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Antimicrobial Peptides Targeting Gram-negative Pathogens, Produced and Delivered by Lactic Acid Bacteria

Katherine Volzing[1], **Juan Borrero**[1], **Michael J. Sadowsky**[2,3], and **Yiannis N. Kaznessis**[1,3]

[1]Department of Chemical Engineering and Materials Science, University of Minnesota, Minneapolis, MN 55455, USA

[2]Department of Soil, Water, and Climate, University of Minnesota, St. Paul, MN 55108, USA

[3]BioTechnology Institute, University of Minnesota, St. Paul, MN 55108, USA

Abstract

We present results of tests with recombinant *Lactococcus lactis* that produce and secrete heterologous antimicrobial peptides with activity against Gram-negative pathogenic *Escherichia coli* and *Salmonella*. In an initial screening, the activities of numerous candidate antimicrobial peptides, made by solid state synthesis, were assessed against several indicator pathogenic *E. coli* and *Salmonella* strains. Peptides A3APO and Alyteserin were selected as top performers based on high antimicrobial activity against the pathogens tested and on significantly lower antimicrobial activity against *L. lactis*. Expression cassettes containing the signal peptide of the protein Usp45 fused to the codon optimized sequence of mature A3APO and Alyteserin were cloned under the control of a nisin-inducible promoter nisA and transformed into *L. lactis* IL1403. The resulting recombinant strains were induced to express and secrete both peptides. A3APO- and Alyteserin-containing supernatants from these recombinant *L. lactis* inhibited the growth of pathogenic *E. coli* and *Salmonella* by up to 20-fold, while maintaining the host's viability. This system may serve as a model for the production and delivery of antimicrobial peptides by lactic acid bacteria to target Gram-negative pathogenic bacteria populations.

Keywords

Alyteserin; A3APO; lactococci; probiotics; food-borne pathogens; salmonella; Escherichia coli

INTRODUCTION

There is growing concern worldwide that extensive use of antibiotics is resulting in the development of antibiotic resistance among pathogenic bacteria. In particular, antibiotic overuse in livestock feeds compromises the effectiveness of current therapies via dissemination of antibiotic resistance genes to both disease-causing and commensal microorganisms[1,2].

Corresponding Author: Yiannis N. Kaznessis, Department of Chemical Engineering and Materials Science, University of Minnesota, 421 Washington Ave. S.E., Minneapolis, MN 55455-0132, Tel: (612) 624-4197, Fax: (612) 626-7246, yiannis@umn.edu.

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Over 80% of the antibiotics produced in the United States are administered in swine, poultry and cattle farming. In addition to their intended use as therapeutics, antibiotics are administered throughout the life of food producing animals, even in the absence of infection to promote animal growth and improve feed efficiency[3,4]. These growth-promoting antibiotics are applied at sub-therapeutic concentrations, establishing the conditions for resistance to antibiotics to develop. Alarmingly, many of the antibiotics used in agriculture have also been listed as critically important for human health by the World Health Organization. Humans depend on many of these same antibiotics as a first line of defense against pathogens like *Escherichia coli* O157:H7, *Salmonella typhimurium, Staphylococcus aureus*, *Streptococcus*, and *Pseudomonas aeruginosa*[5]. Therefore, there is a pressing need for new therapeutic agents with activity against pathogenic bacteria and alternative technologies for application in agriculture, such that front line therapeutics can be reserved for effectively treating infections in humans.

One promising alternative to traditional antibiotic molecules are antimicrobial peptides (AMPs). AMPs are small, often positively-charged, peptides with high antimicrobial activity. The activity of AMPs can be broad, efficiently acting on many Gram-positive and Gram-negative bacteria species. There are however AMPs with very specific activity, targeting one particular bacteria species or even a specific subspecies of a given genus[6–11].

The current production, purification and delivery methods available for these peptides have numerous limitations. For example, solid state peptide synthesis and peptide production and purification from cell culture are both costly and time consuming[11–13]. Additionally, the subsequent targeted delivery of active amounts of these compounds can be challenging. Generally, AMPs cannot be administered orally as they are quickly degraded before they are able to reach their target. AMPs cannot be administered systemically either, as they are rapidly identified and targeted for clearance by the immune system before they can reach the site of infection[11]. Moreover, high peptide concentrations are required to achieve a therapeutic effect which would be cost-prohibitive and would, more importantly, cause severe toxic side-effects. Taken together, these limitations have thus far stifled the development of AMP- based therapeutics[11].

