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Random antimicrobial peptide mixtures as non-antibiotic antimicrobial agents for cultured meat industry

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ABSTRACT

Antibiotics, commonly used in cell culture studies to prevent microbial contamination, cannot be employed in Cultured meat (CM) due to potential residues in the final food products. Hence, there is an urgent need to develop novel and safe non-antibiotic antimicrobial agents. Here, we investigated the potential of random antimicrobial peptide mixtures (RPMs) as non-antibiotic antimicrobial agents. RPMs are synthetic peptide cocktails that have previously shown strong and broad antimicrobial activity; however, their use in cell culture media and their effect on mammalian cells have not yet been explored. Here we show that RPMs had no significant impact on mesenchymal stem cells (MSCs) at concentrations that effectively inhibit bacterial growth. RPMs displayed strong bactericidal activity against Gram-positive bacteria, achieving a 6-log reduction of *L. monocytogenes* in cell culture medium without any cytotoxicity. Additionally, RPMs showed a low occurrence of resistance development with no significant resistance developed in compared with a combination of penicillin and streptomycin. Moreover, LK20 mixture was rapidly digested ~~and a rapid digestion~~ in a simulated digestion model. Our results indicate that RPMs have great potential to serve as safe and effective non antibiotic antimicrobial agents in cultured meat industry.

1. Introduction

Cultured Meat, also known as cell-based meat or lab-grown meat, is part of the growing field of cellular agriculture. It involves using cell-based biotechnology to grow animal tissues *in vitro* for human consumption. CM production begins with extracting tissue from an animal donor and isolating the desired cells from it. Stem cells are often considered an effective cell isolate for cultured meat production due to their self-renewal and differentiation capabilities (Choi et al., 2021; Kadim et al., 2015; Reiss et al., 2021). Mesenchymal stem cells may be especially effective choice due to their differentiation capabilities to both muscle and fat tissues (Klatt et al., 2024; Lee et al., 2023). After isolation, the cells are transferred into a suitable growth medium that contains nutrients, energy sources, growth factors, and other components necessary for their growth and differentiation into mature selected tissue (O'Neill et al., 2020). After proliferation and differentiation, the cells are harvested and processed into the final edible product using various tissue engineering (Ng & Kurisawa, 2021) or 3D printing methods (Handral et al., 2022). These methods involve shaping and organizing the cultured cells into structures that resemble traditional meat products, such as steaks, burgers, or nuggets.

Compared with traditional meat production, CM offers several advantages, mainly in environmental and health aspects. CM has the potential to mitigate greenhouse gas emissions, as it generates lower carbon emissions compared with traditional livestock farming (Tuomisto & de Mattos, 2011). Reducing reliance on animal agriculture and feed production will dramatically reduce the use of arable land and water, and minimize deforestation, habitat destruction, and water pollution associated with conventional meat production. The shift from conventional meat production will contribute significantly to

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sustainable practices and preserve natural resources for future generations.

CM also has the potential to promote public health. Animal farming plays a significant role in the spread of infectious diseases, both directly through farming practices and indirectly through activities like deforestation. Approximately 75 % of infectious diseases are zoonotic, which can be transmitted from animals to humans. These zoonoses result in around one billion cases of illness and millions of deaths annually, and they have been implicated in major pandemics such as COVID-19 and the Ebola virus (Espinosa et al., 2020; Heredia & García, 2018).

The intensive use of antibiotics in the traditional meat industry is also a significant concern. This widespread use of antibiotics poses a major risk to human health and contributes to the emergence of antibiotic-resistant strains of bacteria, which is a significant public health crisis (Silbergeld et al., 2008; Van Boeckel et al., 2019). CM, produced in a controlled sterile environment, appears to offer a promising solution to minimize antibiotic usage drastically (Srutee et al., 2022). However, like any other cell culture, CM is susceptible to microbial contamination, leading to the common addition of antibiotics to the culture medium (Nims & Price, 2017).

Adding antibiotics to the culture medium raises several concerns for public health, including further antibiotic resistance development and antibiotic residues in the final product; that can either directly harm consumers' health due to antibiotic toxicity or indirectly disrupt the microbiome (Broucke et al., 2023; Chen et al., 2019a, 2019b). Antibiotics can also impair cell culture quality by affecting cell viability, proliferation, gene expression, and stem cell differentiation (McNamara & Bomkamp, 2022; O'Neill et al., 2021) and is a major setback for consumer acceptance of CM (Bryant et al., 2020; Szejda et al., 2021). In order to avoid antibiotic usage in CM production with current technologies, it would have to be produced in the most sterile environment possible and ensure frequent testing and monitoring of infections (Ong et al., 2021), restricting the process's scalability.

Currently, cultured meat companies do not use antibiotics, but as consumption increases and the need for higher productivity rises, maintaining completely sterile conditions will become more challenging. Consequently, any contamination could result in significant financial losses. We anticipate that similar to the traditional meat industry, where rising production led to increased density in farms and subsequently more antibiotic use, the same trend will eventually occur in cultured meat production. Therefore, developing robust and effective strategies for preventing microbial contamination without antibiotics is a crucial step in the development of this field.

In recent years antimicrobial peptides (AMPs) have been proposed and explored as an alternative agent to traditional antibiotics (Boparai & Sharma, 2019; Ciumac et al., 2019; Fry, 2018). AMPs serve as part of the innate immune response to bacterial infection in various organisms (Kumar et al., 2018) and present a promising alternative as food preservatives (Rai et al., 2016). They are effective against Gram-positive and Gram-negative bacteria and their mode of action mainly involves targeting the bacterial membrane. AMPs tend to bind to the prokaryotic membrane rather than the eukaryotic membrane due to cationic and hydrophobic residues commonly found in AMPs. Their overall net positive charge attracts them to the negatively charged prokaryotic membrane, and the hydrophobic residue interacts with the lipid bilayer of the membrane and disrupts it (Matsuzaki et al., 1995).

Although AMPs are promising antimicrobials, their production demands a long and expansive purification process, which increases their cost production. Our group has developed a novel and effective method to synthesize a mixture of AMPs that is easy and cheap to produce and does not require any purification process termed Random Peptide Mixtures (RPMs). RPMs are cocktails of synthetic peptides consisting of one type of hydrophobic amino acid and one type of cationic amino acid in a random sequence (Amso & Hayouka, 2019; Hayouka et al., 2013). In each coupling step, a mixture of the two amino acids is introduced, resulting in different amino acid addition to each peptide chain. The result product is a mixture of random sequences, each peptide in the mixture has the same chain length, determined by the number of coupling steps, while each mixture has 2^n different sequences. RPMs have shown strong and broad antimicrobial activity compared with natural AMPs, and due to the diverse number of peptide sequences within the mixture, resistance development towards them might be more challenging to occur (Maron et al., 2022). Two types of RPMs have been developed: 20-mer RPMs and 5-mer lipo-RPMs resulting from N′-palmitoylation of the N′ terminus of the short RPMs (Topman-Rakover et al., 2020). Both are easy and cheap to synthesize and have shown strong and broad antibacterial activity. They are non-toxic, safe, highly active *in vitro* and in mouse models (Bennett et al., 2021; Lau et al., 2023) so they are potentially suitable for human consumption as well.

