> A49594 (4b)	+	+	+
<i>Listeria monocytogenes</i> 311 WT	+	+	+
<i>Escherichia coli</i> O157:H7	+	+	+

^a Alternate ATCC designations; [^] National Collection of Type Cultures (NCTC) isolate; * American Type Culture Collection (ATCC) isolate. Key to table symbols: - no zone of inhibition; + 5 mm mean zone diameter; ++ 13.5 mm mean zone diameter; +++ 18 mm mean zone diameter.

Table 5. Minimum inhibitory concentrations (MIC) of selected AMP against *Campylobacter jejuni*, *coli*, and *lari* isolates.

Target <i>Campylobacter</i> spp. Isolate	C ₁₂ K-2β ₁₂ MIC (μg/mL)	Cecropin A–Magainin 2 MIC (μg/mL)	RL-37 MIC (μg/mL)
<i>Campylobacter jejuni</i> 14118	3.1	50	1.6
<i>Campylobacter jejuni</i> 81-116	1.6	12.5	3.1
<i>Campylobacter jejuni</i> 81-176 ^a	3.1	50	3.1
<i>Campylobacter jejuni</i> 11168 ^{^a}	3.1	25	3.1
<i>Campylobacter jejuni</i> RM1221 ^a	1.6	50	1.6
<i>Campylobacter jejuni</i> A74C	1.6	50	3.1
<i>Campylobacter jejuni</i> A49943 [*]	3.1	25	3.1
<i>Campylobacter jejuni</i> A33250 [*]	3.1	100	3.1
<i>Campylobacter jejuni</i> A29428 [*]	3.1	100	6.3
<i>Campylobacter coli</i> Epi 33-WT	1.6	50	1.6
<i>Campylobacter coli</i> A49941 [*]	1.6	100	1.6
<i>Campylobacter coli</i> A33559 [*]	3.1	100	1.6
<i>Campylobacter lari</i> RM2100	1.6	25	1.6
<i>Campylobacter lari</i> A35221 [*]	3.1	100	3.1
<i>Campylobacter lari</i> “slaughter beach”	3.1	100	3.1

^a Alternate ATCC designations; [^] National Collection of Type Cultures (NCTC) isolate; ^{*} American Type Culture Collection (ATCC) isolate.

4. Discussion

AMP display tremendous chemical diversity in nature, causing difficulty in their classification [53,54]. Amino acid sequence, net charge, secondary structural motif, and the abundance of specific amino acids may all differ between types of AMP [55]. However, AMP also maintain some common features, and classification of AMP may be based on source, activity, structural characteristics, and amino acid-rich species [54]. For example, most AMP are cationic in nature with net charges ranging from +2 to +9. This gives an electrostatic advantage to the AMP in binding to the negatively charged bacterial membranes [56]. Many AMP are membrane-acting peptides and have an amphipathic structure with hydrophobic and hydrophilic regions which play an integral part in interactions with target bacterial membranes, leading to cell lysis through a variety of proposed mechanisms including the toroidal pore or wormhole model, the barrel-stave model, or the carpet-like model [57,58]. Indeed, some AMP are non-membrane-acting and do not cause cell lysis, but rather pass through the intact bacterial lipid bilayer through endocytosis and, once in the cytoplasm, inhibit nucleic acid biosynthesis, protease activity, or DNA replication [51]. A number of peptide databases exist and are available for mining [5,59,60]. We initially chose 11 AMP for evaluation representing a variety of different sources, modes of action, and activities frequently discussed in the literature (Table 1). The selected AMP represent examples from insects, bacteria, amphibians, fish, mammals, and a case of *de novo* synthetic AMP. They also represent membrane-acting and non-membrane-acting peptides.

While very few reports exist in the literature specifically describing inhibition of campylobacters by AMP, *C. jejuni* has been reported to be highly susceptible to chicken host-defense peptides such as cathelicidin [61]. We therefore chose to evaluate RL-37, a 37-residue AMP of the cathelicidin family which is expressed in bone marrow of the rhesus monkey and utilizes an amphipathic α -helical structure to form pores and destabilize the membrane lipid bilayer of target bacteria. RL-37 is reported to inhibit Gram-negative organisms such as *E. coli* and *Pseudomonas* [48] and we found it to be quite efficacious against the Gram (–) campylobacters we tested, achieving MIC ranging from 1.6 to 6.3 μg/mL (Table 5). It was also reported to inhibit Gram (+) organisms such as *Listeria monocytogenes* and this was observed in our study as well (Table 4). We chose several additional α -helical AMP for evaluation including dermaseptin, NRC-Pleurocidin, Paracin I, and Temporin L. Dermaseptin, a 34-residue peptide isolated from frog skin (*Phyllomedusa*), demonstrates broad-spectrum lytic activity against Gram-positive and Gram-negative bacteria, yeast, and

