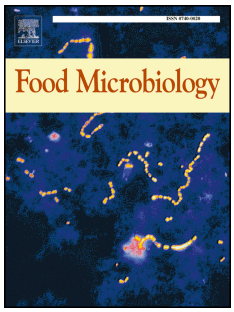

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Antimicrobial peptide cocktail activity in minced turkey meat

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Abstract:

Meat products contain valuable nutrients that are important for human health and development but are also highly susceptible to colonization by microorganisms. This can lead to spoilage and serious foodborne illnesses. Natural antimicrobial peptides, produced by many organisms as part of their innate immune system to fight microbial infections, have great potential as food preservatives. In this study, we explored the effect of ternary antimicrobial random peptide mixtures (RPMs) on food spoilage bacteria in minced turkey meat. Amendment of RPMs to meat led to significant reductions in bacterial abundance in experimental tests, and RPMs worked synergistically with nitrite to reduce bacterial loads. Using high-throughput 16S ribosomal RNA gene amplicon sequencing, we characterized the effect of RPMs and nitrite on meat microbial community structure before and during incubation under refrigerated conditions. Our findings reveal strong antimicrobial activity for RPMs against spoilage bacteria in meat, including *Listeria monocytogenes* and *Pseudomonas putida*. These results demonstrate the potential of RPMs as a safer preservative for reducing spoilage in meat and other food products.

1. Introduction:

Meat products are part of the recommended human diet and contain valuable nutrients that are important for health and development (Hyldgraad, *et al*. 2015). However, microorganisms easily colonize and proliferate in fresh meat due to an excess of nutrients and a moist environment, which leads to spoilage (Hyldgraad, *et al*. 2015; Lamas, *et al*. 2016). The contamination and spoilage of food products is a problem of global concern, since the growth and metabolism of microorganisms can cause serious foodborne illnesses and food loss (Böhme, *et al.* 2012; Borch, *et al.* 1996*)*. Therefore, maximum allowable levels of mesophilic aerobic and facultative anaerobic microorganisms have been mandated by health agencies worldwide (Stieglmeier, *et al*. 2009).

To suppress microbial growth in meat, preservatives such as sodium nitrite are used (Lamas, *et al*. 2016; Serrano, *et al*. 2012; Müller-Herbst, *et al*. 2016). As a food additive, sodium nitrite has three key functions: (i) contributing to flavor by inhibiting the development of rancid off-flavors; (ii) preserving the strong pink color of meat via reactions with myoglobin; and (iii) preventing the growth of pathogenic bacteria such as the toxin-forming *Clostridium botulinum (*Cammack, *et al*. 1999; Crosby, *et al* 1976). Although the activity of sodium nitrite has been extensively studied, its mode of action is still not completely understood. Inhibition of respiration has been proposed as one possible mode of action of nitrite toward *C. botulinum (*McMindes, *et al* 1988*)*. Nitric oxide (NO), formed via nitrite reduction, has been suggested as the primary bacteriostatic compound in nitrite-amended food. NO interacts with the iron-sulfur proteins of bacteria (*e.g.*, cytochromes), which are important for microbial energy metabolism (Cammack, *et al.* 1999*;* Tompkin, *et al.* 1978). Nitrite may also inhibit pyruvate-ferredoxin reductases, leading to bacterial cell death *(*McMindes, *et al* 1988*)*.

Although the preservative function of sodium nitrite has been well established, there has recently been a greater focus on its toxicity to humans. Nitrites are toxic at high concentrations as are *N*-nitroso compounds (nitrosamines) which form when nitrites react with secondary amines in the acidic conditions of the stomach. Compounds such as *N*-nitrosodimethylamine have been shown to be carcinogenic in several animal species (Lamas, *et al*. 2016; Cammack, *et al*. 1999; Honikel, 2008). Thus, there is an urgent need to replace nitrite in the meat industry with safer preservatives (Rydlo, *et al*. 2008; Anderson, *et al*. 2004).

Natural antimicrobial peptides (AMPs) and host defense peptides (HDPs) are produced by eukaryotic innate immune systems. Their biological role in eukaryotic organisms is to eliminate Gram-positive and Gram-negative bacteria, as well as fungi and viruses. In bacterial infections, these compounds are a component of the host immune response, and act primarily by disrupting bacterial cell membranes. As a result, these classes of peptides have great potential as effective and safe preservatives (Diamond, *et al*. 2009; Malheiros, *et al*. 2010; Nakatsuji, and Gallo, 2012; Cleveland, *et al*. 2001; Rathinakumar, *et al.* 2009). Most AMPs possess common structural features such as positive charge and moderate hydrophobicity (~50%). This amphipathicity enables them to interact with and permeabilize negatively charged membranes of bacteria, resulting in cell membrane disruption (Nakatsuji, and Gallo, 2012; Rathinakumar, *et al.* 2009; Brogden, 2005; Wimley and Hristova 2001; Hancock, 2001). Previous studies have evaluated AMPs as preservatives (Anderson, *et al*. 2004). For example, an analogue of magainin (an AMP isolated from frog skin) possessed strong antimicrobial activity against 13 pathogenic bacterial strains associated with foodborne illnesses (Abler, *et al*. 1995). Elsewhere, the activity of a synthetic peptide bearing six leucine and eight lysine residues was studied against a range of foodborne microorganisms including *Listeria monocytogenes (*Aiyegoro, 2014; Appendini, and Hotchkiss, 2000).

Despite the promise of AMPs as safer meat preservatives, there are still several challenges that must be addressed: (i) they must be effective against a diverse array of microorganisms; (ii) phospholipids or proteins can potentially suppress their antimicrobial activity ; (iii) AMPs can be degraded rapidly by proteases (Anderson, *et al*. 2004; Malheiros, *et al*. 2010); (iv) rapid development of antimicrobial resistance can occur (Mayrhofer, *et al*. 2004; Dobson, *et al*. 2014; Perron, *et al*. 2006; Pränting, et al. 2008; Habets *et al*. 2012; Dobson, *et al*. 2013); and (v) cost of manufacture (Wimley and Hristova. 2011). Although these challenges are daunting, there is already one AMP preservative on the market which indicates feasibility. The antimicrobial peptide-based preservative nisin (produced by certain strains of *Lactococcus lactis*) has been approved by the FDA (Cleveland, *et al*. 2001) and is effective against Gram-positive bacteria, including spores, but shows very low activity against Gram-negative bacteria, yeasts and molds[28]. Nisin has been widely used as an exogenous addition to a variety of food products around the world and is also naturally present in many dairy products (Rydlo, et al. 2008; Müller-Auffermann, *et al*. 2015). Nisin has a dual mechanism of action, which is facilitated by binding to the peptidoglycan precursor, lipid II. At lower concentrations, nisin interferes with cell wall synthesis and at higher concentrations it forms pores that disrupt the proton motive force in bacterial membranes (Müller-Auffermann, *et al*. 2015). When examined as a meat preservative, nisin displayed strong antimicrobial activity in inoculated minced beef against Gram-positive *L. monocytogenes*; conversely, application of nisin in minced sheep meat showed no antimicrobial activity against *Salmonella* Enteritidis (Solomakos, *et al*. 2008; Govaris, *et al*. 2010).