In recent years probiotic bacteria have emerged as useful tools for effectively boosting overall human and animal health[14]. Probiotics are typically Gram-positive, bile-resistant, bacteria that either colonize or transiently inhabit the gastrointestinal (GI) tract of a host. When administered in adequate amounts they confer health benefits by improving nutrient absorption and decreasing the relative abundance of potentially pathogenic bacteria[15,16]. Lactic acid bacteria (LAB), which include microbes in the genera *Lactobacillus* and *Lactococcus*, and *Bifidobacterium*, are currently the bacteria most commonly employed as probiotics[14,16]. A number of probiotic bacteria are currently in use as nutritional supplements for humans and animals[17–23]. In addition, recombinant LAB are also significant therapeutic delivery vectors. They are presently being tested as candidates for the delivery of therapeutics inside the GI tract of humans for the treatment of inflammatory bowel syndrome and Crohn’s disease[24–26]. Although the therapeutic promise of modified LAB is yet to be realized in these cases, there have been numerous demonstrations that LAB can be used as vehicles to delivery proteins inside the GI tracts of hosts.

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Here we report the results of experimental efforts to design, build, and characterize AMP production and delivery systems founded on probiotic bacteria. Our approach is based on *Lactococcus lactis*, a non-invasive and non-pathogenic LAB, generally recognized as safe by the FDA, with probiotic properties that is amenable to heterologous protein over-expression[27,28]. Since *L. lactis* is able to survive in the GI tract of both humans and animals, this bacterium is an excellent candidate to deliver health benefits to the targeted host organism[28]. Over the last two decades several genetic tools have been developed for *L. lactis*[29–32], making it suitable to engineer as an efficient cell-based protein expression factory[31,33–35]. Based on these attributes, *L. lactis* may be an ideal vehicle for producing and delivering AMPs to the site of GI infection [36].

The systems that we report constitute *L. lactis* strains that have been engineered to inducibly express and secrete the AMPs Alyteserin-1a and A3APO, both peptides that are active against Gram-negative pathogenic *Escherichia coli* and *Salmonella* strains. Our systems have been engineered with the intention of future application as an alternative to antibiotics in agriculture.

RESULTS AND DISCUSSION

Motivated by the current state of antibiotic overuse and the rapid emergence of bacterial strains with resistance to antibiotics molecules, the overall goal of this work was to engineer a LAB strain to inducibly express and secrete AMPs with high activity against important Gram-negative pathogens. In summary, this was achieved by first screening AMPs for high activity against pathogenic *E. coli* and *Salmonella* strains and low activity against LAB. Genes encoding peptides that displayed the most favorable activity were then included in expression cassettes for use in *L. lactis.* In the following, we detail how we 1) selected the AMPs of interest, 2) engineered *L. lactis* to express the heterologous peptides and 3) tested the recombinant expression systems. To our knowledge this is one of the first works in which LAB have been engineered to express and secrete AMPs that are specifically active against Gram-negative pathogens.

Screening of AMPs with activity against Gram-negative bacteria

Recently discovered AMPs that have been introduced into clinical practice largely display activity against Gram-positive organisms while being ineffective against Gram-negative bacteria[37]. This is due in part to the unique cell wall and membrane structure of these two classes of bacteria[38,39]. However there are a few exceptional AMPs that show high specific activity against Gram-negative bacteria[40,41]. To select top candidate AMPs to use in our study, we initially searched the literature for functional peptides fulfilling the following requirements: 1) lack of post-translational modifications and disulfide bonds, 2) activity against Gram-negative bacteria at low concentrations (≤500 μg/ml) and 3) no or significantly less activity against Gram-positive bacteria, in particular against the *L. lactis* host LAB. From this first screen, numerous candidates were chosen (data not shown) and chemically synthesized as described in Methods. The minimum inhibitory concentration (MIC) of the synthetic peptides was evaluated against a panel of pathogenic strains of *E. coli* and *Salmonella* and against *L. lactis*.

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Two peptides, Alyteserin-1a (Alyteserin) and A3APO, emerged as promising candidate AMPs. Alyteserin was previously identified in the secretions on frog skin while A3APO was discovered in a synthetic peptide library screen[42,43]. The MIC of pure Alyteserin was 500 μg/ml against indicator *E. coli* and *Salmonella* strains. Additionally, *L. lactis* remained viable despite treatment with up to 1 mg/ml Alyteserin (Figure 1a). Inhibition of *E. coli* by A3APO was observed only at a concentration of 1 mg/ml. However, *Salmonella* growth was inhibited by 30 μg/ml Alyteserin, reducing viability through the highest concentration tested (1 mg/ml). Similarly, *L. lactis* growth was not inhibited with the different A3APO concentrations tested (Figure 1b).

The two AMPs chosen in this study did not require post-translational modifications for activity. The heterologous production of proteins that require post-translational modifications to be active can be problematic when using LAB as hosts. There are several examples where recombinant peptides that required post-translational modifications were being produced at high concentrations but had low specific activity[44]. This has been attributed to a variety of causes, such as non-efficient disulfide bond formation, problems in protein folding, and protein aggregation[45].