All these characteristics make them promising non-antibiotic antimicrobial agents to be used in the food industry. Previous studies have demonstrated the ability of RPMs to inhibit bacterial growth in both bovine milk (Stern Bauer & Hayouka, 2018) and turkey minced meat (Palman et al., 2020). Our objective in the current study is to incorporate RPMs into CM growth media and explore their potential as antimicrobial agents in CM production by investigating their antimicrobial activity in cell culture media and their effect on bovine mesenchymal stem cells (MSCs) as a model for CM. In addition, we will explore the digestibility of RPMs and the bacterial resistance occurrence towards them compared to traditional antibiotics.

2. Results

2.1. Lead RPMs synthesis

We have synthesized the most potent RPMs that were developed so far, FK20 (20-mer RPMs composed from Phenylalaninene:Lysine in 1:1 M ratio), LK20 (20-mer RPMs composed of Leucine:Lysine 1:1 M ratio), p-FK5 (5-mer Lipo-RPMs composed of Phenylalanine:Lysine 1:1 M ratio) and p-LK5 (5-mer Lipo-RPMs composed of Leucine:Lysine 1:1 M ratio) both conjugated to palmitic acid at their free amino group as described at (Amso & Hayouka, 2019; Hayouka et al., 2013; Topman-Rakover et al., 2020).

2.2. RPMs antimicrobial activity in cell culture media

The antimicrobial potential of RPMs in cell culture media (DMEM supplemented with 10 % inactivated FBS and 2 mM L-Gln) was evaluated for the first time against two bacterial models: *E. coli* NR (Gram Negative model) and *L. monocytogenes* (Gram Positive model), through a minimum inhibitory concentration (MIC) assay and bacterial killing assay (Fig. 1) to characterize their bactericidal activity as well. Our findings showed that all RPMs preserved their antimicrobial activity in DMEM media, with varying efficacy against the two model bacteria. Notably, RPMs displayed greater activity against *L. monocytogenes*, a Gram-positive bacterium (Fig. 1A), compared with *E. coli*, a Gram-negative bacterium (Fig. 1B) under the current condition. The 20-mer RPMs demonstrated higher antimicrobial potency than the 5-mer lipo-RPMs against both *L. monocytogenes* and *E. coli*, with MIC values estimated to be 4–8 times lower than those of the 5-mer lipo-RPMs under these conditions. Among the tested RPMs, LK20 exhibited the highest effectiveness, inhibiting *E. coli* growth at a concentration of 25 μg/mL and *L. monocytogenes* growth at 3.125 μg/mL.

Furthermore, all RPMs demonstrated potent bactericidal activity in DMEM media (Fig. 1C, D) against *L. monocytogenes*, with effective concentrations corresponding to the MIC values. However, against *E. coli*, the activity of all peptides and especially of the lipo-RPMs was notably reduced, suggesting a more potent bacteriostatic mode of action against gram-negative bacteria in DMEM. Notably, the bactericidal activity was sustained even after three cycles of inoculations (Supplementary Fig. S1), demonstrating RPMs' endurance and potential to treat multiple infections.

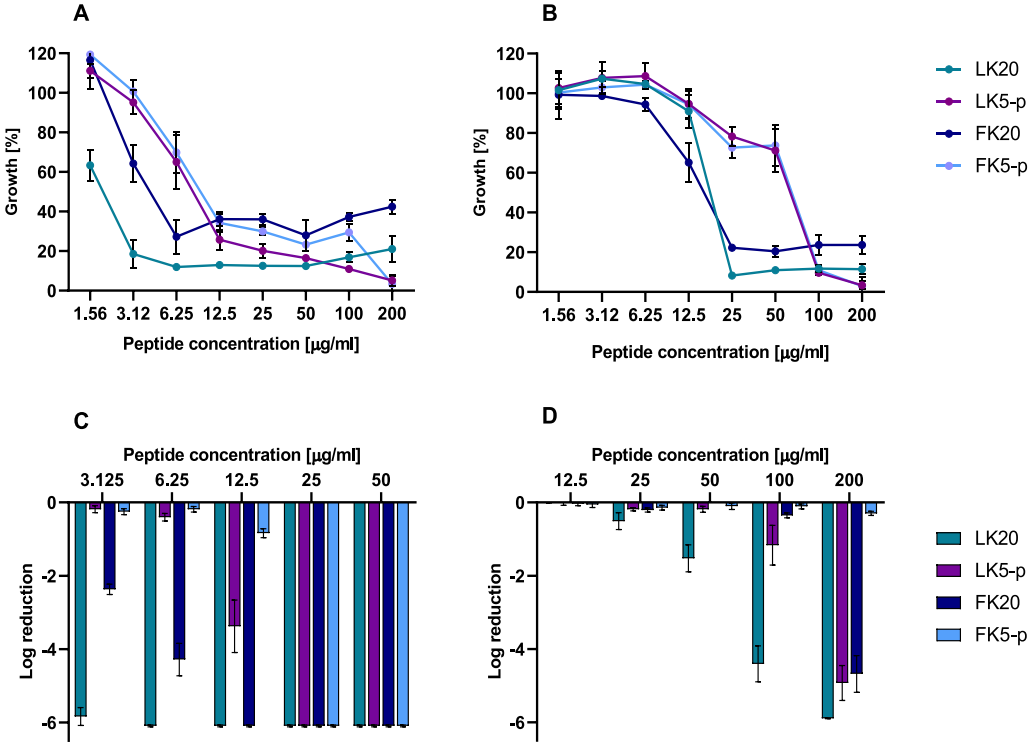

Fig. 1. Antimicrobial activity of RPMs in tissue culture media. MIC values of various RPMs were examined against **A)** *L. monocytogenes* and **B)** *E. coli* NR. 10^7 and 10^6 CFU/mL of bacteria (respectively) were incubated with RPMs in DMEM for 24 h at 37 °C. Bacterial growth values were measured (595 nm) in a plate reader (Tecan) and normalized against the growth of untreated bacteria. The results summarize three independent triplicates ±SEM. A bacterial killing assay was conducted against **C)** *L. monocytogenes* and **D)** *E. coli* NR with an initial bacterial load of 10^6 CFU/mL. The bacteria were incubated with RPMs in DMEM for 1 h at 37 °C, then serially diluted in PBS and plated on LB-agar plates. The results summarize CFU count of three independent triplicates ±SEM.

2.3. RPMs safety towards bovine mesenchymal stem cell culture

To evaluate the impact of the lead RPMs on the viability of MSCs, two primary cell lines were exposed to varying RPM concentrations for 24 h. Subsequently, a cell viability assay was conducted using the Resazurin method, which measures mitochondrial activity (Braissant et al., 2020). Our findings showed variability in the tolerance of MSCs to RPMs. The first cell line (umbilical cord-derived bovine MSCs) (Shimoni et al., 2020) demonstrated higher endurance against RPMs, tolerating concentrations lower than 100 μg/mL, with no significant changes between treatments (Fig. 2A). The second cell line (Wharton jelly-derived bovine MSCs) was more sensitive and exhibited different tolerance levels to different RPMs (Fig. 2B). Specifically, the Leucine-lysine RPMs (LK20 at 50 μg/mL and p-LK5 at 25 μg/mL) were less cytotoxic than the phenylalanine-lysine RPMs. Beyond these tolerable concentrations, cell viability declined in a concentration-dependent manner.

The most active and less cytotoxic RPM of each RPM group, were chosen for a comprehensive further investigation to assess their impact on the cells' viability and proliferation rate (Fig. 3).