The structural diversity of AMPs suggests that their activity is not tightly linked to a specific amino acid sequence (Rathinakumar, *et al.* 2009). This observation led to the development of random peptide mixtures (RPMs) as antimicrobial agents (Hayouka, *et al.* 2013). During peptide synthesis, instead of using one amino acid at each coupling step, a mixture of two or

more amino acids (at a known stoichiometry) are used. The result is $2^n/3^n$ (where n represents the peptide chain length equal to the number of coupling steps) sequences of random peptides composed of hydrophobic and cationic amino acids but with controlled chain length and stereochemistry. This novel AMP synthesis strategy may overcome some difficulties associated with specific sequence of AMPS (Hayouka, *et al.* 2013; Topman, *et al*. 2018; Stern, *et al*. 2016; Amso and Hayouka 2019), as this approach is cheaper and may confound bacterial attempts to develop resistance. The aim of the current study was to investigate the antimicrobial activity of RPMs in food. We have used minced turkey meat as a food model and have coupled cultivation approaches with cultivation-independent molecular characterization of microbial community structure to gain insights into the activity of AMPs in meat.

2. Material and Methods:

2.1 Synthesis of random peptide mixtures

RPMs were synthesized using the traditional solid phase peptide synthesis (SPPS). Synthesis of random peptide mixtures (RPMs) was carried out according to Hayouka et al. (2013). RPMs were synthesized using microwave irradiation on Rink Amide resin (Substitution 0.53 mmol g−1, 25 μmol) in Alltech filter tubes. Coupling reactions were conducted with binary combinations of protected amino acids, with a freshly prepared stock solution that contained the protected amino acids in 1:1 molar ratio of L□Phenylalanine, L-leucine, and L -lysine (25 μmol) of each amino acid, which were used for each coupling step. Upon completion of the synthesis (20 cycles for 20 mer peptide chain length), the RPMs were cleaved from the resin, resuspended in double□distilled water (DDW), frozen on dry ice and lyophilized. RPMs were analyzed by MALDI□TOF to evaluate molecular weight and quality and by amino acid analysis.

2.2 Assessment of minimal inhibitory concentration (MIC) values:

To determine the antimicrobial activity of FLK (L□Phenylalanine, L-leucine, and L -lysine), FK (L□Phenylalanine and L -lysine) and $^{D}F^{D}L^{D}K$ (D□Phenylalanine, D-leucine, and D -lysine) RPMs, MIC values were measured for *B.subtilis* NCIB 3610, *L. monocytogenes* 10403S, *P. putida* KT2440 and *E. coli* rp MG1655 strains (Table 1). MICs were determined by growth in sterile 96□well plates (Corning 3650) by a broth microdilution method as described by Hayouka et al. (2013). Bacteria were grown for 24 h in brain heart infusion broth (BHI, HiMedia Laboratories, India) or Lysogeny broth (LB; BD, USA)) at 30°C or 37°C depended on the bacteria type with shaking (200 rpm). Then, the bacterial cultures were diluted in growth medium to an optical density at 600 nm (OD600) of 0.1 using a ThermoSpectronic (Genesys 10uv) spectrophotometer. 100 µl aliquots were added to 100 µl of growth medium containing RPMs at various concentrations in each well. The plates were then incubated at 30°C or 37°C for 24 h. Bacterial growth was determined by measuring the OD at 595 nm using a Tecan Infinite Pro Plate reader. The MIC values were the lowest concentrations of the peptide mixtures that caused inhibition of bacterial growth (Hayouka, *et al.* 2013*).* MIC values were determined as the average obtained from three independent experiments. The highest concentrations tested were 200 µg/ml for RPMs, 1 mg/ml (14.49 mM) for sodium nitrite and 0.25 mg/ml (0.074 mM) for Nisin.

2.3 Meat preparation:

Fresh minced turkey meat was purchased at a local super-market and immediately transferred to the lab. The meat was ground for a second time in an ethanol cleaned grinder and divided into 40 gr portions. Samples were stored at -20°C. At the beginning of each experiment, a 40 gr portion was defrosted at 4 °C. The portion was ground and divided into 10 gr or 1 gr meat balls, and each meatball was placed in a sterile test tube. 150 µl of double□distilled water

Name	Growth conditions: Media/ Temperature and antibiotic

(DDW) was added to control sample test tubes. For treatment samples, 150 µl of DDW containing each of the different treatment compounds was added to each test tube. For treatments with sodium nitrite, final concentrations of 2.17 /1.08 /0.5 mM were used. For treatments with RPMs of FLK (L□Phenylalanine, L-leucine, and L -lysine) or $^{D}F^{D}L^{D}K$ (D□Phenylalanine, D-leucine, and D -lysine), RPMs were dissolved in double□distilled water (DDW) to final concentration of 0.25, 2, 5, and 7 mg/g or (0.096, 0.76, 1.92, 2.688 mM) except for Nisin, which had a final concentration of 0.074 mM. For the combination of sodium nitrite and FLK, the 150 µl solution added to test tubes contained FLK random peptide mixture dissolved in DDW at a final concentration of 1.92 mM and 5 µl of sodium nitrite dissolved in DDW to arrive at a final concentration of 1.08 mM or 0.5 mM. Samples were mixed and stored under refrigerated conditions at 4 °C for the length of the experiment. Microbiological analyses were performed at 0, 1, 3 and 5 days of storage.

Table S1. Bacterial strains and growth conditions used in this study. All strains were maintained at -80°C in glycerol stock (25% v/v) until use.

L. monocytogenes 10403S	BHI, 37°C, overnight, 100 µg/ml streptomycin
P. putida KT2440	LB, 30°C, overnight, 100 µg/ml ampicillin
B. subtilis NCIB 3610	BHI, 37°C, overnight
E. coli rp	BHI, 37°C, overnight

The *L. monocytogenes* 10403S culture was a generous gift from Prof. Anat Herskovits from Tel Aviv University. The strain (10403S) was modified by deleting the hly gene that codes the listeriolysin O toxin responsible for the species' virulence and has a streptomycin resistance.

2.4 Inoculated meat samples:

1 g samples of minced turkey meat were placed in sterile test tubes and inoculated with single strain of *L. monocytogenes* or *P. putida* separately (ca. 10^4 CFU/g). The bacterial cultures were diluted in the appropriate medium to an optical density at 600 nm (OD600) of 0.1 using a ThermoSpectronic (Genesys 10uv) spectrophotometer. Cultures were diluted to 10^5 CFU/ml in saline solution 0.9% for inoculation of turkey meat samples. Microbial load was determined by serial dilution and plating on BHI agar or LB agar plates. 100 µl/g of the 10^5 CFU/ml bacterial stock was used to inoculate the meat. To ensure proper distribution of the bacteria, the samples were properly mixed before addition of the treatment. Subsequently, sodium nitrite (1.08 mM), FLK (1.92 mM) and their combination were added to the inoculated samples.

2.5 Microbiological analysis:

To monitor the microbial load, we evaluated the samples at different time points 0, 1, 3 and 5 days. After the treatment, a 9 ml saline solution 0.9% was added to each 1 gr minced turkey sample. Samples were vigorously vortexed for 60 seconds at room temperature, and then serially diluted 1:10 in 0.9% saline solution. 100 µl from each sample were spread plated by duplicates on LB agar or BHI agar plate and held at 30 °C for 24 h. Each sample was analyzed with at least three independent repetitions. After 24 h, the microbial load was determined and the average number of CFU per gram was calculated by counting plates containing 20-200 colonies.