Once the two candidate peptides were identified, *L. lactis* was engineered to express Alyteserin and A3APO. Details of this process are presented in the Materials and Methods. The resulting *L. lactis* were cultured to express and secrete the peptides. The cell-free supernatants containing these AMPs were isolated and their effect on *E. coli* and *Salmonella* growth and viability were assessed as also described in the Materials and Methods.

Construction of recombinant L. lactis strains for AMP production

Lactococcus lactis strain IL1403 was engineered to express the AMPs Alyteserin and A3APO as detailed in the Materials and Methods. Briefly, the codon-optimized nucleotide sequences of both peptides were synthesized by GeneArt and fused to the Usp45 secretion signal peptide sequence (SP_{usp45})[46]. The expression cassettes were cloned downstream of the nisin inducible promoter, PnisA, from plasmid pMSP3545[29] resulting in recombinant vectors pMS-Alys and pMS-A3APO, respectively. *L. lactis* IL1403 was transformed with both recombinant vectors, and the resulting *L. lactis* (pMS-A3APO) and *L. lactis* (pMS-Alys) strains, as well as the control *L. lactis* strain (*L. lactis* containing the empty pMSP3545 vector), were cultured to express and secrete each AMP. The AMP-containing supernatants (AMP-SNs: Alys-SN, A3APO-SN and C-SN for supernatants containing AMPs Alyteserin, A3APO and the control supernatant lacking AMPs, respectively) were collected as described in Methods.

Growth of pathogenic and non-pathogenic *E. coli* strains was assessed in medium containing these AMP-SNs while only pathogenic *Salmonella* strains were tested. In all cases, AMP-SNs diluted 7:3 with LB were inoculated with each indicator strain and growth was monitored spectrophotometrically at 600 nm for 15 hr.

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Peptide production and secretion

QPCR was performed to determine the transcript levels of Alysteserin and A3APO genes upon induction. A3APO transcript increased by over 100-fold upon induction while Alyteserin mRNA increased by 30-fold (data not shown). To confirm the production of recombinant Alyteserin and A3APO by *L. lactis*, a His-tag sequence was fused to the C-terminus of both genes. The induced cultures were centrifuged and 2 fractions were collected: the cell pellet and the cell-free supernatant. The cell-pellet was lysed, as described in Methods, obtaining a cell lysate. The cell-free supernatants were treated with ammonium sulfate to 50% saturation to precipitate and concentrate the proteins. The crude supernatants, the ammonium sulfate and the cell-lysate fractions were subjected to dot blotting and immunological detection using Anti- His (C-terminal) antibodies. No signal was detected in any of the fractions obtained from the *L. lactis* control strain. A signal was detected in the crude supernatant samples from the AMP producing *L. lactis* and cell-lysate fractions. Increased signal was detected upon concentration with ammonium sulfate and in the cell-lysate fractions (data not shown). Although this confirms the presence of AMPs in the supernatants, it illustrates that a significant amount of peptide still remains inside the cells. Similar observations have also been observed with other recombinant peptides fused to the Usp45 signal peptide[47]. The protein size and the particular combination of signal peptide and mature protein are factors that may be limiting the secretion of the peptides.

E. coli growth inhibition by Alyteserin produced by L. lactis

As shown in Figure 2, Alys-SN inhibited growth of *E. coli* strains. Culture titer was assessed starting at 30 min post-inoculation and culture density was monitored by OD_{600} 10–15 h post-inoculation. Prior to the 6 h time point, *E. coli* cultures treated with Alys-SN and C-SN were not statistically different (data not shown). However, different culture concentrations were observed beginning 6 h post-inoculation (Figure 2a), and this differential growth pattern was maintained through 15 h (Figure 2b). Upon treatment with Alys-SN, culture concentrations were reduced by over 20-fold relative to those treated with C-SN at 6 h post-inoculation and maintained a density at or less than this value through the 15 h incubation period. The same trends were found with all *E. coli* strains tested. Growth rates for *E. coli*, as shown in Table 1, were calculated based on culture densities 10–15 h post inoculation. There was no significant *E. coli* growth when treated with Alys-SN during this time and the cultures never achieved exponential growth. Thus, Alys-SN effectively inhibited *E. coli* growth during the 15 h culture period.

In contrast, there was no change in *E. coli* culture concentration when strains were cultured with A3APO-SN (data not shown). The MIC value for synthetic A3APO against *E. coli* strains was 1 mg/ml (Figure 1) and it is reasonable that the concentration of active peptide is lower than this in tested supernatant samples.