The maximum concentration tolerated in the 24-h experiment was not endured in long-term culture; however, at concentrations below 25 μg/mL, both LK20 (Fig. 3A, B) and p-LK5 (Fig. 3C, D) did not significantly alter the cells' doubling time, which remained consistent at approximately 1.5 days per doubling. Furthermore, the percentage of

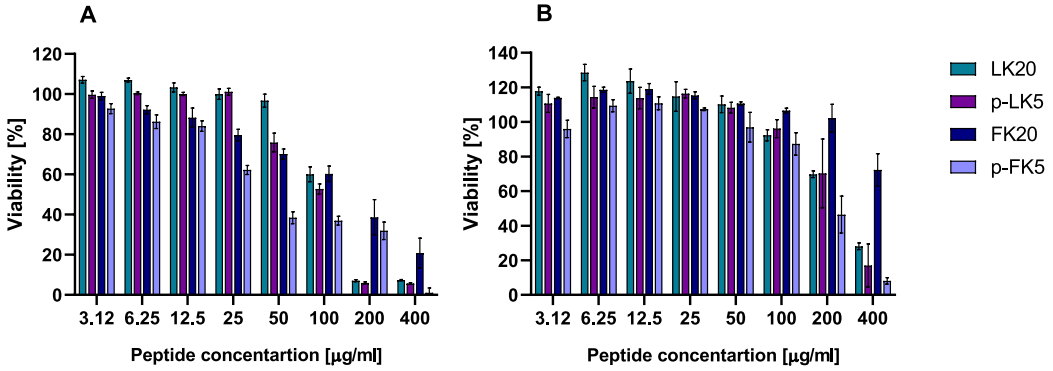

Fig. 2. Resazurin viability assay of MSCs after 24 h of incubation with RPMs. A) umbilical cord derived and **B)** Wharton jelly derived MSCs were incubated with RPMs for 24 h at 37 °C 5 % CO_2. Cells viability values were calculated by fluorescent intensity (560 nm/590 nm) measured in a plate reader (Tecan) and normalized to untreated cells. The results summarize three independent triplicates±SEM.

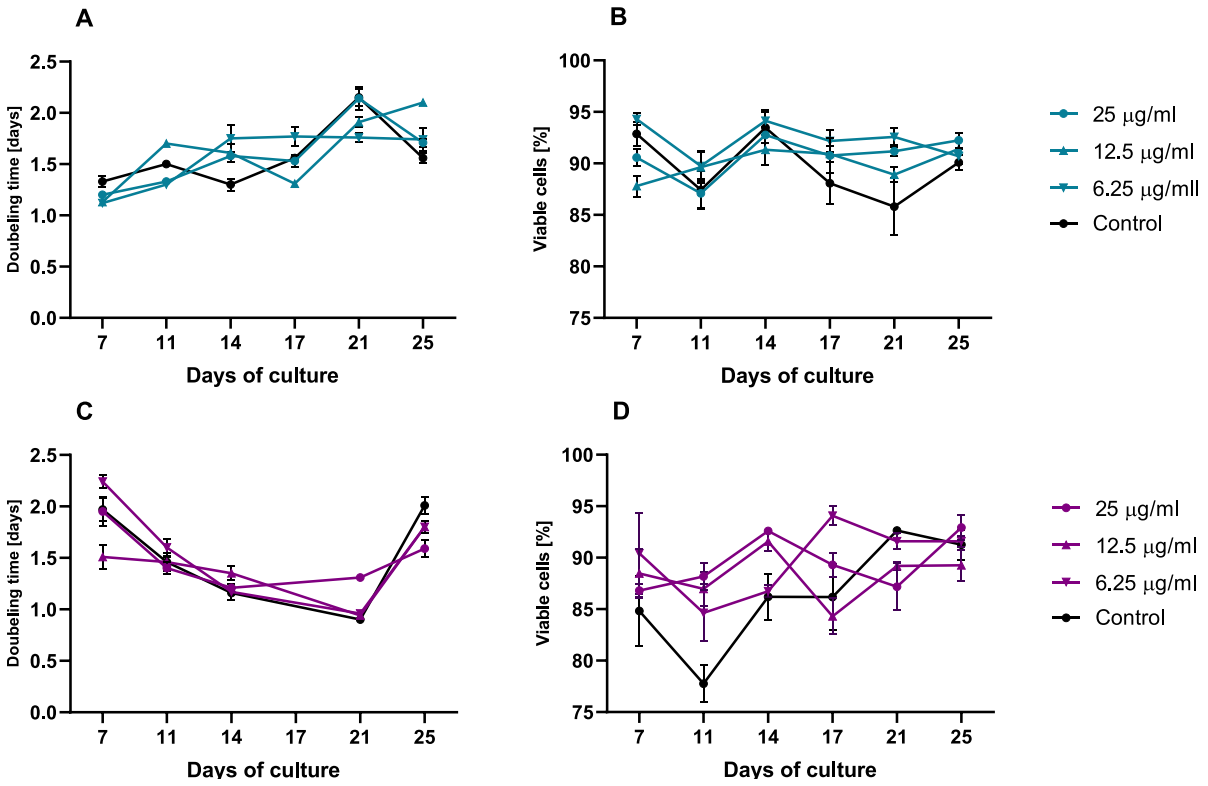

Fig. 3. Long-term effect of RPMs on MSCs doubling time and viability. MSCs were grown in a 6-well plate and passaged every 3–4 days. In each passage, cells died in Trypan Blue 0.4 % and viable cells were counted using an automated cell counter. **A)** Doubling time of MSCs grown with LK20. **B)** Viable cells grown with LK20. **C)** Doubling time of MSCs grown with p-LK5. **D)** Viable cells grown with p-LK5. Doubling time was calculated as $\times \frac{ln(2)}{ln\left(\frac{cells\ seeded}{cells\ counted}\right)}$. The results summarize technical triplicates ±SEM. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