2.6 Statistical analysis

The results are presented as the mean ± SEM. One-way analysis ANOVA of variance followed by Tukey post-hoc analysis was used for statistical analysis. An independent T test analysis which compares the means of the treatments was performed. The results were considered to be statistically significant if $p < 0.05$ or $p < 0.01$ as mentioned for each experiment.

2.7 Cultivation-independent analysis of meat microbial communities

1 gr samples of minced turkey meat were divided into equal portions of 500 mg. Of these portions, one was used for DNA extraction and the other was used for cultivation-based approaches. Samples were amended with the following compounds: double-distilled water (DDW; 'Control'), 1.92 mM (5 mg/ml) of RPMs FLK ('FLK'), 1.08 mM sodium nitrite (75 ppm), and a combination of sodium nitrite (1.92 mM) and FLK (1.08 mM) ('FLK+Nitrite). Samples were stored at 4°C. Samples were taken at day 0 (prior to amendment), after 3 days, and after 5 days. Total genomic DNA (gDNA) was extracted using an Exgene™ Soil DNA Prep Kit (Songpa-gu, Korea), following the manufacturer's standard protocol. Genomic DNA was PCR amplified with primers CS1_515Fb and CS2_806Rb (modified from the primer set

employed by the Earth Microbiome Project (EMP; GTGYCAGCMGCCGCGGTAA and GGACTACNVGGGTWTCTAAT) targeting the V4 regions of microbial small subunit ribosomal RNA genes. Amplicons were generated using a two-stage "targeted amplicon sequencing (TAS)" protocol (Naqib, *et al.* 2018; Bybee, *et al.* 2011*)*. The primers contained 5' common sequence tags (known as common sequence 1 and 2, CS1 and CS2) as described previously (Moonsamy, *et al.* 2013; Green, *et al*. 2015). First stage PCR amplifications were performed in 10 microliter reactions in 96-well plates, using the MyTaq HS 2X mastermix. PCR conditions were 95°C for 5 minutes, followed by 28 cycles of 95°C for 30", 55°C for 45" and 72°C for 60."

Subsequently, a second PCR amplification was performed in 10 microliter reactions in 96-well plates. A mastermix for the entire plate was made using the MyTaq HS 2X mastermix. Each well received a separate primer pair with a unique 10-base barcode, obtained from the Access Array Barcode Library for Illumina (Fluidigm, South San Francisco, CA; Item# 100-4876). These Access Array primers contained the CS1 and CS2 linkers at the 3' ends of the oligonucleotides. Cycling conditions were as follows: 95°C for 5 minutes, followed by 8 cycles of 95°C for 30", 60°C for 30" and 72°C for 30". A final, 7-minute elongation step was performed at 72°C. Samples were pooled in equal volume using an EpMotion5075 liquid handling robot (Eppendorf, Hamburg, Germany). The pooled library was purified using an AMPure XP cleanup protocol (0.6X, vol/vol; Agencourt, Beckmann-Coulter) to remove fragments smaller than 300 bp. The pooled libraries, with a 20% phiX spike-in, were loaded onto an Illumina MiniSeq mid-output flow cell (2x153 paired-end reads). Fluidigm sequencing primers, targeting the CS1 and CS2 linker regions, were used to initiate sequencing. De-multiplexing of reads was performed on instrument. Library preparation, pooling, and sequencing were performed at the University of Illinois at Chicago Sequencing Core (UICSQC).

3. Results and Discussion

3.1 Random peptide design and synthesis

Our aim in this study was to examine the potential of RPMs to inhibit growth of food spoilage bacteria in minced turkey meat. We previously described the antimicrobial activity of different RPMs composed from a binary combination of hydrophobic and cationic residues where the most active mixtures were 20-mers containing L-leucine (L) and L-phenylalanine (F) as their hydrophobic residue with L-lysine (K) as the cationic amino acid (Hayouka, *et al*. 2013). Here, we designed and synthesized for the first time a ternary random peptide mixture by combining the most active cationic amino acid residue (Lysine) with the two most active hydrophobic amino acids residues (Leucine and Phenylalanine). These ternary peptide mixtures FLK was composed of 25% F, 25% L, and 50% K to preserve the optimal 1:1 proportion between cationic and hydrophobic amino acids. To verify the subunit proportion after synthesis, we performed amino acid analysis and determined the molecular weight range of the mixture using MALDI-TOF mass spectrometry (Figure S1). In addition, we synthesized a ternary enantiomer consisting of a D-homochiral random peptide mixture of D-phenylalanine (^{D}F), D-leucine (^{D}L), and D-lysine (^{D}K) to evaluate the effect of stereochemistry on bioactivity.

To determine the antimicrobial activity of the ternary RPMs, we performed minimal inhibition concentration (MIC) assays using *Bacillus. subtilis* and *Listeria. monocytogenes* as model Gram-positive bacteria, and *Pseudomonas . putida* and *E. coli* as model Gram-negative bacteria (**Table 1**). The new FLK RPM showed broad antimicrobial activity toward all tested bacteria. The MIC values for *B. subtilis*, *L. monocytogene* and *P. putida* were 13 µg/ mL; for *E. coli* the MIC value was 25 µg/mL. Both FLK and ^{D}F^{D}L^{D}K possessed strong

bacteriostatic activity against the tested bacteria as compared to FK peptide mixtures and similar activity to LK peptide mixtures.

Table 1. Minimum inhibitory concentration (MIC) values for LK, FK, FLK $^{D}F^{D}L^{D}K$ RPMs, nisin, and sodium nitrite. Values, in the units of μg/ml, represent the median value obtained from at least three independent repetitions for each bacterial strain with tests performed *in vitro*.

Treatment	***B. subtilis***	***L. monocytogenes***	***P. putida***	***E. coli* rp**
Leucine: Lysine (LK)	13	13	13	6
Phenylalanine: Lysine (FK)	50	25	25	50
Phenylalanine :Leucine:Lysine (FLK)	13	13	13	25
D-Phenylalanine : D-Leucine: D-Lysine ($^{D}F^{D}L^{D}K$)	13	13	13	13
Nisin	3	3	>1000	>1000
Sodium nitrite	>1000	>1000	>1000	>1000

We also compared the activity of our RPMs with nisin (Table 1), and our findings confirm previous results showing that Nisin has no antimicrobial activity against Gram-negative bacteria. Prior studies have shown that RPMs such as LK 20-mer and FK 20-mer can be active against both Gram-positive and Gram-negative bacteria and towards mature biofilms (Hayouka, *et al.* 2013; Topman, *et al*. 2018; Stern, *et al*. 2016; Amso and Hayouka 2019), and we observed that our RPMs were indeed active against both Gram-negative and -positive bacteria. No inhibition of bacterial growth was observed *in vitro* when sodium nitrite was added (14.49 mM, 1 mg/mL), despite this concentration being significantly higher than

mandated maximum quantity allowed for these additives in meat (2.17 mM, 150 μg/mL). As the FLK RPM was an effective antimicrobial agent with broad-spectrum activity toward the tested bacteria, these compounds were further tested in a controlled food model system.