Salmonella growth inhibition by Alyteserin-1a and A3APO produced by L. lactis

Alys-SN and A3APO-SN inhibited growth of pathogenic *S. typhimurium* and *S. infantis* as shown in Figure 3a. As before, the culture titer was assessed beginning 30 min post-inoculation and culture density was monitored by OD_{600} from 10 to 15 h. By 2 h post-inoculation, a differential titer between cultures treated with Alys-SN and A3APO-SN was

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observed (Figure 3a). Relative to culture growth with C-SN treatment, growth of *S. infantis* treated with Alys-SN was reduced by about one-half while *S. typhimurium* was reduced by 10-fold. Moreover, A3APO-SN reduced the culture density of *S. infantis* by over 20-fold relative to C-SN while *S. typhimurium* culture density was reduced by 4-fold. The inhibition of *Salmonella* by Alys-SN was maintained through 15 h, while culture density with A3APO-SN was the same as C-SN by 10 h post-inoculation (Figure 3b). At 15 h post inoculation, the Alys-SN maintained *Salmonella* culture densities at only 25% relative to the same strains treated with the C-SN. These trends were consistent across both the *S. infantis* and *S. typhimurium* strains tested.

The strong activity shown by A3APO-SN against *Salmonella* is consistent with the significantly lower MIC value observed (30 μg/ml) for synthetic A3APO peptide against *Salmonella*.

As shown in Table 1, growth rates for *Salmonella* were calculated based on culture densities 10 to 15 h post inoculation. In contrast to what was observed with *E. coli*, there was significant *Salmonella* growth in the presence of Alys-SN and the cultures achieved exponential growth. However, growth rates were reduced by 15% relative to cultures grown in C-SN during this time. Additionally, although the densities of the *Salmonella* cultures grown in A3APO-SN were significantly reduced at earlier time points, relative to C-SN, no differences were observed by 10 h after inoculation.

Improving active peptide production

Currently, the factors that most significantly improve the antimicrobial activity of Alyteserin and A3APO produced by recombinant *L. lactis* are difficult to determine. We postulate that the use of SP_{usp45} to produce and secrete these peptides is likely to be one such factor as there are significant context dependencies between a secretion peptide and the molecule for which they are driving secretion[48]. Peptide translation, targeting the Sec-dependent protein to the membrane, the translocation process itself, and the peptide's subsequent processing by a signal peptidase likely represent the major bottlenecks for efficient translocation, and thus production of heterologous proteins[49]. Since there are no good prediction methods for determining the right combination of secretion peptide and target protein to achieve a high protein production system[44,50], screening for a more efficient secretion peptide and protein combinations for overproduction and secretion may still further improve active peptide secretion[49,51]. Several strategies have been used in this direction. Using different signal petides[44], modifying the amino acids of the N-terminus of the signal peptide[52] or adding a propeptide between the signal peptide and the mature protein may help increase peptide secretion[53]. Further experiments must be performed in order to maximize the secretion of AMPs and further increase the antimicrobial activity of the supernatants. Improper protein folding may also account for compromised antagonistic activity. Thus, the use of chaperones, increasing specific peptide activity through rounds of mutagenesis, and increasing peptide gene copy number are approaches currently being pursued to improve these expression systems[54].

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Summary

In this study we report that *L. lactis* can be used to produce and secrete the antimicrobial peptides Alyteserin-1a and A3APO, with sufficient activity to inhibit pathogenic *E. coli* and *Salmonella* strains, while maintaining the host's viability. Previous studies have reported the production and secretion of AMPs by recombinant *L. lactis* [44,45,55]. However, these peptides have frequently displayed antimicrobial activity against Gram-positive bacteria but either no or poor activity against Gram-negative indicators[56,57]. While the activity of A3APO and Alyteserin in the supernatants of recombinant *L. lactis* is still not at the level of many small molecule antibiotics, to our knowledge this is the first time that a synthetic or animal-origin AMP has been produced by *L. lactis* with activity against Gram-negative bacteria pathogens. This opens up possibilities for the design of new synthetic peptides and the engineering of known AMPs to improve their antimicrobial activity and spectrum of action. Although beyond the scope of the present work, it is interesting to identify the peptide sequence and structure features that are responsible for the specificity against Gram-negative bacteria. With such features known, engineering of new peptides may be rationalized, as was recently accomplished with Alyteserin[58]. An important hypothesis that has been tested with relative success for various classes of antimicrobial peptides is that they act by binding and permeabilizing the membranes of bacteria[59]. It is well established that the inner lipid bilayer membranes differ substantially between Gram-positive and Gram-negative bacteria, mostly in their content of phosphoethanolamine (PE)[60]. PE changes the electrostatic and the mechanical properties of lipid bilayers. Understanding of exactly how is currently lacking but may in the future guide engineering peptides that preferentially bind and insert into the membranes of Gram-negative bacteria.

Lactic acid bacteria are bile-resistant, generally considered safe to consume organisms that may take hold in the gastrointestinal tract of animal or human hosts. As such, they can be considered as promising delivery vehicles for AMPs to the site of gastrointestinal infections. By making and delivering peptides to the site of *E. coli* or *Salmonella* infections, AMP-producing organisms may circumvent previous limitations of the short half-lives that are characteristic of AMPs and the high production and purification costs also associated with peptides[11–13].