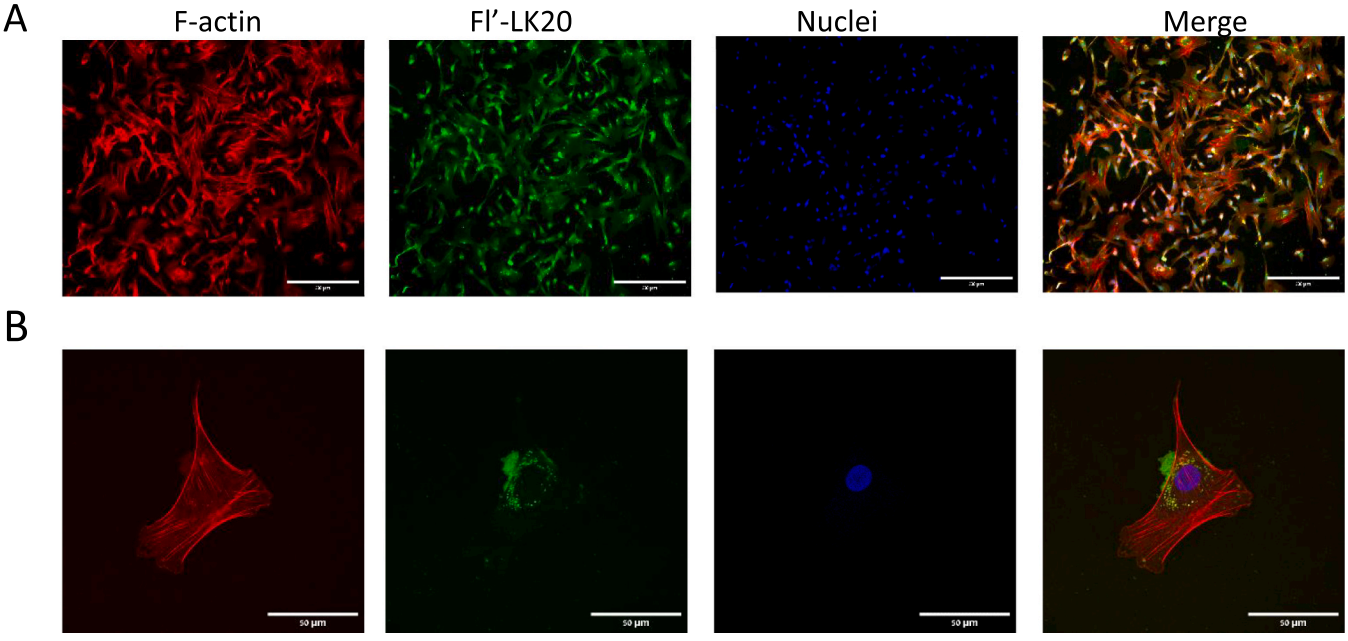

Fig. 4. Fluorescent label RPM penetrates MSCs. Umbilical cord derived MSCs were incubated with fluorescently labeled (6-carboxyfluorescein) LK20 (25 μg/mL) in a 24-well plate. Cells were fixed using 4 % paraformaldehyde and stained with DAPI and Phalloidin. **A)** Fl'-LK20 penetration after 5 of hours incubation. Images were captured using Evos imaging system. **B)** Fl'-LK20 penetration to a single MSC after 24 h of incubation using Leica Stellaris 5 FLIM STED confocal microscope, magnified x10 (A), x40(B).

viable cells in the culture was consistently high, hovering around 90 % throughout the 25-day experiment.

2.4. RPMs penetration to MSCs

Fluorescently labeled RPMs were synthesized to investigate the penetration of RPMs into MSCs. Fl'-LK20 displayed good penetration capabilities with no noticeable changes in cell morphology (Fig. 4); the cells maintained their characteristic shape without showing signs of cellular distress or abnormalities. This observation is particularly encouraging as it suggests that the presence of LK20 did not compromise the cells' structural integrity or overall health under the tested conditions.

2.5. RPMs Immunomodulatory activity

To further investigate the potential impact of RPMs on MSCs, we evaluated their immunomodulation ability. We have conducted a gene expression analysis targeting three key pro-inflammatory cytokines: tumor necrosis factor-α (TNFα), interleukin-1β (IL-1β), and interleukin-6 (IL-6) and one housekeeping gene b-actin (Table. S1). Expression levels were measured using real-time PCR and compared to untreated cells using the ΔΔCT method. First, MSCs were exposed to two RPM concentrations (50 and 25 μg/mL of LK20) for 3 h. No significant changes in cytokine expression or MSC marker CD90 were observed (Supplementary Fig. 2). Consequently, the cells were treated with lipopolysaccharide (LPS) at 0.1 μg/mL for 6 h incubation to induce inflammatory response by cytokine expression.

Our results demonstrated (Fig. 5) that RPMs appeared to prevent the cellular stress induced by LPS exposure. Specifically, p-LK5 was more effective than LK20 in reducing the expression of IL-1β and IL-6 to basal levels, which dramatically increased upon LPS exposure. Polymyxin B was used as a positive control due to its known immunomodulatory activity and resemblance in structure to p-LK5, both showed similar effectiveness. TNFα expression did not increase upon LPS exposure, nor was it affected by RPM treatment.

2.6. RPMs activity in bacteria-infected MSCs culture

To evaluate the impact of MSCs on the efficacy of selected RPMs, both MIC and killing assays were conducted in a co-culture system with the two model bacteria. In each experiment, MSC culture was inoculated with model bacteria, and afterwards exposed to RPMs. We showed that the distinct environment in the co-culture conditions, influenced by MSCs metabolites, had varying effects on the activity of different RPMs (Fig. 6). While the MIC and effective bacterial killing concentration of p-LK5 increased against *L. monocytogenes*, LK20 maintained its strong activity profile. Conversely, against *E. coli*, the MIC and killing concentrations of p-LK5 remained unchanged, whereas the MIC value of LK20 was increased.

MSCs were examined under the microscope (Fig. 6,3S) to assess the culture conditions 24 h after bacterial inoculation. Corresponding with the viability test results and the bacterial MIC, RPMs had a specific range of effective concentrations (6–25 μg/mL for LK20 and 12–25 μg/mL for p-LK5) in which we observed great protection effect: the RPMs inhibited the bacterial growth while the cells maintained their healthy morphology. The cells exhibited damage at high concentrations due to RPMs' toxicity, while at low concentrations, damage occurred due to bacterial infection. This observation highlights the importance of optimizing RPM concentrations to balance antimicrobial efficacy and cytotoxicity.

2.7. RPMs immunomodulatory activity upon bacterial infection

To further investigate the immunomodulatory effects of RPMs, we conducted gene expression analysis of pro-inflammatory cytokines induced by bacterial infection (Fig. 7). MSC cultures containing RPMs were inoculated with bacteria for 6 h. Gene expression analysis was then performed as previously described. Our results demonstrated that *E. coli* infection substantially increased pro-inflammatory cytokines expression response, which was significantly reduced by treatment with both RPMs. RPMs also attenuated the expression induced by *L. monocytogenes*, except for TNFα, which did not show an increase in expression due to the infection.

2.8. In vitro digestion

To assess the digestibility of RPMs, an *in vitro* digestion assay was conducted following the protocol outlined by Minekus et al. (2014) with minor adjustments. The RPM sample (LK20, 500 μg/mL) was first digested by porcine mucosa pepsin (0.5 μg/mL) in simulated gastric fluid (SGF). Subsequently, the mixture was titrated to pH 7 to inactivate the pepsin and to simulate the small intestine conditions. The mixture was then digested with Porcine trypsin (0.5 μg/mL). Throughout the experiment, samples were taken every 10 min to evaluate peptide degradation using MALDI-TOF-MS and protein gel electrophoresis SDS-PAGE gel (Fig. 8). The results showed rapid RPM digestion in the SGF environment. Within less than an hour, most of the LK20 mixture was cleaved into shorter peptides, chain lengths consisting of 6–10 amino acids. In the small intestine simulation, both the left original mixture and the small peptides produced in the previous treatment were fully digested whithin 10 min.