Measuring the efficacy of antimicrobial agents in a food system holds several challenges. A decrease in antimicrobial activity is usually observed, due to the interaction of the agent with other components in the food matrix such as lipids, proteins, and sugars (Rydlo, *et al*. 2006). To determine the effective concentration of FLK in meat, various concentrations were added and the microbial load was quantified. A dose-dependent relationship was observed (Figure 1), whereby FLK RPM concentrations of 1.92 mM and 2.69 mM exhibited the greatest antimicrobial activity. FLK at 1.92 mM concentration was therefore used for subsequent experiments.

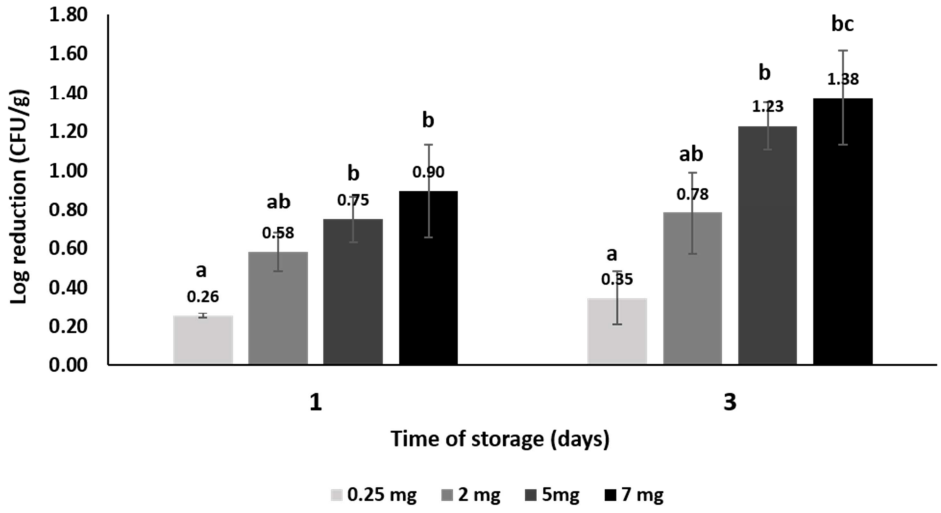


Figure 1: The effect of varying FLK concentration (mg/ml) on total aerobic heterotrophic bacterial abundance in minced turkey meat during storage at 4°C. The y-axis represents the decrease in cfu/g between the control and treatment samples (mean ± SEM, n = 9). Samples were diluted, plated, and counted on after 0, 1, and 3 days of storage. a,b,c $p < 0.01$ indicates a statistically significant difference between the treatments at the day of treatment.

The antimicrobial activity of both FLK and $^{D}F^{D}L^{D}K$ (1.92 mM) were compared to nisin (0.074 mM, Figure 2) and sodium nitrite (2.17 mM, Figure 3) in minced turkey meat. The microbial load of the minced turkey meat was assessed at 1, 3 and 5 days post-inoculation, and compared to the microbial loads at day 0 (Figure 3). FLK displayed strong antimicrobial activity and stability even after 5 days of storage, and there was no substantial advantage in using the $^{D}F^{D}L^{D}K$ (Figure S2). After 3 days of incubation the antimicrobial activity of FLK and nitrite was similar (~1 log CFU/g reduction). After 5 days FLK displayed strong antimicrobial activity, representing an average log reduction of ~1.55 CFU/g, while sodium nitrite displayed stronger antimicrobial activity of ~3 log CFU/g reduction. Nisin at this level showed weak antimicrobial activity toward meat bacterial population with a log reduction of maximum 0.5 log CFU/g during the entire storage period (Figure S3). This poor antimicrobial activity of nisin in meat has been reported previously (Cleveland, *et al*. 2001; Solomakos, *et al.* 2008; Govaris, *et al*. 2010).

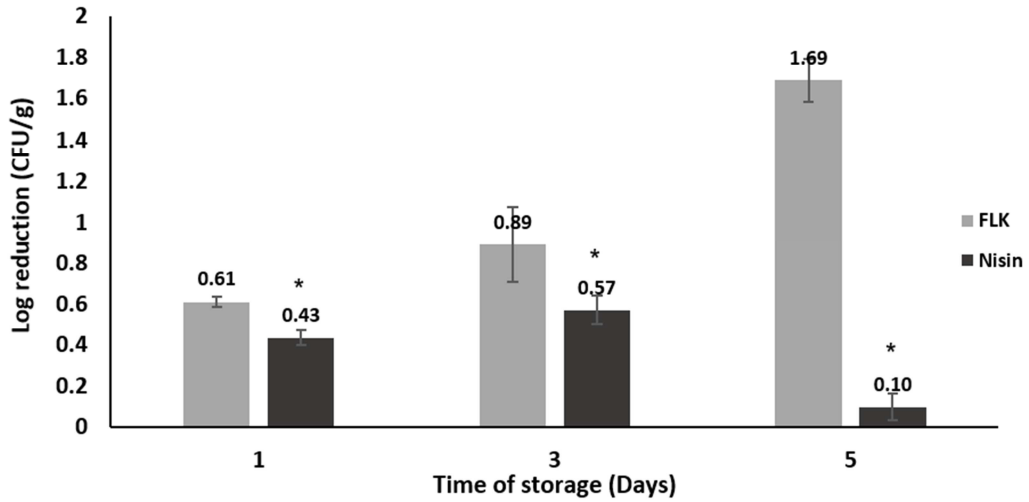


Figure 2: Comparing the antimicrobial activity of RPMs with Nisin. The antimicrobial activity of Phe-Leu-Lys (FLK, 1.92 mM) and Nisin (0.074 mM) against meat bacterial population in minced turkey meat during storage at 4 °C. Samples were diluted, plated, and counted at different time points; 0, 1, 3 and 5 days of storage (mean ± SEM, n = 17,7,8). *$p < 0.01$ indicates a statistically significant difference between the RPMs and Nisin treatments at the day of treatment.

3.2 The effect of combining FLK and sodium nitrite

Despite the health concerns regarding sodium nitrite usage, it remains one of the most common meat preservatives. The use of nitrite is primarily due to its strong antimicrobial activity against *C. botulinum*, a heat-resistant, spore-forming, toxin producer that causes botulism (Lamas, *et al.* 2016; Cammack, *et al*. 1999). The efficacy of antimicrobial compounds can sometimes be potentiated by utilizing them in combination (Marquette and Bechinger 2018). Mixtures of AMPs and conventional antibiotics have shown synergistic activity (Rank, *et al*. 2017; Kim, *et al.* 2017; Chou, *et al*. 2016), typically due to two different modes of action (Marquette and Bechinger 2018). For example, AMPs that cause damage to bacterial cell membranes (which does not necessarily result in cell death) will increase membrane permeability, which could lead to improved efficacy of sodium nitrite. For this reason, the combination of sodium nitrite and RPMs could lead to an improvement in antimicrobial activity whilst reducing the amount of sodium nitrite required to suppress bacterial growth, and hence its associated health risks. Therefore, we monitored microbial growth in turkey meat with a combination of FLK (1.92 mM) and sodium nitrite at concentrations of 1.08 mM (half dose) and 0.5 mM (quarter dose, Figure 4). The treatment regimen with the half dose of sodium nitrite resulted in significantly lower microbial load after both 3 and 5 days, compared to the individual treatments. By combining RPM with nitrite, we were able to reduce the effective concentration of nitrite by 50%. We further reduced the nitrite concentration to a quarter dose and still observed a synergistic effect. After 5 days of storage at 4°C the antimicrobial effect was maintained, with a significant log reduction of ~2.5 log CFU/g or greater at both nitrite concentrations.