METHODS

Synthetic peptide synthesis

The synthetic AMPs used in this study (Table 2) were synthesized by solid-phase methods at the BioMedical Genomics Center at the University of Minnesota (20 mg aliquots at 99% purity).

Bacterial strains and growth conditions

The bacteria used in this study are listed in Table 3. *L. lactis* IL1403 was cultured at 30°C in M17 broth (Oxoid Ltd., Basingstoke, UK) supplemented with 0.5% (w/v) glucose (GM17). The *E. coli* and *Salmonella* strains were grown in LB broth (Fisher Scientific, Fair Lawn, NJ, USA) at 37°C, with shaking. Agar plates were made by the addition of 1.5% (wt/vol) agar (Oxoid) to the liquid media. When necessary, erythromycin (Sigma Chemical Co., St.

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Louis, MO, USA) was added to the cultures at 200 μg/ml and 5 μg/ml, for *E. coli* and *L. lactis* respectively.

Molecular Biology

The amino acid sequences of the peptides Alyteserin and A3APO (Table 2) were used as templates for design of the synthetic genes. The nucleotide sequences for each peptide were then based on the preferred codon usage for expression by *L. lactis*. The nucleotide sequences of the synthetic expression cassettes contained the Usp45 signal peptide nucleotide sequence (SP_{usp45}) (Table 4) and a 5′-nucleotide extension containing a *Nco*I restriction site at the N-terminus. It also included and a 3′-nucleotide extension with the stop codon (TAA) and the *Xba*I restriction site. All synthetic genes were supplied by GeneArt® (Life Technologies, Paisley, UK).

Molecular cloning techniques were performed according to Sambrook et al[61] and all DNA restriction enzymes were supplied from New England BioLabs (Beverly, MA, USA) and used as recommended by the supplier. Ligations were performed with the T4 DNA ligase (New England Biolabs). *E. coli* JM109 competent cells were transformed as described by the supplier, and electrocompetent *L. lactis* cells were transformed with a Gene Pulser XCell™ (Bio-Rad Laboratories, Hercules, CA, USA) as described previously[32].

Construction of expression vectors

The plasmids and synthetic genes used in this study are listed in Table 4. The SP_{usp45}:Alyteserin and SP_{usp45}:A3APO containing *Nco*I-*Xba*I fragments were obtained from the digestion of the Geneart vectors pMK-RQ-Alys and pMK-RQ-A3APO, respectively. These fragments were inserted into plasmid pMSP3545, in frame with the strong inducible Nisin A (PnisA) promoter, obtaining plasmids pMS-Alys and pMS-A3APO, respectively.

Protein production

Recombinant *L. lactis* were induced to produce both AMPs upon reaching an OD_{600} of 0.5, using nisin A (Sigma) at a final concentration of 25 ng/ml as the inducer. Cell-free culture supernatants were obtained by centrifugation of cultures at 12,000 × *g* at 4°C for 10 min and filtering through 0.2 μm pore-size filters (Whatman Int. Ltd., Maidstone, UK.). Supernatants were stored at −20°C until use.

Peptide transcript quantification by qPCR

A3APO and Alyteserin production was induced as described above. 3 hrs post-induction mRNA was isolated using the RNeasy kit and RNAprotect Bacteria Reagent (Qiagen). cDNA libraries were made from each RNA sample using SuperScript II reverse transcriptase (Life Technologies) as directed and qPCR was performed using Brilliant III Ultra-Fast SYBR Green QPCR Master Mix (Agilent Technologies) as directed using the internal ROX dye as a reference. Primers Alys-qPCR-F (CGTTGTCAGGTGTTTACGCTGGTTT) and Alys-qPCR-R (CGTTTAATTAGCAACATGAGCAGCAA) were designed to amplify a 95 bp product of Alyteserin gene. Primers A3APO-qPCR-F

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(TTTTAATGTCTACAGTGATACTTTCTGCTGC) and A3APO-qPCR-R (ATACGTTTAACGAACTGGACGTGGTG) were designed to amplify a 122 bp product of A3APO gene. Primers Tuf-qPCR-F (GCGTTCTGGAGTTGGGATGT) and Tuf-qPCR-R (CCTCTTGAGCGAATACGATT) were designed to amplify a 149 bp product of the elongation factor Tu gene (*tuf*), which was used as an internal control. Relative transcript increases upon induction were calculated for both AMPs from CT values.