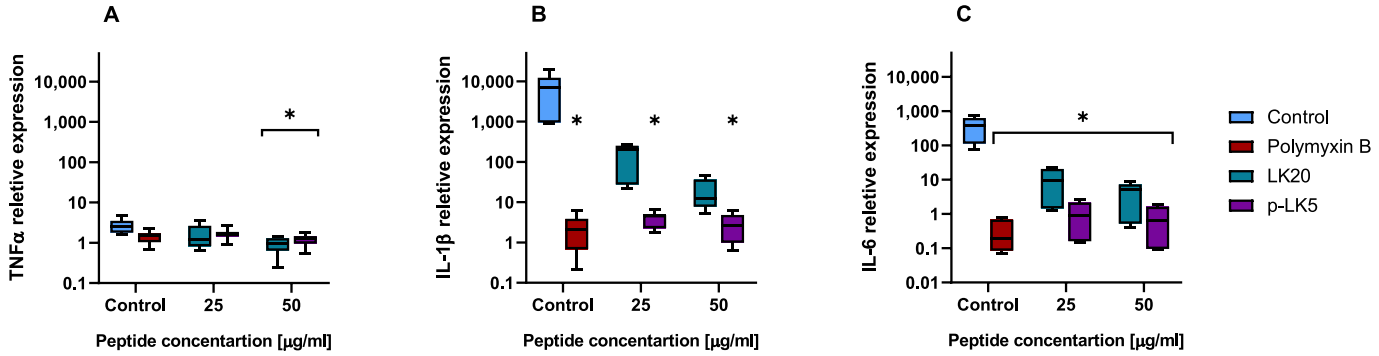

Fig. 5. Pro-inflammatory cytokines relative expression. Wharton-jelly derived bovine MSC's pro-inflammatory cytokines expression was induced with 0.1 μg/mL LPS. The RPMs were added with the LPS for 6 h incubation at 37 °C 5 % CO_2. Polymyxin B was used at 25 μg/mL as a positive control. A) TNFα B) IL-1β and C) IL-6 expression was measured by real-time PCR and compared to untreated cells using the ΔΔCT method with β-Actin as a house-keeping gene. The results summarize three independent triplicates ±SEM. Statistical analysis was conducted using the Kruskal-Wallis multiple comparison test. Asterisks (*) indicate statistically significant results with a *p*-value of less than 0.05 ($\alpha < 0.05$).

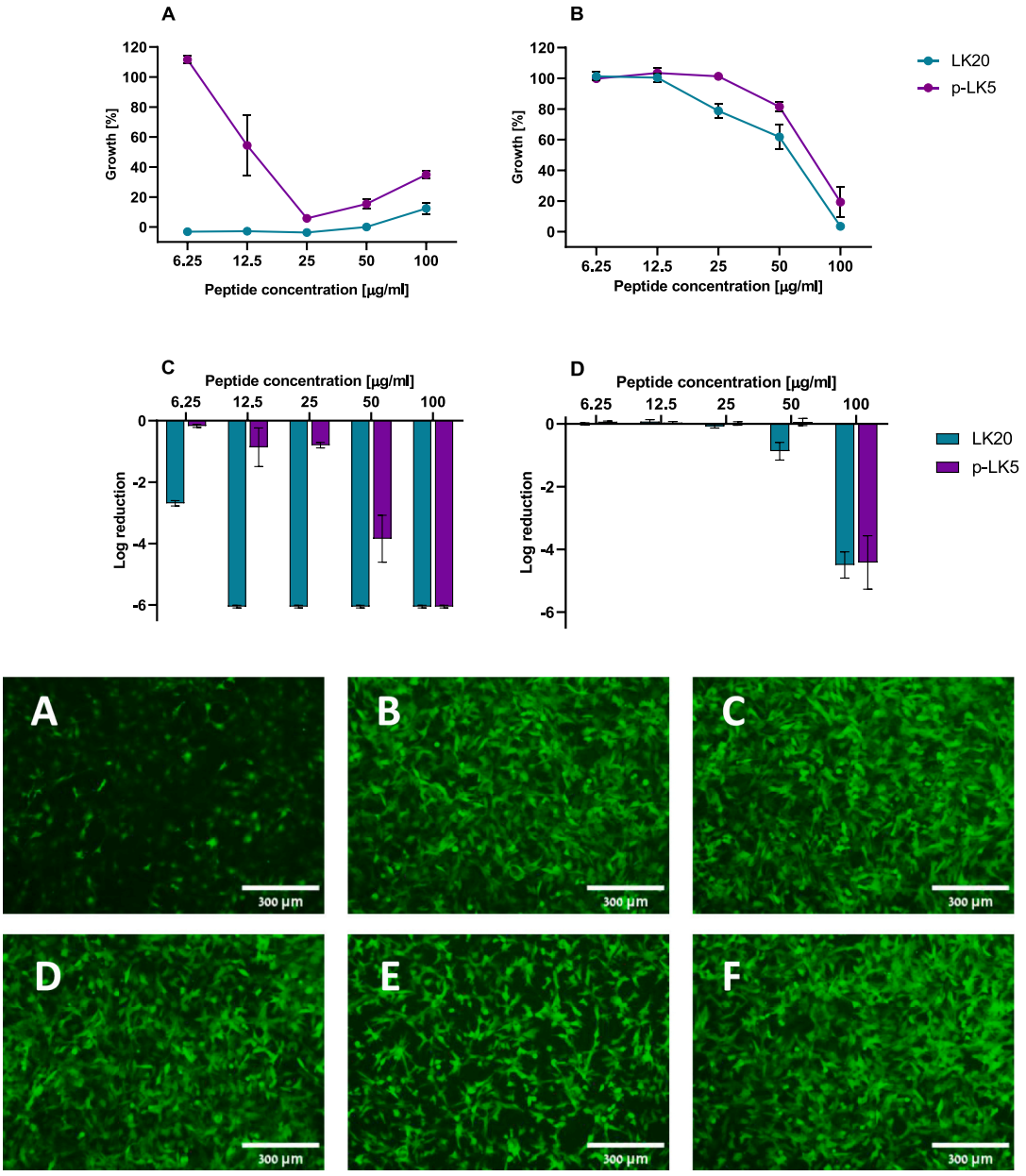

Fig. 6. Antimicrobial activity of RPMs in co-culture with MSCs. MIC values of various RPMs were examined against A) *L. monocytogenes* and B) *E. coli* NR. 10^6 CFU/mL of bacteria were incubated with RPMs in Wharton jelly derived MSC culture for 24 h at 37 °C, 5 % CO_2. Bacterial growth values were measured (595 nm) in a plate reader (Tecan) and normalized against the growth of untreated bacteria. The results summarize three independent triplicates ±SEM. A bacterial killing assay was conducted against C) *L. monocytogenes* and D) *E. coli* NR with an initial bacterial load of 10^6 CFU/mL. The bacteria were incubated with RPMs in cell culture for 1 h at 37 °C, 5 %CO_2, then serially diluted in PBS and plated on LB-agar plates. The results summarize the CFU count of three independent triplicates ±SEM.Wharton jelly-derived MSCs morphology after 24 h incubation with E) *L.monocytogenes* and LK20 F) 6.25 µg/mL G) 12.5 µg/mL H) 25 µg/mL I) 50 µg/mL J) Control.

2.9. Resistance occurrence towards RPMs

Antibiotic resistance poses a significant public health threat. Thus, the development of new antimicrobial agents must consider the potential for resistance to emerge. An experimental evolution study was conducted to evaluate the development of resistance in response to RPM exposure compared with traditional antibiotics in DMEM media. The experiment was conducted with some modifications based on the protocol outlined by (Maron et al., 2022), involved maintaining three bacterial lines of each species over 28 days, using sub-MIC concentrations of each treatment (Supplementary Fig. 4). *E coli* lines were maintained in DMEM (10 % inactivated FBS, L-Gln) at 37 °C. *L. monocytogenes* lines were maintained in LB. All MIC experiments were performed in DMEM (10 % inactivated FBS, L-Gln).

The findings revealed (Fig. 9) that bacteria exposed to a combination of penicillin and streptomycin, commonly used in cell culture, exhibited significant resistance development, with a 4–8-fold MIC increase for *L. monocytogenes* and an 8–32-fold MIC increase for *E. coli*. Strains treated with rifampicin showed a remarkable increase in MIC values, with over a 2000-fold change for *L. monocytogenes* and a 128-fold change for *E. coli*. In contrast, no resistance was developed against LK20 in both *E. coli* and *L. monocytogenes*. Resistance was slightly developed to a minimal extent, up to 2-fold, against p-LK5.