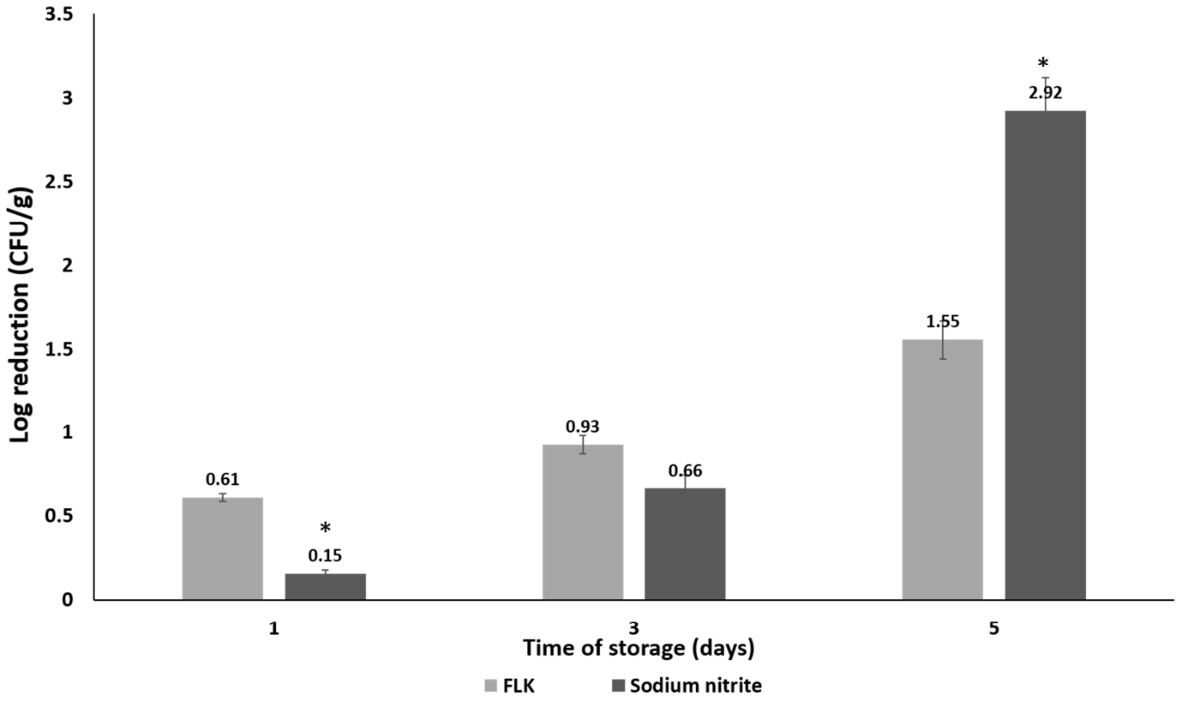


Figure 3: Antimicrobial activity of FLK (1.92 mM) and nitrite (2.17 mM / 150 ppm) in minced turkey meat stored at 4°C. The y-axis represents the decrease in total aerobic heterotrophic bacterial CFU/g between control and treatment samples (mean ± SEM, n = 9). Meat samples were diluted, plated, and counted after 1, 3 and5 days of storage. *p < 0.01 indicates a statistically significant difference between the RPMs treatments and sodium nitrite treatments at the day of treatment.

3.3 The effect of treatment on meat microbial community structure

Rapid microbial growth in meat contributes to the development of an off flavor and/or color, leading to meat that is unappealing and unsuitable for human consumption. The diversity and composition of meat microbial communities is dependent on the storage conditions and competition between organisms present in the meat (Hyldgaard, *et al*. 2015; Doulgeraki, *et al*. 2012). Therefore, the effect of treatment on the microbial community structure in turkey meat was determined, via a combination of cultivation-dependent and cultivation-independent analyses. Cultivation-independent analyses were performed by DNA extraction and 16S rRNA gene amplicon sequencing, and these analyses generated information regarding the relative abundance of microbial taxa in samples. Prior studies have shown that

the diversity of microorganisms in fresh meat, fish, and environmental samples decreases during spoilage (Filippis, *et al*. 2018); this loss of diversity was observed in control turkey meat samples analyzed after five days of incubation (Figure 5).