protozoa [43,62]. We found it to inhibit some, but not all, of our test isolates (Table 3). Since it did not inhibit both of our initial campylobacter isolates used for screening, we did not include it in further MIC evaluations. NRC-Pleurocidin, a 23-residue peptide isolated from the American plaice winter flounder has been reported to rapidly kill G (–) *Pseudomonas aeruginosa* and G (+) Methicillin-resistant *S. aureus* (MRSA) [45]. We found it capable of lysing all our test isolates except for *C. perfringens* 39. However, it was excluded from further analysis due to its moderate cytotoxicity. Paracin I is a 19-residue peptide isolated from catfish skin and has been reported to inhibit G (+) and G (–) organisms including *E. coli* and *Salmonella* [46]. However, in the concentrations tested in our assay, Paracin I failed to inhibit any of our test isolates. Temporin L is a 13-residue peptide from European red frog skin and is among the shortest natural AMP known [49,63]. We found it to be efficacious against all our test organisms but because it is reported to be hemolytic and requires dimethylsulfoxide (DMSO) as a solvent rather than water, we did not think it practical for our purposes and did not include it in further analysis.

Some AMP contain a high content of certain amino acid residues such as proline, tryptophan, glycine, or histidine. These AMP are frequently extended with no regular structure [56]. Proline-rich AMP (Pr-AMPs) are cationic peptides which usually enter the cell through translocation of the inner membrane using a transporter-mediated uptake mechanism and do not lyse the cell. Pr-AMPs usually kill bacteria by targeting intracellular components. Pyrrhocoricin is a 20-amino acid residue Pr-AMP isolated from the European sap-sucking bug (*Pyrrhocoris apterus*). Pyrrhocoricin interferes with protein biosynthesis in target bacteria by binding to the 70S ribosomal subunit and inhibiting heat-shock proteins such as DnaK, preventing protein folding [64]. In the concentrations tested in our assay, Pyrrhocoricin failed to inhibit any of our test isolates. Apidaecin 1B is 18-residue Pr-AMP isolated from honeybees. Apidaecin 1B is known to inhibit a large number of plant-associated bacteria and human pathogens via a bactericidal pathway without cell lysis [39]. Apidaecin 1B inhibited *Salmonella enteritidis* serovars Typhimurium and Heidelberg and *E. coli* O157:H7 in our assay, but did not kill *Campylobacter jejuni*, *Clostridium perfringens*, or *Listeria monocytogenes* isolates.

Bacteriocins are AMP produced by bacteria. Carnobacteriocin B2 is a Class II cationic bacteriocin which can form α -helices and lyse specific target bacteria [41]. Under the conditions of our spot-on-lawn assay, none of our target bacteria were affected by Carnobacteriocin B2. Dermcidin DCD is a 48-residue peptide isolated from human sweat glands. This anionic AMP forms oligomeric ion channels in lipid bilayers of specific target bacteria. This AMP was also unable to form any zones of inhibition against any of the bacteria utilized in our assay.

Despite the large number of natural AMP identified, isolated, and characterized, there are some inherent disadvantages associated with natural AMP. For example, the limited quantities of AMP isolated from natural sources, the uneconomic synthesis of longer AMP sequences, folding issues associated with natural AMP, short half-lives due to protease degradability, and difficulties in site-directed delivery [56]. This has led to a research emphasis on synthetic AMP in recent decades to overcome shortcomings of natural AMP. Several approaches have been employed for the design of improved synthetic AMP including template-based *de novo* synthesis based on natural AMP to improve activity and proteolytic stability (often through truncation), biophysical modeling in hydrophobic membrane environments, and virtual screening of peptide libraries to identify potential AMP sequences [56].

Various chemical modifications of AMP have been utilized to improve potency while decreasing cytotoxicity to mammalian cells, or improving peptide resistance to protease digestion, high temperatures, and low pH. Cecropin A (from the Cecropia moth) and Magainin 2 (from the African clawed frog, *Xenopus Laevis*) both individually exhibit potent antimicrobial effects against a broad spectrum of bacteria [65]. Shin and co-workers designed an engineered hybrid AMP composed of residues 1–8 of Cecropin A fused to residues 1–12 of Magainin 2 [66]. Cecropin–Magainin had greater antimicrobial activity

against bacteria than either of the AMP individually while maintaining low toxicity. It has been suggested that the flexibility induced by Gly-Ile-Gly or Pro residues in the central part of Cecropin–Magainin may be important in the electrostatic and hydrophobic interactions of the N and C terminus with cell membrane surface and cell membrane, respectively [42]. We included Cecropin–Magainin in our spot-on-lawn assays and found it to inhibit all the bacterial species in our test, including the 15 *Campylobacter* spp. isolates, but not *L. helveticus* or *C. perfringens* 39 isolates.

