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

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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 PnisA 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

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 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 subtherapeutic 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 cultures 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

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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 \textit{Lactobacillus} and \textit{Lactococcus}, and \textit{Bifidobacterium} are currently the bacteria most commonly employed as probiotics.\(^{2,4,6}\) 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 in 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 deliver proteins inside the GI tracts of hosts.

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 \textit{Lactococcus lactis}, a noninvasive and nonpathogenic LAB, generally recognized as safe by the FDA, with probiotic properties that are amenable to heterologous protein overexpression.\(^{27,28}\) Since \textit{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 \textit{L. lactis},\(^{29−32}\) making it suitable to engineer as an efficient cell-based protein expression factory.\(^{33−35}\) On the basis of these attributes, \textit{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 \textit{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 \textit{Escherichia coli} and \textit{Salmonella} strains. Our systems have been engineered with the intention of future application as an alternative to antibiotics in agriculture.

\section*{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 \textit{E. coli} and \textit{Salmonella} strains and low activity against LAB. Gene-encoding peptides that displayed the most favorable activity were then included in expression cassettes for use in \textit{L. lactis}. In the following, we detail how we (1) selected the AMPs of interest, (2) engineered \textit{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.

\textbf{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 \textit{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 \textit{E. coli} and \textit{Salmonella} and against \textit{L. lactis}.

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 \textit{E. coli} and \textit{Salmonella} strains. Additionally, \textit{L. lactis} remained viable despite treatment with up to 1 mg/mL Alyteserin (Figure 1a).

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{figure1.png}
\caption{Synthetic AMP screen against \textit{E. coli}, \textit{Salmonella}, and \textit{L. lactis}. (a) Alyteserin and (b) A3APO were diluted and applied to \textit{E. coli}, \textit{Salmonella}, and \textit{L. lactis} 1403. Inhibitory concentrations for \textit{E. coli} and \textit{Salmonella} are emphasized in red, the smallest of which is the MIC observed for each Gram-negative pathogen and AMP combination. \textit{L. lactis} growth inhibition was not achieved by any AMP concentration tested.}
\end{figure}

Inhibition of \textit{E. coli} by A3APO was observed only at a concentration of 1 mg/mL. However, \textit{Salmonella} growth was inhibited by 30 μg/mL Alyteserin, reducing viability through the highest concentration tested (1 mg/mL). Similarly, \textit{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 produced at high concentrations but had low specific activity.\(^{44}\) This has been attributed to a variety of causes, such as nonefficient disulfide bond formation, problems in protein folding, and protein aggregation.\(^{45}\)

Once the two candidate peptides were identified, \textit{L. lactis} was engineered to express Alyteserin and A3APO. Details of this process are presented in Methods. The resulting \textit{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 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 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). The expression cassettes were cloned downstream of the nisin inducible promoter, PnisA, from plasmid pMSP3545, 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 (SN) (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 nonpathogenic *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 h.

**Peptide Production and Secretion.** qPCR was performed to determine the transcript levels of Alyteserin and A3APO genes upon induction. A3APO transcript increased by over 100-fold upon induction, while Alyteserin mRNA formation to determine the transcript levels of Alyteserin and A3APO genes upon induction. A3APO transcript increased over 100-fold upon induction, while Alyteserin mRNA 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 h.

**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 postinoculation, and culture density was monitored by OD_{600} 10–15 h postinoculation. 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 postinoculation calculated based on culture densities 10–15 h postinoculation. 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.

**Salmonella Growth Inhibition by Alyteserin-1a and A3APO Produced by *L. lactis*.** Alyteserin-1a and A3APO-SNs were assayed against *Salmonella* strains as described in Methods. As shown in Figure 2, Alys-SN inhibited growth of *Salmonella* strains, while only nonpathogenic *E. coli* were tested. Growth rates for *E. coli*, as shown in Table 1, were

Table 1. Growth Rates and Relative Culture Growth of *E. coli* and *Salmonella* with AMP Treatment

<table>
<thead>
<tr>
<th></th>
<th><em>E. coli</em></th>
<th><em>Salmonella</em></th>
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<tbody>
<tr>
<td>C-SN</td>
<td>0.73</td>
<td>0.66</td>
</tr>
<tr>
<td>Alys-SN</td>
<td>0</td>
<td>0.57</td>
</tr>
<tr>
<td>A3APO-SN</td>
<td>&gt;0.8</td>
<td>0.7</td>
</tr>
</tbody>
</table>