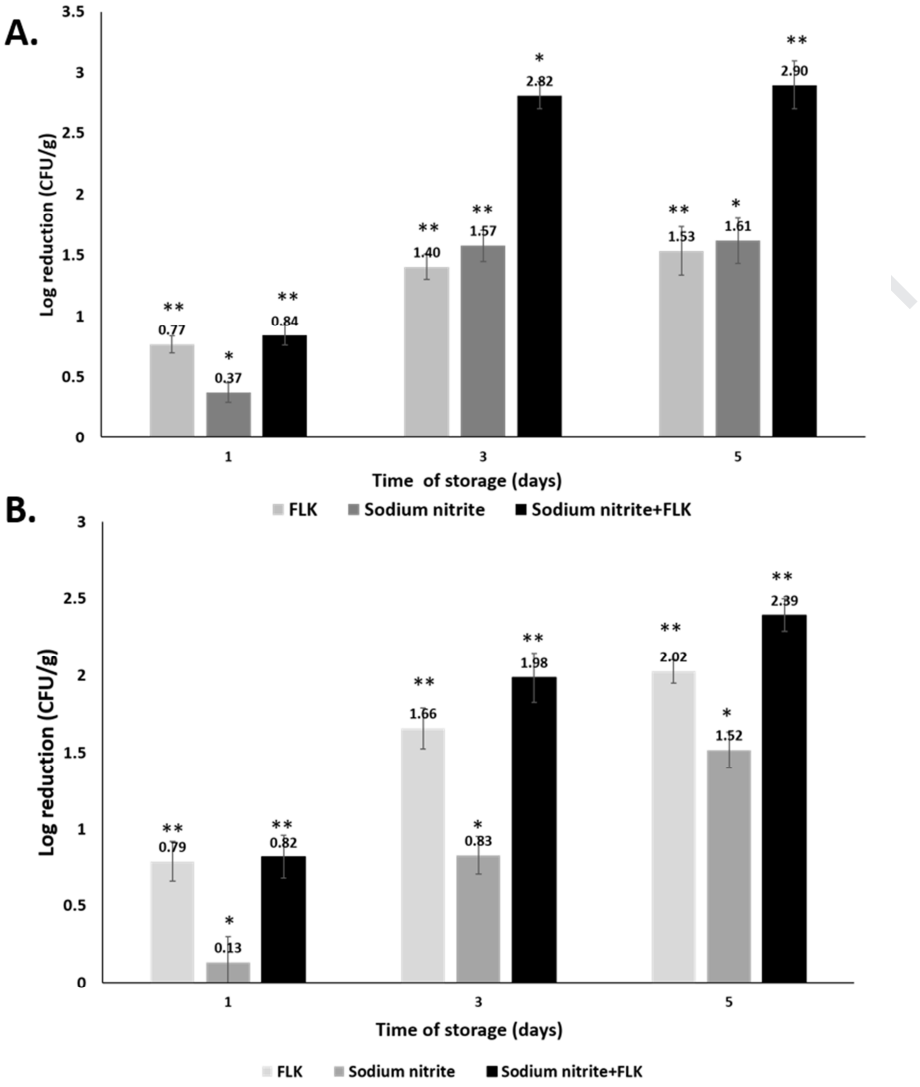


Figure 4: Antimicrobial activity of FLK (1.92 mM) and Sodium nitrite alone and in combination at different concentrations in minced turkey meat stored at 4°C. The y-axis represents the decrease in total aerobic heterotrophic bacterial CFU/g between control and treatment samples (mean ± SEM, n = 9). (A) Sodium nitrite concentration of 1.08 mM (75 ppm); and (B) sodium nitrite concentration of 0.5 mM (35 ppm). Samples were diluted, plated, and counted at 0, 1, 3 and 5 days (mean ± SEM, n = 17, 10, 14). *,**$p < 0.01$ indicates a statistically significant difference between treatments and the control at the same day.

Microbial communities changed in all treatments during the five days of incubation, though the magnitude of the effect differed between treatments (Figures 5 and 6). Alpha diversity (within-sample diversity) was calculated using the Shannon index (a measure of microbial richness and evenness) at the taxonomic level of genus (Figure 5). Microbial diversity in the control treatment at day 5 was significantly lower than the baseline diversity (Mean Shannon Index, 1.78 vs 2.37; Tukey's test p=0.029). In addition, microbial diversity (Shannon Index) in the control treatment at day 5 was significantly lower than the diversity of sodium nitrite treatment and the sodium nitrite/FLK combined treatment at day 5 (Tukey's test p<0.015). The microbial diversity of the sodium nitrite and nitrite/FLK combined treatment was not significantly different from that of the baseline microbial diversity.

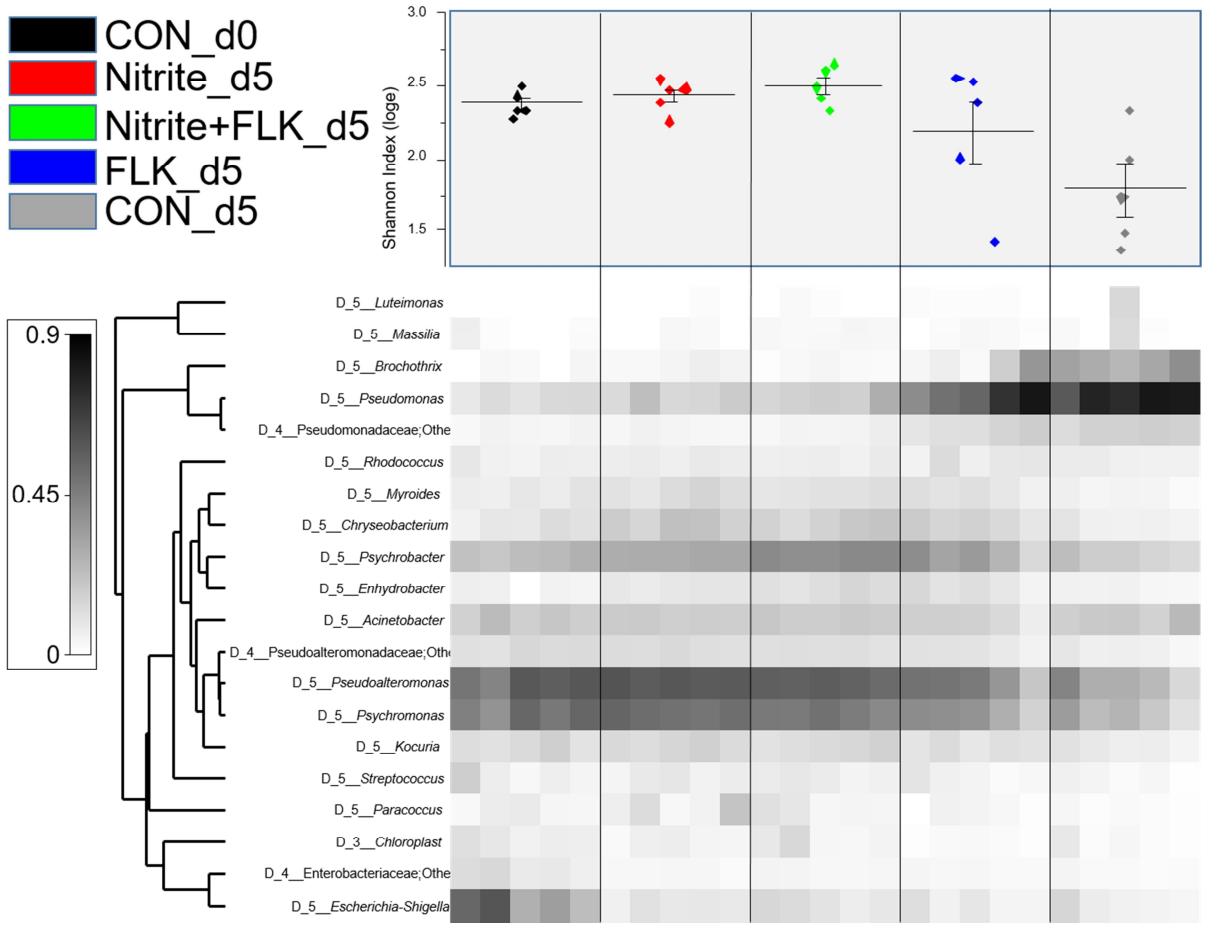
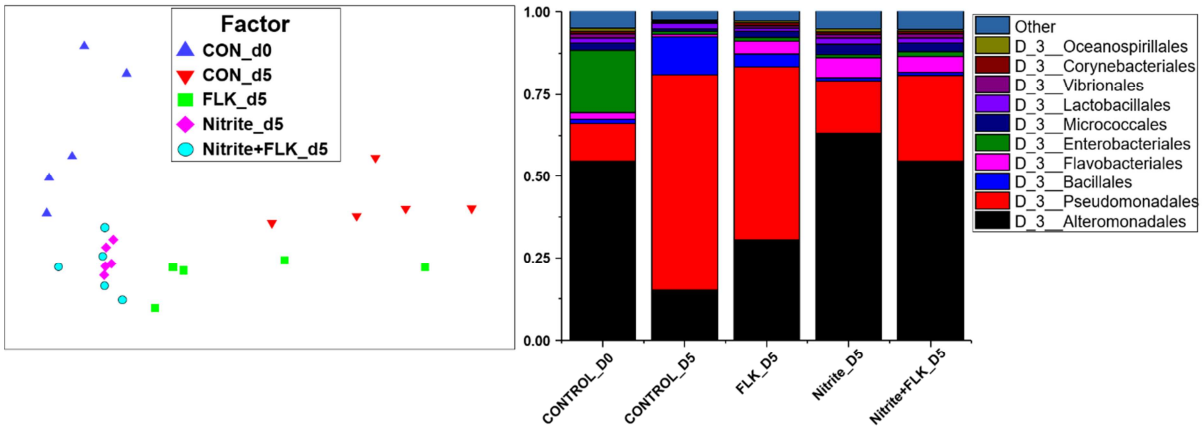


Figure 5: Shade plot of 20 most abundant microbial taxa based on genus-level annotations. Sample grouping was retained, and the scale is the square root of relative abundance. Bactria from the genus *Pseudomonas* were most abundant in FLK (day 5 or d5) and control (d5) samples, while *Escherichia-Shigella* were only present substantially in the control (day 0, d0) samples. Bacteria from the genera *Pseudoalteromonas*, *Psychromonas* and *Psychrobacteria* were abundant in samples treated with nitrite at d5. Above the shade plot is a box plot of Shannon index values (genus-level) for five replicates of each group. By ANOVA, group means were significantly different at the 0.05 level. Tukey's test indicated that the diversity of the day 5 control samples was significantly different than that of the d0 control, d5 nitrite treatment, and d5 nitrite + FLK treatment samples, but not the d5 FLK treatment samples. Diversity indices were generated from datasets rarefied to 17,000 sequences/sample.