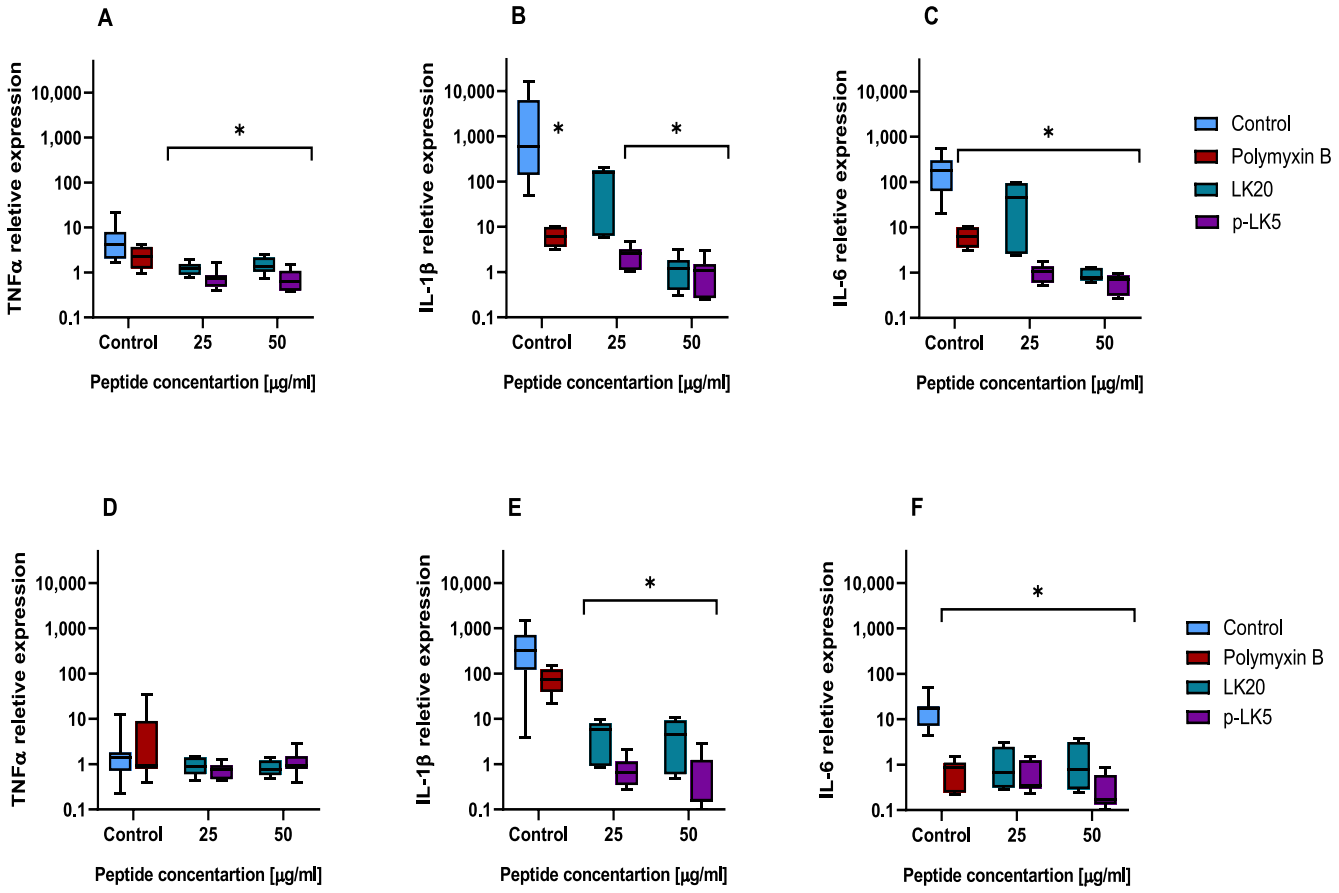

Fig. 7. Gene expression analysis of bacterial-induced pro-inflammatory cytokines. Wharton-jelly derived MSC were inoculated with 10^5 CFU/mL of *E. coli* (A-C) and 10^6 CFU/mL of *L. monocytogenes* (D—F) and incubated with RPMs for 6 hours. Polymyxin B was used as positive control. A, D) TNFα B, E) IL-1β and C, F) IL-6 expression was measured by real-time PCR and analyzed using the ΔΔCT method. The results summarize three independent triplicates ±SEM. Statistical analysis was conducted using the Kruskal-Wallis multiple comparison test. Asterisks (*) indicate statistically significant results with a *p*-value of less than 0.05 ($\alpha < 0.05$).

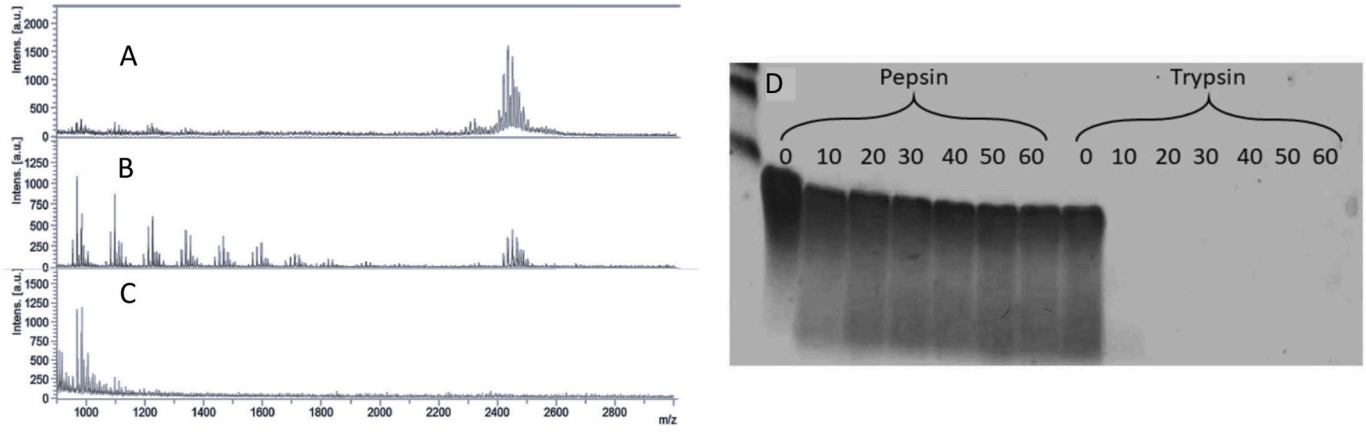

Fig. 8. RPMs digestion in *in vitro* Digestion. RPM (LK20) (500 µg/mL) was incubated for 1 h in a simulated gastric fluid (pH = 3) with porcine pepsin (0.5 µg/mL) followed by 1 h incubation with porcine Trypsin (pH = 7) at 37 °C. After incubation, the reaction was inactivated using heat treatment (90 °C). Every 10 min a small sample was inactivated using NaOH and analyzed. Samples analyzed in MALDI-TOF MS are depicted in A) RPM mixture before digestion. B) After pepsin digestion and C) After Trypsin Digestion. RPMs digestion throughout the experiment is depicted in D) protein electrophoresis gel showing a decrease in the original mixture and an increase in smaller peptides in pepsin digestion and a complete breakdown in trypsin digestion.