*Salmonella* growth was inhibited by ≈100% when cultured in Alyteserin supernatant (Alys-SN) (red ▲) relative to the control supernatant (black ●) through 15 h postinoculation. (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 postinoculation 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...
Inhibited growth of pathogenic *S. typhimurium* and *S. infantis*, as shown in Figure 3a. As before, the culture titer was assessed beginning 30 min postinoculation and culture density was monitored by OD$_{600}$ from 10 to 15 h. By 2 h postinoculation, a differential titer between cultures treated with Alys-SN and A3APO-SN was 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 postinoculation (Figure 3b). At 15 h postinoculation, 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 postinoculation. 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 at 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 SF$_{wsp45}$ 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. 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. 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, screening for a more efficient secretion peptide and protein combinations for overproduction and secretion may still further improve active peptide secretion. Several strategies have been used in this direction. Using different signal peptides, modifying the amino acids of the N-terminus of the signal peptide or adding a propeptide between the signal peptide and the mature protein may help increase peptide secretion. 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.

### 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*. However, these peptides have frequently displayed antimicrobial activity against Gram-positive bacteria but either no or poor activity against Gram-negative indicators. 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. 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. 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). PE changes the electrostatic and the mechanical properties of lipid bilayers. An 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 gastrointestinal 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.\textsuperscript{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, U.K.) 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 Company, St. Louis, MO) was added to the cultures at 200 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\textsubscript{usp45}) (Table 4) and a 5′-nucleotide extension containing a NcoI restriction site at the N-terminus. It also included a 3′-nucleotide extension with the stop codon (TAA) and an XhoI restriction site. All synthetic genes were supplied by GeneArt (Life Technologies, Paisley, U.K.).

Molecular cloning techniques were performed according to Sambrook et al.\textsuperscript{61} and all DNA restriction enzymes were supplied from New England BioLabs (Beverly, MA) and used cultured at 30 °C in M17 broth (Oxoid Ltd., Basingstoke, U.K.) 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 Company, St. Louis, MO) was added to the cultures at 200 and 5 μg/mL, for *E. coli* and *L. lactis*, respectively.

**Table 2. Synthetic Peptides Used in This Study**

<table>
<thead>
<tr>
<th>synthetic peptides</th>
<th>description</th>
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<tbody>
<tr>
<td>A3APO (single chain)\textsuperscript{a}</td>
<td>RPDKPRPYLPRRPFPVR</td>
</tr>
<tr>
<td>Alyteserin-1a\textsuperscript{b}</td>
<td>GLDIFKAGLGSLVKGIAAHVAN</td>
</tr>
</tbody>
</table>

\textsuperscript{a}Ref 42. \textsuperscript{b}Ref 43.

**Table 3. Strains Used in This Study**

<table>
<thead>
<tr>
<th>strains</th>
<th>description</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>E. coli</em> JM109\textsuperscript{a}</td>
<td>selection of recombinant plasmids</td>
</tr>
<tr>
<td><em>L. lactis</em> IL1403\textsuperscript{b}</td>
<td>plasmid-free strain, non-bacteriocin producer</td>
</tr>
</tbody>
</table>

**Table 4. Plasmids and Synthetic Genes Used in This Study**

<table>
<thead>
<tr>
<th>plasmids</th>
<th>description</th>
<th>reference</th>
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</thead>
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<tr>
<td>pMK-RQ-A3APO</td>
<td>Kan r, pMK-RQ plasmid carrying SP\textsubscript{usp45}:A3APO</td>
<td>GeneArt</td>
</tr>
<tr>
<td>pMK-RQ-Alys</td>
<td>Kan r, pMK-RQ plasmid carrying SP\textsubscript{usp45}:Alys</td>
<td>GeneArt</td>
</tr>
<tr>
<td>pMSP3545</td>
<td>Em r; expression vector</td>
<td>29</td>
</tr>
<tr>
<td>pMS-A3APO</td>
<td>Em r; pMSP3545 derivative carrying SP\textsubscript{usp45}:A3APO</td>
<td>this work</td>
</tr>
<tr>
<td>pMS-Alys</td>
<td>Em r; pMSP3545 derivative carrying SP\textsubscript{usp45}:Alys</td>
<td>this work</td>
</tr>
<tr>
<td>pMS-A3APO:HIS</td>
<td>Em r; pMSP3545 derivative carrying SP\textsubscript{usp45}:A3APO:HIS</td>
<td>this work</td>
</tr>
<tr>
<td>pMS-Alys:HIS</td>
<td>Em r; pMSP3545 derivative carrying SP\textsubscript{usp45}:Alys:HIS</td>
<td>this work</td>
</tr>
</tbody>
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\textsuperscript{a}Ref 62. \textsuperscript{b}Ref 63. \textsuperscript{c}Ref 64. \textsuperscript{d}UMN Collection.
as recommended by the supplier. Ligation 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), as described previously.22