The change, or lack thereof, in microbial alpha diversity by these treatments was consistent with the observed microbial community structure. Baseline microbial communities were largely comprised of bacteria from the genera *Escherichia-Shigella*, *Psychromonas*, *Pseudoalteromonas*, *Psychrobacter* and *Pseudomonas* (Figure 5). Bacteria from the genera *Escherichia-Shigella* (order *Enterobacteriales*) were abundant in the baseline samples (average relative abundance of 17.6%) and were much lower in all treatments at day 5 (average relative abundance of 1.0%). Although the microbial structure of each treatment was significantly different from all other treatments using analysis of similarity (ANOSIM $R>0.448$; $p<0.032$), day 5 microbial communities in treatments containing nitrite (Nitrite, Nitrite+FLK) were the most similar to each other. They were dominated by bacteria from the genera *Psychromonas*, *Pseudoalteromonas* and *Psychrobacter*, with low relative abundance of bacteria from the genus *Pseudomonas* (order Pseudomonadales; Figures 5 and 6). Conversely, the relative abundance of bacteria from the genera *Pseudomonas* and *Brochothrix* was high in samples from the control treatment at day 5. At day 5, the FLK treatment was intermediate between the control and treatments with nitrite; three replicates were comparable to treatments with nitrite, while two replicates were more similar to control treatment samples (Figure 6).



406

Figure 6: (**Left panel**) Non-metric multi-dimensional (nMDS) plot of microbial communities in meat samples. 407
Data were rarefied to 17,000 sequences per sample, and analysis was performed at the taxonomic level of genus. 408
Data were square-root transformed. The 2D stress of the nMDS plot is 0.07. Analysis of similarity (ANOSIM) 409
analyses demonstrated that all groups were significantly different than each other (P=0.008 to 0.032; R values 410
ranged from 0.448 to 1). (**Right panel**) Average abundance of the ten most abundant bacterial orders in the data 411
set, representing >94% of all sequences from all samples. 412

Bacteria from the order *Pseudomonadales* were most abundant in d5 control and d5 FLK 413
samples. Thus, we confirm in this study that spoilage leads to a significant decrease in alpha 414
diversity and represents a large increase in the relative abundance of bacteria from two 415
genera, *Pseudomonas* and *Brochothrix*. In the presence of nitrite or nitrite/FLK, microbial 416
community composition was only modestly altered relative to the time 0 control, likely due to 417
the absence of substantial microbial growth. In all samples, the relative abundance of bacteria 418
from the genera *Escherichia-Shigella* decreased dramatically from time 0 to day 5, regardless 419
of treatment. 420

Cultivation-based analyses demonstrated a significant reduction in the absolute abundance of 421
bacteria in meat treated with sodium nitrite and synthetic RPMs. Cultivation-independent 422
analyses demonstrated that in the presence of nitrite, microbial community structure was 423
similar to that of the baseline, despite a decrease in the absolute abundance of viable cells. 424
This is consistent with broad-spectrum bactericidal activity. The FLK-only treatment, which 425

did reduce the absolute abundance of viable cells, had poor activity against bacteria from the genus *Pseudomonas*, as indicated by the elevated relative abundance of *Pseudomonas* in FLK-only samples. At day 5, the observed microbial community structure in the FLK-only treatment was intermediate between treatments with nitrite and the control and demonstrated greater within-treatment variability in the observed microbial community.

Numerous bacterial taxa have been previously described in meat spoilage systems, including bacteria from the phyla Firmicutes, Proteobacteria and Bacteroidetes. These organisms have been shown to be responsible for the effects on sensorial properties (Hyldgaard, *et al*. 2015; Raimondi, *et al*. 2018; Thomas, *et al*. 2011; Benson, *et al*. 2011). In the control treatment, the relative abundance of both *Pseudomonas* and *Brochothrix* increased significantly compared to the baseline and other treatments at day 5 (Figure S3). *Brochothrix* was partially inhibited by FLK RPM treatment, with significantly lower relative abundance in the day 5 FLK treatment relative to the day 5 control (Figure S4). *Brochothrix* is a significant contributor to the spoilage of cooked meat products (Nychas, *et al.* 2008) and aerobically spoiled meat that has been stored under refrigerated conditions (Kilcher, *et al.* 2010; Russo, *et al*. 2006). We observed that the relative abundance of *Brochothrix* was significantly lower in the presence of nitrite and moderately so with FLK alone (Figure S5). However, the shift in relative abundance of bacteria from the genus *Pseudomonas* was most strongly associated with spoilage (*i.e.*, control treatment, day 5) in this study and in prior studies (Nychas, *et al.* 2008). The growth of *Pseudomonas* was inhibited in treatments containing nitrite, and with FLK alone a moderate reduction was observed compared to the control treatments at day 5. Other bacteria, such as those from the genera *Carnobacterium* and *Lactobacillus*, are potential spoilers of poultry meat (Rouger, *et al*. 2017) but the relative abundance of these two taxa was extremely low in this study, never exceeding 0.5% of the observed microbial community in any sample.

3.4 Antimicrobial activity in inoculated minced turkey meat

Cultivation-independent microbiome analyses demonstrated that one of the main contributors to meat spoilage was *Pseudomonas* spp. Bacteria from the species *P. putida* are also often isolated from aerobically spoiled meat (Hyldgaard, *et al.* 2015; Doulgeraki, *et al*. 2012), even when stored at 4°C. Previous studies have shown that ε-Poly-L-lysine, a cationic peptide produced by *Streptomyces albulus*, has weak antimicrobial activity against *P. putida* when added to the meat alone. In our study we evaluated the antimicrobial activity of FLK (1.92 mM) against a representative food spoilage bacterial strain, *P. putida* KT2440, as part of a combined treatment with sodium nitrite (1.08mM) (Figure 7).