3. Discussion

Microbial contaminations are a major setback for the upscaling and cost reduction of CM production. Traditional antibiotics are not allowed to be used in large-scale production, mainly due to public health concerns arising from the consumption of antibiotics and the emergence of antibiotic-resistant bacteria. Thus, the cultured meat industry requires novel, cost-effective, "resistance proof", and safe non-antibiotic

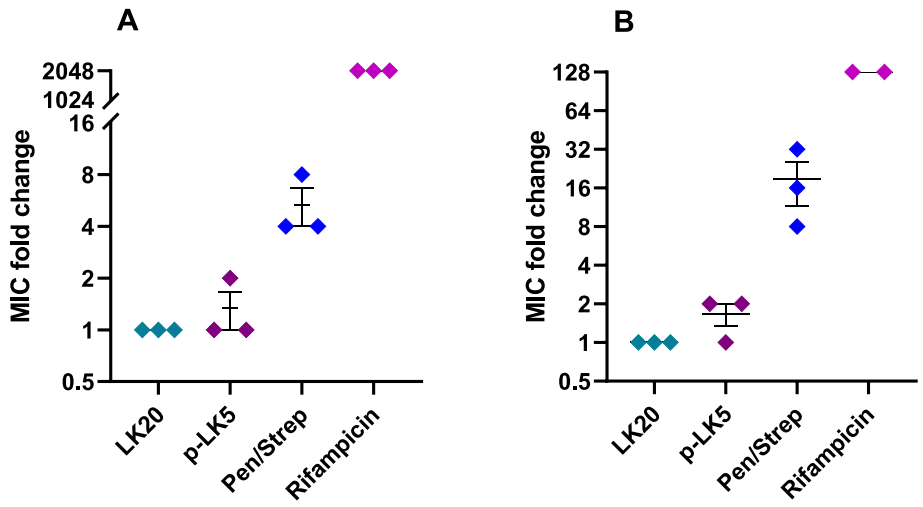

Fig. 9. Resistance evolution procedure. Every day 10 μL of the last day culture transferred into a new plate with fresh medium and RPM/antibiotics treatment. Every 4 days, bacteria that grew in the highest RPM/antibiotic concentration were selected and transferred into four new concentrations of the line. RPM/antibiotic concentrations doubled when growth was observed in 2 out of 3 lines and quadrupled when growth was observed in all the lines. The experiment continued for 28 days. Evolved strains were isolated and the resistance rate was determined by MIC assay of each evolved strain towards the corresponding RPM/antibiotics. Results are shown as fold change of ancestor MIC values of **A)** *L.monocytogenes* and **B)** *E. coli* NR, were obtained from three independent experiments, with three replicates.

antimicrobial agents to protect cell cultures during large-scale production. Random peptide mixtures (RPMs) have previously shown strong antimicrobial activity and low cytotoxicity. Their inexpensive and easy synthesis further highlights their potential as a promising opportunity for this application.

In this study, we thoroughly investigated the potential of RPMs to serve as effective and safe non-antibiotic antimicrobial agents in CM production, revealing several promising characteristics. Firstly, RPMs did not affect mesenchymal stem cells' (MSCs) viability or proliferation rate in long-term culture, indicating that they do not impair cell growth. Moreover, no activation of inflammatory genes was observed. Secondly, RPMs exhibited strong and broad antimicrobial activity against both *E. coli* NR and *L. monocytogenes*. Notably, RPMs were particularly effective against *L. monocytogenes*, a gram-positive pathogen. However, optimizing RPMs for greater efficacy against gram-negative bacteria is recommended to widen the margin between the effective antimicrobial concentration and the maximum concentration tolerated by the MSCs. The strong antimicrobial activity further underscores the potential of RPMs as versatile antimicrobial agents in ensuring the safety and integrity of CM production. RPMs have also shown immunomodulatory capabilities by mitigating pro-inflammatory cytokines induced by bacteria and LPS. The absence of induced expression of pro-inflammatory cytokines further supports the lack of adverse effects of RPMs on mammalian cells. Additionally, their immunomodulatory activity suggests that RPMs may help mitigate stress caused by toxins released into the culture media by bacteria eradicated by RPMs.

As food preservatives, RPMs are expected to be fully digestible and absorbable in the digestive system without causing damage to consumers or their microbiome. Furthermore, our study highlighted that RPMs showed a reduced likelihood of bacterial resistance compared with traditional antibiotics as we have demonstrated recently under different conditions (Maron et al., 2022). These findings indicate that RPMs have great potential as a safe and "resistance-proof" additive to cell growth media in the cultured meat industry, preventing microbial contamination without compromising cellular integrity. Collectively, these findings suggest that RPMs have the potential to revolutionize food preservation strategies in the context of CM and in the cellular agriculture in general by providing an effective and safe alternative to traditional antibiotics to prevent microbial contamination and to support the scale up of the processes.

4. Materials and methods

4.1. Solid phase peptide synthesis methodology (SPPS)

20-mer RPMs were synthesized by a standard Fmoc-based solid-phase peptide synthesis (SPPS) on Rink Amide resin (substitution 0.6 mmol/g), using an automated microwave peptide synthesizer (Liberty Blue, CEM, USA). Amino acids were dissolved with dimethylformamide (DMF) to a final concentration of 0.2 M. Fmoc deprotection step was conducted by 20 % piperidine in DMF (*v*/v). Coupling step was conducted by Ethyl cyanohydroxyiminoacetate (Oxyma) (0.1 M) and N,N′-diisopropylcarbodiimide DIC (0.1 M). At the end of the synthesis, the peptides were cleaved from the resin by adding a solution containing 95 % trifluoroacetic acid (TFA), 2.5 % double-distilled water (DDW) and 2.5 % triisopropylsilane (TIPS) and stirred for 3 h. The mixture was then filtered, and the peptides precipitated by the addition of 40 mL cold diethyl ether to the TFA solution and centrifuged. The supernatant was then removed and the peptide pellet dried, dissolved in 20 % acetonitrile in DDW, frozen with liquid nitrogen and lyophilized. The synthesis was validated by MALDI-TOF mass spectrometry (Bruker Daltonik, Germany).

Lipo-5-mer RPMs were manually synthesized using a microwave (MARS 6, CEM, USA). A mixture of the designated amino acid was dissolved in DMF (0.15 M for each AA), then the reaction was activated by hexaflourophosphate benzotriazole tetramethyl uranium (HBTU) (0.15 M) and N,N-Diisopropylethylamine (DIEA) (0.13 M). F-moc deprotection step was conducted by 20 % piperidine in DMF (v/v). Palmitic acid was dissolved in DMF to a final concentration of 0.06 M, conjugation was activated using HBTU and DIEA (0.06 M and 0.075 M respectively). After an overnight incubation (room temperature, shaking at 25 RPM), the peptides were cleaved from the resin as described above. RPMs were lyophilized and stored at −20 °C.

To fluorescently label the RPM, 5 (6)-carboxyfluorescein was conjugated to the N-terminal of the peptides. After synthesis and prior to resin cleavage, 0.25 mmol of RPM was incubated overnight (room temperature, shaking at 25 RPM) with 4 equivalents (1 mmol) of 5-(6)-carboxyfluorescein, DIEA hydroxybenzotriazole (HOBT), (DIC), and dichloromethane (DCM) in DMF (final volume of approximately 4 mL). After incubation, the peptides were cleaved from the resin as described above.