Production and immunodetection of His-tagged proteins

To confirm the production of recombinant Alyteserin and A3APO by *L. lactis* using immulogical technics, a 6xHis-tag sequence was fused to the C-terminus of the cloned genes. Primers SPUsp45-F (ACTCATCATGAAAAAAAAGATTATCTCAGC) and A3APO-HIS-R (GATCTAGATTAGTGATGGTGATGGTGATGACCACCACGAACTGGACGTGGTGG) were used in a PCR reaction to amplify a *BspHI*/*Xba*I 180 bp fragment containing SP_{usp45}:A3APO fused to a Cterminal 6xHis-tag (fragment SP_{usp45}:A3APO:HIS). Primers SPUsp45-F and Alys-HIS-R (GATCTAGATTAGTGATGGTGATGGTGATGACCACCATTAGCAACATGAGCAGC) were used in a PCR reaction to amplify a *BspHI*/*Xba*I 192 bp fragment containing SP_{usp45}:Alys fused to a C-terminal 6xHis-tag (fragment SP_{usp45}:Alys:HIS). Fragments SP_{usp45}:A3APO:HIS and SP_{usp45}:Alys:HIS were digested with the indicated restriction enzymes and inserted into pMSP3545, digested with *Nco*I and *Xba*I. The ligation mixtures were used to transform *L. lactis* IL1403 competent cells. The plasmid derivatives pMS-Alys:HIS and pMS-A3APO:HIS, respectively, were checked by PCR and sequencing of the inserts.

L. lactis IL1403 (pMSP3545), *L. lactis* IL1403 (pMS-A3APO:HIS) and *L. lactis* IL1403 (pMS-Alys: HIS) strains were grown in 100 ml of GM17 medium and induced with nisin A at an OD_{600} of 0.5 as previously described. 3 hours after induction the cultures were centrifuged at 12,000 × g at 4°C for 15 min. 50 ml of the supernatants (SN) were stored at −20 °C until use, while the remaining 50 ml were subjected to precipitation with ammonium sulphate (50%) and resuspended in 1 ml phosphate-buffered saline (PBS) (AS-SN). The cell-pellets where washed with PBS and resuspended in 2 ml of ice-cold PBS. Cells were lysed in a Fast-prep apparatus (Biospec) using 0.1 mm glass beads and 6 cycles of 45 s (speed 6.0), with cooling intervals of 45 s on ice. The unbroken cells, cell debris and glass beads were separated from the cell lysate (CL) by centrifugation at 16,100 × g at 4°C for 30 min. 20 µl of SN, AS-SN and CL were spotted into a Amersham Hybond-P PVDF membrane (GE Healthcare) as indicated by the manufacturer. After transfer of the proteins onto the membranes, a dot blots analysis was performed using the Chemiluminescent Western Breeze kit (Invitrogen, Carlsbad, CA, USA). For detection of Alys:His and A3APO:His, an anti-His (C-term) mouse monoclonal antibody (Invitrogen) was used as recommended by the manufacturer.

Bioassays for antimicrobial activity

MICs of the synthetic AMPs were determined in triplicate by a liquid growth inhibition microdilution assays in flat-bottom sterile polypropylene 96-well plates (Maxisorp, Nunc,

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Roskilde, Denmark), in a final volume of 150 µl. The bacteria were diluted 2% in fresh media and grown to an OD_{600} = 0.5 ± 0.05 (OD_{600} = 1 ≈ 10^9 cells/ml). The cells were diluted 50-fold to 10^7 cells/ml in fresh media. The AMP stocks were serially diluted (150 µl/well) in liquid growth media in a 96-well plate, covering a concentration range of 1,000 µg/ml – 1 ng/ml. Briefly, 150 µl of the serially diluted AMPs were inoculated with 5 µl of the bacterial strains to achieve a final indicator concentration of 3×10^5 cells/ml. For each strain a row with no peptide was included as growth control, and for each test a row of medium-only wells was included as a sterility control. Plates were then incubated at 37°C for 16–20 h without shaking, and growth inhibition was assessed measuring OD_{600} using a microplate reader (SpectraMax® Plus384; Molecular Devices, Sunnyvale, CA, USA). MICs were identified as the lowest antimicrobial concentration where the OD_{600} just exceed that of the control.

The loss of cell viability was monitored to determine the antimicrobial activity of the supernatants from the recombinant *L. lactis* strains. Briefly, 0.3 ml of fresh medium, individually inoculated with the target strains, was added to tubes containing 0.7 ml of the supernatants, to reach a final concentration of 1×10^3 cells/ml. As a control, a supernatant sample from the *L. lactis* strain containing only the expression vector pMSP3545 (C-SN) was used. Tubes were incubated at 37°C with agitation. Samples were taken at 30 min, 2 and 6 h, and the number of colony forming units per ml (CFU/ml) was determined by plating 25 µl on LB agar plates. The plates were incubated for 16 h at 37°C, and the number of viable cells was assessed by counting CFUs. Additionally, the OD_{600} was monitored up to 15 h post-inoculation to follow the influence of AMPs on culture growth.