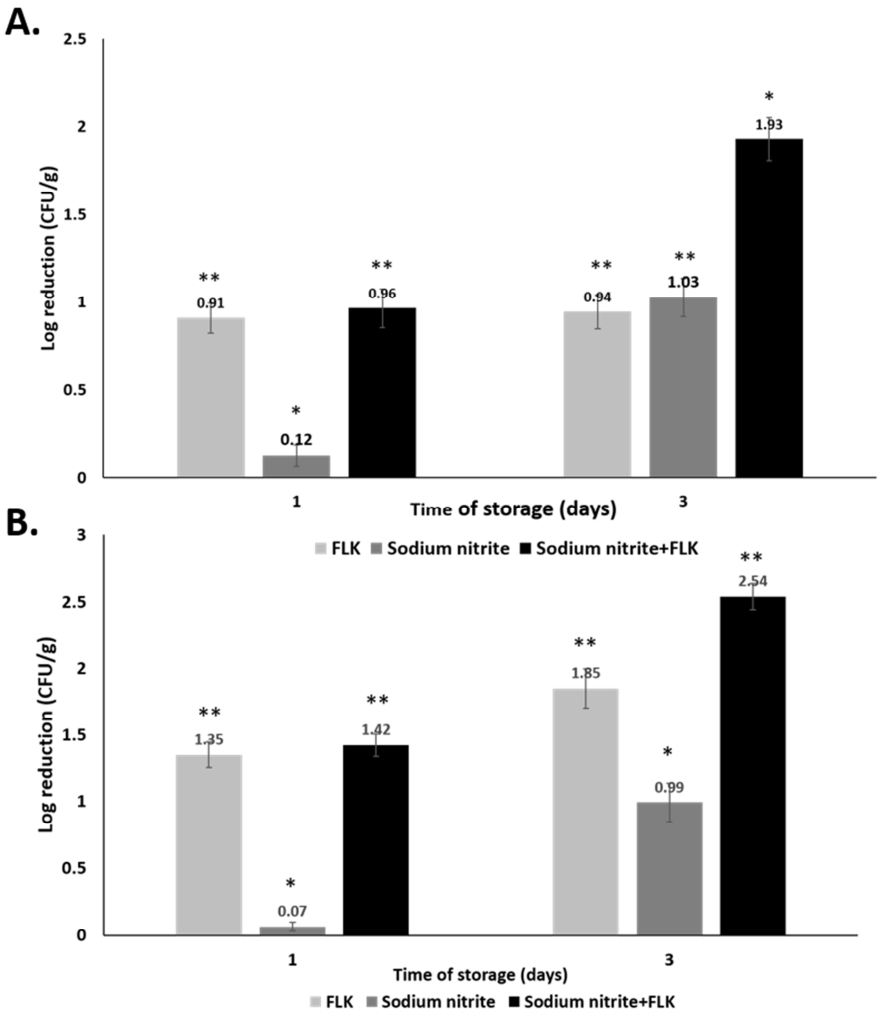


Figure 7: Antimicrobial activity of FLK (1.92 mM) and sodium nitrite (1.08 mM) alone and in combination against *P. putida* and *L. monocytogenes* in minced turkey meat stored at 4°C. The y-axis represents the decrease in total aerobic heterotrophic bacterial CFU/g between control and treatment samples (mean ± SEM, n = 9). Initial microbial loads of (A) *P. putida* (10^4 CFU/mL) and (B) *L. monocytogenes* (10^4 CFU/mL) were used. Samples was diluted, plated and counted at time point 0, 1 and 3 days of storage (mean ± SEM, n =9 for both bacteria inoculation). *,**$p < 0.01$ indicates a statistically significant difference between treatments at the day of measurement.

Due to their ability to proliferate at low temperature, bacterial strains in the control samples increased significantly during the storage period. Initial populations of ~10^4 CFU/g increased to approximately 10^6 CFU/g by the end of 3 days at 4°C in control treatments. When FLK was added to the inoculated meat, an approximate 0.9 log CFU/g reduction of *P. putida* was observed relative to the control after 3 days. A combination of FLK and sodium nitrite (1.08 mM) was found to be most effective for inhibition of bacterial growth, and compared to the control treatment, a combination of FLK and nitrite led to an approximately 1.9 log CFU/g reduction in cell numbers after 3 days of incubation, and this was significantly greater than for FLK or nitrite treatment alone (Figure 7A). Similar findings were observed for the foodborne pathogen *Listeria monocytogenes*, a Gram-positive pathogenic bacterium that has been associated with several outbreaks of foodborne disease over the past decade due in part to a wide temperature range for survival and growth (1–44°C) (Solomakos, *et al*. 2008; Farber and Peterkin 1991). This non-spore forming intracellular bacteria causes listeriosis, which can lead to septicemia, meningitis, gastroenteritis and fetal death. In meat incubated with *L. monocytogenes*, FLK alone led to a significant reduction in microbial growth, by a decrease of 1.85 CFU/g and was superior to nitrite alone (Figure 7B). However, a combination of FLK and sodium nitrite improved the antimicrobial effect against *L. monocytogenes* even after 5 days of storage (Figure 7B).

4. Conclusion

Bacteria in food can lead to its spoilage and is therefore a significant economic burden and a major public health concern to society. The use of preservatives is essential to mitigate spoilage but there are toxicity issues associated with current antimicrobial agents such as sodium nitrite. Therefore, there is a need to develop new bactericidal agents and food preservative regimens that are less toxic to humans. In this study, we evaluated the potential of antimicrobial RPMs as effective and less toxic food preservatives. Our findings reveal strong antimicrobial activity for 20-mer RPMs that consist of randomized combinations of the amino acids phenylalanine, leucine and lysine against multiple strains of spoilage bacteria. We also evaluated the antimicrobial activity of RPMs in combination with sodium nitrite. By using this approach, we were able to lower the concentration of nitrite required to suppress the spoilage of the meat and the growth of selected bacteria such as *P. putida* and *L. monocytogenes*.

Treating food products with antimicrobial agents can trigger microbiota shifts, as they have the potential to inhibit or even eliminate certain populations which can create new opportunities for the growth of other spoilage organisms or even pathogens. To address this concern, we performed non-targeted analysis of microbial community structure in meat samples incubated with and without antimicrobial compounds. We observed that microbial diversity in the control treatment after 5 days was significantly lower than the baseline diversity due to the growth of bacteria from the genera *Pseudomonas* and *Brochothrix*. However, the microbial diversity of the nitrite and nitrite/FLK treated meat was not significantly different from that of the baseline microbial diversity. In addition, community structure of nitrite and nitrite/FLK samples was comparable after five days of incubation, and most similar to the baseline community structure. Cultivation-based analyses determined that

a significant reduction in the absolute abundance of bacteria in meat treated with sodium nitrite and/or RPMs had occurred. Our findings demonstrate the great potential of RPMs as safe and effective food preservatives. Ultimately, their usage could lead to a significant improvement in the economics of food production and better outcomes for human health.

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Highlights:

- Random peptide mixtures, composed of Phenylalanine:Leucine:Lysine (FLK) showed broad and strong antimicrobial activity.
- Addition of random antimicrobial peptide mixtures (FLK) to turkey minced meat led to significant reductions in bacterial abundance in experimental tests.
- Random antimicrobial peptide mixtures (FLK) showed high synergistic activity when were combined with sodium nitrite to reduce bacterial loads.
- Sodium Nitrite required concentration was dramatically reduced to prevent toxic effect.
- Using high-throughput 16S ribosomal RNA gene amplicon sequencing, we showed strong antimicrobial activity for random antimicrobial peptide mixtures against spoilage bacteria in meat.
- Random antimicrobial peptide mixtures have great potential as safer preservatives for reducing spoilage in meat and other food products.