4.2. MSCs maintenance

MSCs primary culture was maintained in low glucose (1 g/L) DMEM (Gibco) supplemented with 10 % fetal bovine serum (FBS), 1 % L-Glutamine (Biological Industries) and 1% Penicillin streptomycin at 37 °C, 5 % CO_2. Approximately once a week, when the culture reached ~90 % confluence, the cells were washed with 5 mL phosphate buffer saline (PBS) and harvested using 0.25 % trypsin (1.5 mL, 4 min incubation). Cells were diluted in 10 mL DMEM to a concentration of 4×10^5 cells/mL and seeded on a new 10-mm plate (4×10^5/plate).

4.3. MSCs viability assessment

Resazurin cell viability assay: RPMs were dissolved in DMSO (40 mg/mL) and diluted 1:100 in a 96-well plate containing 50 µL of DMEM (no P/S). The RPMs were then serially diluted two-fold. MSCs (P.6–8) were added to the plate (50ul, 4×10^4 cells/well) and incubated for 24 h at 37 °C, 5 % CO_2. The next day, Resazurin (Sigma) was introduced to the culture (final volume of 150 u DMEM, 25 µg/mL Resazurin), and the culture was then incubated for 4 h. Fluorescence intensities (560/590) were measured in a plate reader (Tecan).

Trypan blue viability assay: In each cell passage cells were dyed using Trypan blue 0.4 % to evaluate the percentage of dead cells in the culture, using an automated cell counter.

4.4. Fluorescent imaging

MSCs were incubated with RPM (LK20, 25µg/mL) on a glass slide in a 24-wells plate (5×10^5 cells/well) overnight. The next day the cells were washed with PBS and were fixed using 4 % paraformaldehyde in PBS (300ul, 10 min). Next, cells were lysed with 0.5 % triton in PBS (300uL, 5 min). The cells were then incubated for 1 h in TBST based blocking solution containing 1 % bovine serum albumin (BSA), 0.1 % phalloidin and 0.13 % DAPI. The cells were washed again 3 times in PBS and dried, slides were attached to cover slips using mounting. Images captured using EVOS cell imaging system (Thermo Fischer Science) at 10×, or confocal laser scanning microscope at 40X (Leica SP8).

4.5. Gene expression analysis

Wharton-jelly derived MSCs were seeded in a 6-well plate ate cell density of 2×10^5 Cells/well and allowed to grow for 48 h to reach approximately 90 % confluency. The culture was then introduced to RPMs and inoculated with 10^5 CFU/mL *E.coli* or 10^6 CFU/mL *L. monocytogenes* for 6 h incubation at 37 °C, 5 % CO_2. RNA was extracted using NucleoSpin RNA extraction kit followed by cDNA synthesis (Quantabio) following kits instructions. RT-PCR was performed on a BioRad 148-well plate RT-PCR system using Luna® Universal One-Step RT-qPCR Kit. CD90 was used as MSC markers, the primer sequences used for real-time PCR analysis of β-Actin, TNFα, IL-1β, and IL-6 were obtained from Lu et al. (2021).

4.6. Antimicrobial activity assays

Assessment of minimal inhibitory concentration (MIC): An overnight culture of bacteria was diluted 1:50 in 5 mL fresh LB media and allowed to grow in a shaker incubator (200 RPM) at 37 °C until reaching an OD of 0.1 (595 nm). The diluted culture was washed thrice with DMEM (no phenol-red). RPMs were dissolved in DMSO and diluted 1:100 in a 96-well plate containing 100 µl of the medium. The RPMs were then serially diluted two-fold. 100 µL of the bacteria culture was added to each well, resulting in a final volume of 200 µL DMEM with bactrial load of $5x10^5$ CFU/mL containing <0.5 % DMSO. The plates were incubated at 37 °C for 24 h. Growth was determined by measuring OD using a plate reader at 595 nm.

Bacterial killing assay: MSCs were seeded in a 24-wells plate (10^5 cells/well) overnight. The next day, MSCs were inoculated with 10^6 CFU/mL of bacteria, which had been prepared as described previously. The infected co-culture was incubated for 1 h at 37 °C, 5 % CO2. RPMs were added for another 1-h incubation (final volume of 500 µL 1 % DMSO) at different concentrations. The culture media was then serially diluted in PBS and plated on LB agar plates.

Re-inoculation bacterial killing assay: Bacteria were grown as described earlier to reach an OD of 0.1 and then washed 3 times with PBS to inhibit additional growth. For each inoculation, 1 mL of the bacteria stock (10^8 CFU/mL) was transferred to a 1.5 mL Eppendorf tube and centrifuged at 7400 *g*, at 4 °C for 4 min. The PBS was replaced with RPM-containing high glucose (4.5 g/mL) DMEM. Tubes were vortex and incubated in a shaker incubator at 37 °C, 200 RPM for 1 h. After incubation, the tubes were centrifuged again, and the RPM-containing media was transferred to a new tube containing a fresh bacteria pellet for another 1 h of incubation. The post-incubation pellet was diluted in 1 mL PBS to restore the initial bacterial load and then serially diluted in PBS and plated on an LB-agar plate. This process was repeated three times, resulting in three inoculations and killing assays.

4.7. In vitro digestion assay

Simulated gastric fluid was prepared as described in Minekus et al. (2014). LK20 was dissolved in 3 mM HCl solution, the mixture was then mixed 1:1 (*v*/v) with simulated gastric fluid (pH = 3) containing porcine mucosa pepsin. The simulated gastric digestion was conducted at concentrations of 500 µg/mL LK20 and 0.5 mg/mL pepsin, at 37 °C, 150 RPM for 1 h. After incubation, the enzyme was inactivated by adding 1 M NaOH to pH 7 then porcine trypsin was added for simulated intestine digestion. After 1 h of incubation under the same conditions, the enzyme was inactivated by NaOH to reach pH 13. The samples were then analyzed using MALDI-TOF MS and protein gel electrophoresis.

4.8. Experimental evolution

The procedure was based on a previous study by Maron et al. (2022) with minor adjustments. Before evolution, the MIC values of each treatment was evaluated. Our experimental evolution procedure was designed to exert selective pressure yet to avoid extinction of the bacteria. Therefore, each line was exposed to 4 concentrations of RPM/antibiotic according to its MIC as follows: 1.5×, 1×, 0.5×, and 0.25× MIC. The plate contained another 8 wells with bacteria only as a positive control, and 4 wells with medium only as a negative control to indicate contaminations. Three lines that evolved with rifampicin were used as a positive control for resistance evolution. Every day, 10 uL of the previous plate was replicated into 200uL of fresh medium and RPM/antibiotic. Every 4 days, bacteria grown at the highest concentration of each treatment were selected and transferred into 4 new-MIC-based concentrations in the new plate. MIC was doubled when growth was observed in 2 out of 3 lines and quadrupled when growth was observed in all lines in MIC or higher. Growth was defined as an OD of 0.1. The experimental evolution was carried out for 28 transfers. Before every selection or MIC increase, samples were taken to make glycerol stocks (25 %) and preserved in −80 °C to avoid line extinction. Spot plating was performed on LB agar to indicate growth before selection.

CRediT authorship contribution statement

Idan Yakir: Writing – review & editing, Writing – original draft, Methodology, Investigation. **Einav Cohen:** Project administration. **Sharon Schlesinger:** Writing – review & editing, Methodology, Conceptualization. **Zvi Hayouka:** Writing – review & editing, Writing – original draft, Visualization, Supervision, Project administration, Methodology, Investigation, Funding acquisition, Formal analysis, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.fochms.2025.100240.

Data availability

All the data is shared in the paper.

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