**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 Ncol-XbaI 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 12000 g at 4 °C for 10 min and filtering through 0.2 μm pore-size filters (Whatman Int. Ltd., Maidstone, U.K.). Supernatants were stored at −20 °C until use.

**Peptide Transcript Quantification by qPCR.** A3APO and Alyteserin production was induced, as described above. Three hours postinduction 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 (CTTTATCAGGTTTACGGTCTGTT) and Alys-qPCR-R (CGTTAATGCAACATGCGACGGAA) were designed to amplify a 95 bp product of Alyteserin gene. Primers A3APO-qPCR-F (TTTTAATGTCTACAGTGATACTTTCTGCTG) and A3APO-qPCR-R (ATACGTTTAACGAACTGGACGTGG-R) were designed to amplify a 122 bp product of A3APO gene. Primers Tuf-qPCR-F (GCATTTCTGAGGATTGATGATCC) and Tuf-qPCR-R (CCTCCTGAGGATACGATT) 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 immunochemical technics, a 6×His-tag sequence was fused to the C-terminus of the cloned genes. Primers SP\(_{up45}\)-F (ACTCTACATGAAAAAAGATTAT-CTCAGC) and A3APO-HIS-R (GATCTAGATTAGTGA-TGGTATGTTGATGACACCACCCACACTGGACGCTGG-TG) were used in a PCR reaction to amplify a *BspH*I/XbaI 180 bp fragment containing SP\(_{up45}\)-A3APO fused to a C-terminal 6×His-tag (fragment SP\(_{up45}\)-A3APO-HIS). Primers SP\(_{up45}\)-F and Alys-HIS-R (GATCTAGATTAGTGGATGGATG-TGATGACACCACCCACACTGGACGCTGG-TG) were used in a PCR reaction to amplify a *BspH*I/XbaI 192 bp fragment containing SP\(_{up45}\)-Alys fused to a C-terminal 6×His-tag (fragment SP\(_{up45}\)-Alys-HIS). Primers SP\(_{up45}\)-F and A3APO-HIS-R (GATCTAGATTAGTGGATGGATG-TGATGACACCACCCACACTGGACGCTGG-TG) were used in a PCR reaction to amplify a *BspH*I/XbaI 192 bp fragment containing SP\(_{up45}\)-A3APO fused to a C-terminal 6×His-tag (fragment SP\(_{up45}\)-A3APO-HIS). Fragments SP\(_{up45}\)-Alys-HIS and SP\(_{up45}\)-A3APO-HIS were digested with the indicated restriction enzymes and inserted into pMSP3545 and digested with Ncol and XbaI. 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. Three hours after induction, the cultures were centrifuged at 12000 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 sulfate (50%) and resuspended in 1 mL phosphate-buffered saline (PBS) (AS-SN). The cell pellets were 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 16100g at 4 °C for 30 min. Twenty microliters of SN, AS-SN, and CL were spotted into an 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). 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, 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 ± 10⁹ cells/mL). The cells were diluted 50-fold to 10⁹ 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 1000 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 × 10⁵ 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\(_{600}\) at 480 nm using a microplate reader (SpectraMax Plus384; Molecular Devices, Sunnyvale, CA). MICs were identified as the lowest antimicrobial concentration, where the OD\(_{600}\) just exceeds 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, initially inoculated with the target strains, was added to tubes containing 0.7 mL of the supernatants, to reach a final concentration of 1 × 10⁷ 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 at 37 °C for 16 h, and the number of viable cells was assessed by counting CFUs. Additionally, the OD\(_{600}\) was monitored up to 15 h postinoculation to follow the influence of AMPs on culture growth.

**AUTHOR INFORMATION**

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