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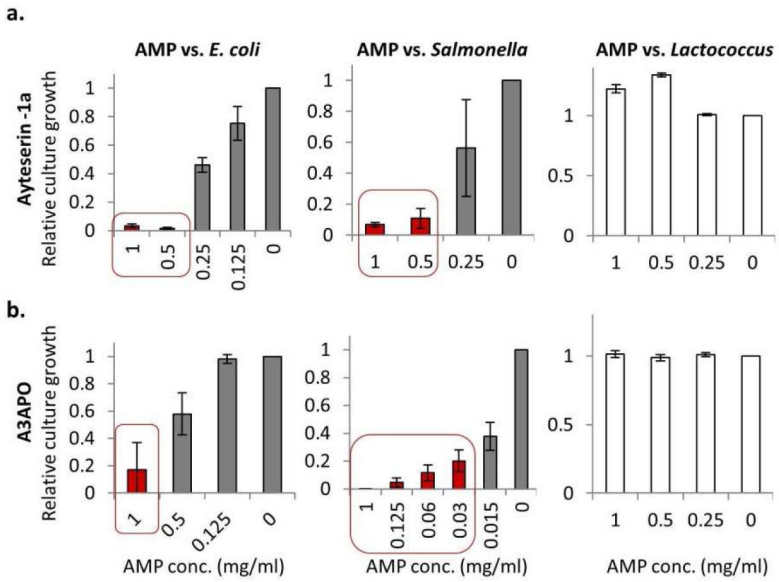

Figure 1. Synthetic AMP screen against *E. coli, Salmonella* and *L. lactis*

(a) Alyteserin and **(b)** A3APO were diluted and applied to *E. coli*, *Salmonella* and *L. lactis* 1403. Inhibitory concentrations for *E. coli* and *Salmonella* are emphasized in red, the smallest of which is the MIC observed for each Gram-negative pathogen and AMP combination. *L. lactis* growth inhibition was not achieved by any AMP concentration tested.

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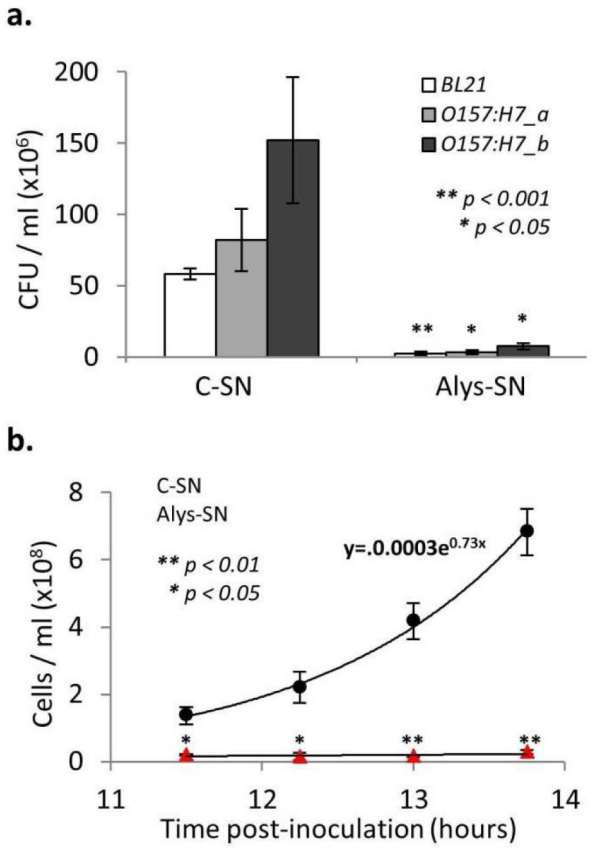

Figure 2. *E. coli* growth inhibition by Alyteserin

(a) Pathogenic (grey bars) and non-pathogenic (white bars) *E. coli* are inhibited by Alyteserin. Cultures grown in the presence of Alyteserin achieved a density 20-fold lower than the cultures grown in control supernatant (C-SN). **(b)** Pathogenic and non-pathogenic *E. coli* growth was inhibited by ≈100% when cultured in Alyteserin supernatant (Alys-SN) (red triangles) relative to the control supernatant (black dots) through 15 h post-inoculation.