Many naturally occurring AMP have rather long AA sequences which can limit their application as commercial drugs due to the high cost of producing them on an industrial scale [67]. Shorter synthetic AMP could potentially lower cost of synthesis and may be more resistant to proteolysis. Shorter synthetic AMP also could be less cumbersome to express in natural production vectors such as *E. coli* or *Bacillus subtilis* [68,69]. Oligomers of acylated lysines (OAKs) constitute a novel class of synthetic AMP made up of alternating amino acyl chains and cationic amino acids arranged in such a way as to create an optimal molecular charge and hydrophobicity for increased potency [70–72]. The synthetic OAK hexamer C₁₂K-2β₁₂ exhibits inhibitory activity in vitro against Gram-negative bacteria such as the gastric pathogen *Helicobacter pylori* [40]. Because *H. pylori* has some structural characteristics in common with campylobacters, we chose to evaluate C₁₂K-2β₁₂ in our spot-on-lawn assay. C₁₂K-2β₁₂ was very active and inhibited growth of all target bacterial isolates in our assay. The bactericidal effects of C₁₂K-2β₁₂ are exhibited by a dual mode of action. The OAK causes pore formation and cell lysis at higher concentrations while, at lower concentrations, it binds nucleic acids and proteins [55]. It demonstrated the lowest MIC of any of the AMP we tested against 15 isolates of *Campylobacter* representing *jejuni*, *coli*, and *lari* species, with a range of 1.6 to 3.1 ug/mL. C₁₂K-2β₁₂ is only 8 amino acid residues in length and is reported to be stable at low pH and after exposure to extreme temperatures [40]. These are all important considerations for an AMP to be potentially administered to poultry. This AMP also inhibited other pathogenic bacterial species associated with poultry (*Salmonella*, *C. perfringens*). These characteristics make C₁₂K-2β₁₂ a strong candidate for further investigation and potential development into a practical intervention for reduction of human foodborne pathogen populations associated with broiler chickens.

It is interesting that the AMP investigated in this study and found to be efficacious against *Campylobacter* spp. (NRC-13 Pleurocidin, RL-37, Temporin L, Cecropin–Magainin, Dermaseptin, and C₁₂K-2β₁₂) are all cationic AMP hypothesized to form amphipathic α-helical structures to initiate pore formation in target bacteria and subsequent cell lysis. It is also interesting that Pyrrhocoricin, which is also a cationic, amphipathic α-helical AMP, did not inhibit *Campylobacter* spp. There is evidence in the literature suggesting that the selectivity and potency of a specific AMP is determined primarily by the chemical composition of the target membrane [1]. It has likewise been hypothesized that differences in membrane composition between different strains of bacteria are responsible for the diversity in the potency and selectivity exhibited by a particular AMP against different strains of bacteria [73].

One of the most important factors in reducing the burden of foodborne disease has been identified as development of effective control measures [74]. By providing novel alternatives to antibiotic usage in poultry, the overall impact of our long-term research goals will be a reduction in bacterial pathogens associated with chickens and safer products for human consumption. Our working hypothesis is that AMP that inhibit the growth of *Campylobacter* spp. can be identified and subsequently utilized to reduce the *Campylobacter* load among commercially produced chickens. Identifying non-hemolytic AMP, such as C₁₂K-2β₁₂, RL-37, and Cecropin–Magainin, capable of killing campylobacters in vitro is a positive first step toward this goal. An obvious second step will be producing efficacious AMP in an economical fashion. Microbiological production of AMP through fermentation is becoming a feasible and economic method of production. Recent studies have described the development of a *Bacillus subtilis* culture expressing chicken NK-2 peptide [69]. Oral

delivery of the *B. subtilis* culture can protect against *Eimeria acerouline* infection in broiler chickens. We are currently working to similarly express effective AMP in a microbial vector for in vitro analysis and subsequent oral delivery to chickens to protect against colonization by *Campylobacter* spp.

5. Conclusions

Selected AMP produced obvious zones of inhibition against growth of *C. jejuni* isolates. These AMP included: NRC-13 Pleurocidin, RL-37, Temporin L, Cecropin A–Magainin 2 hybrid, Dermaseptin, and C₁₂K-2β₁₂. MIC for C₁₂K-2β₁₂ ranged from 1.6 to 3.1 µg/mL with no discernable differences between strains of campylobacter. MIC for RL-37 varied from 1.6 to 6.3 µg/mL, also with no obvious differentiation between campylobacter strains. The MIC for Cecropin–Magainin were significantly higher, ranging from 12.5 to 100 µg/mL. No significant differences between campylobacter stains were observed in response to the Cecropin–Magainin AMP. The most efficacious of the AMP tested, the synthetic OAK C₁₂K-2β₁₂, is also heat- and acid-stable, making it an attractive subject for future study and potential in vivo delivery to poultry.

Author Contributions: Conceptualization, J.E.L., B.S.S., and J.K.G.; methodology, J.E.L., B.S.S., and J.K.G.; software, J.E.L.; validation, J.K.G.; formal analysis, J.E.L.; investigation, J.E.L. and J.K.G.; resources, J.E.L.; data curation, J.K.G.; writing—original draft preparation, J.E.L.; writing—review and editing, J.E.L., B.S.S., and J.K.G.; visualization, J.E.L.; supervision, J.E.L.; project administration, J.E.L.; funding acquisition, J.E.L. All authors have read and agreed to the published version of the manuscript.

Funding: This research was funded by USDA, ARS Project Number 6040-32000-079-000D.

Institutional Review Board Statement: Not applicable.

Informed Consent Statement: Not applicable.

Data Availability Statement: Data is contained within the article.

Conflicts of Interest: The authors declare no conflict of interest.

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