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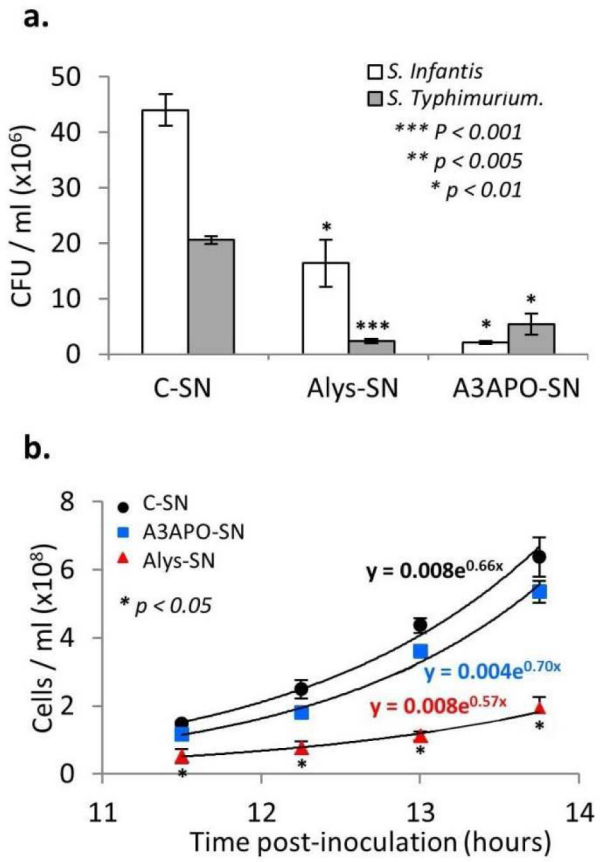

Figure 3. *Salmonella* growth inhibition by Alyteserin and A3APO

(a) *Salmonella infantis* (grey bars) and *typhimurium* (white bars) are both inhibited by Alyteserin and A3APO. *S. infantis* culture density, in the presence of Alyteserin, was reduced by about one-half and *S. typhimurium* was reduced by 10-fold. *S. infantis* cultures grown in the presence of A3APO achieved a density 20-fold less than the cultures in the control supernatant while *S. typhimurium* culture density was reduced by 4-fold. **(b)** *Salmonella* growth is inhibited by 15% when cultured in the presence of Alyteserin (red triangles) relative to the control supernatant (black dots) through 15 h post-inoculation. Growth inhibition by A3APO is not observed at these longer times however (blue squares).

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Table 1

Growth rates and relative culture growth of *E. coli* and *Salmonella* with AMP treatment

	E. coli	***Salmonella***
	Growth rate (hr^{-1})	
C-SN	0.73	0.66
Alys-SN	≈0	0.57
A3APO-SN	>0.8	0.7
	Relative Growth rate (%)	
C-SN	100	100
Alys-SN	≈0	86
A3APO-SN	>100	>100

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Table 2

Synthetic peptides used in this study

Synthetic peptides	Description
A3APO (single chain)[a]	RPDKPRPYLPRPRPPRPVR
Alyteserin -1a[b]	GLKDIFKAGLGSLVKGIAAHVAN

[a]42

[b]43

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Table 3

Strains used in this study

Strains	Description
E. coli JM109[a]	Selection of recombinant plasmids
L. lactis IL1403[b]	Plasmid-free strain, non bacteriocin producer
Indicator organisms	
E. coli BL21[c]	AMP indicator
E. coli 0157-H7	
2026[d]	AMP indicator
2031[d]	AMP indicator
S. infantis 129B[d]	AMP indicator
S. typhimurium 2219B[d]	AMP indicator

[a] 62

[b] 63

[c] 64

[d] UMN Collection

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Table 4

Plasmids and synthetic genes used in this study

Plasmids	Description	Reference
pMK-RQ-A3APO	Kan^r, pMK-RQ plasmid carrying SP$_{usp45}$:A3APO	Geneart
pMK-RQ-Alys	Kan^r, pMK-RQ plasmid carrying SP$_{usp45}$:Alys	Geneart
pMSP3545	Em^r; expression vector	[29]
pMS-A3APO	Em^r; pMSP3545 derivative carrying SP$_{usp45}$:A3APO	This work
pMS-Alys	Em^r; pMSP3545 derivative carrying SP$_{usp45}$:Alys	This work
pMS-A3APO:HIS	Em^r; pMSP3545 derivative carrying SP$_{usp45}$:A3APO:HIS	This work
pMS-Alys:HIS	Em^r; pMSP3545 derivative carrying SP$_{usp45}$:Alys:HIS	This work

Synthetic genes	Nucleotide sequence
SP$_{usp45}$:A3APO	**ATGGATGAAAAAAAAGATTATCTCAGCTATTTTAATGTCTACAGTGATACTTTCTGCTGCAGCCCCGTTGTCAGGTGTTTACGCT**CGTCCAGATAAACCACGTCCATATTTACCACGTCCACGTCCACCACGTCCAGTTCGT
SP$_{usp45}$:Alys	**ATGAAAAAAAAGATTATCTCAGCTATTTTAATGTCTACAGTGATACTTTCTGCTGCAGCCCCGTTGTCAGGTGTTTACGCT**GGTTTAAAAGATATTTTTAAAGCTGGTTTAGGTTCATTAGTTAAAGGTATTGCTGCTCATGTTGCTAAT