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Antimicrobial peptide cocktail activity in minced turkey meat

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Abstract:

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Meat products contain valuable nutrients that are important for human health and 11 development but are also highly susceptible to colonization by microorganisms. This can lead 12 to spoilage and serious foodborne illnesses. Natural antimicrobial peptides, produced by 13 many organisms as part of their innate immune system to fight microbial infections, have 14 great potential as food preservatives. In this study, we explored the effect of ternary 15 antimicrobial random peptide mixtures (RPMs) on food spoilage bacteria in minced turkey 16 meat. Amendment of RPMs to meat led to significant reductions in bacterial abundance in 17 experimental tests, and RPMs worked synergistically with nitrite to reduce bacterial loads. 18 Using high-throughput 16S ribosomal RNA gene amplicon sequencing, we characterized the 19 effect of RPMs and nitrite on meat microbial community structure before and during 20 incubation under refrigerated conditions. Our findings reveal strong antimicrobial activity for 21 RPMs against spoilage bacteria in meat, including Listeria monocytogenes and Pseudomonas 22 putida. These results demonstrate the potential of RPMs as a safer preservative for reducing 23 spoilage in meat and other food products. 24

<u>1. Introduction:</u>

Meat products are part of the recommended human diet and contain valuable nutrients that 27 are important for health and development (Hyldgraad, et al. 2015). However, microorganisms 28 easily colonize and proliferate in fresh meat due to an excess of nutrients and a 29 moist environment, which leads to spoilage (Hyldgraad, et al. 2015; Lamas, et al. 2016). The 30 contamination and spoilage of food products is a problem of global concern, since the growth 31 and metabolism of microorganisms can cause serious foodborne illnesses and food loss 32 (Böhme, et al. 2012; Borch, et al. 1996). Therefore, maximum allowable levels of mesophilic 33 aerobic and facultative anaerobic microorganisms have been mandated by health agencies 34 worldwide (Stieglmeier, et al. 2009). 35

To suppress microbial growth in meat, preservatives such as sodium nitrite are used (Lamas, 36 et al. 2016; Serrano, et al. 2012; Müller-Herbst, et al. 2016). As a food additive, sodium 37 nitrite has three key functions: (i) contributing to flavor by inhibiting the development of 38 rancid off-flavors; (ii) preserving the strong pink color of meat via reactions with myoglobin; 39 and (iii) preventing the growth of pathogenic bacteria such as the toxin-forming Clostridium 40 botulinum (Cammack, et al. 1999; Crosby, et al 1976). Although the activity of sodium nitrite 41 has been extensively studied, its mode of action is still not completely understood. Inhibition 42 of respiration has been proposed as one possible mode of action of nitrite toward C. 43 botulinum (McMindes, et al 1988). Nitric oxide (NO), formed via nitrite reduction, has been 44 suggested as the primary bacteriostatic compound in nitrite-amended food. NO interacts with 45 the iron-sulfur proteins of bacteria (e.g., cytochromes), which are important for microbial 46 energy metabolism (Cammack, et al. 1999; Tompkin, et al. 1978). Nitrite may also inhibit 47 pyruvate-ferredoxin reductases, leading to bacterial cell death (McMindes, et al 1988). 48

Although the preservative function of sodium nitrite has been well established, there has 49 recently been a greater focus on its toxicity to humans. Nitrites are toxic at high 50 concentrations as are N-nitroso compounds (nitrosamines) which form when nitrites react 51 with secondary amines in the acidic conditions of the stomach. Compounds such as 52 N-nitrosodimethylamine have been shown to be carcinogenic in several animal species 53 (Lamas, et al. 2016; Cammack, et al. 1999; Honikel, 2008). Thus, there is an urgent need to 54 replace nitrite in the meat industry with safer preservatives (Rydlo, et al. 2008; Anderson, et 55 al. 2004). 56

Natural antimicrobial peptides (AMPs) and host defense peptides (HDPs) are produced by 57 eukaryotic innate immune systems. Their biological role in eukaryotic organisms is to 58 eliminate Gram-positive and Gram-negative bacteria, as well as fungi and viruses. In 59 bacterial infections, these compounds are a component of the host immune response, and act 60 primarily by disrupting bacterial cell membranes. As a result, these classes of peptides have 61 great potential as effective and safe preservatives (Diamond, et al. 2009; Malheiros, et al. 62 2010; Nakatsuji, and Gallo, 2012; Cleveland, et al. 2001; Rathinakumar, et al. 2009). Most 63 AMPs possess common structural features such as positive charge and moderate 64 hydrophobicity (~50%). This amphipathicity enables them to interact with and permeabilize 65 negatively charged membranes of bacteria, resulting in cell membrane disruption (Nakatsuji, 66 and Gallo, 2012; Rathinakumar, et al. 2009; Brogden, 2005; Wimley and Hristova 2001; 67 Hancock, 2001). Previous studies have evaluated AMPs as preservatives (Anderson, et al. 68 2004). For example, an analogue of magainin (an AMP isolated from frog skin) possessed 69 strong antimicrobial activity against 13 pathogenic bacterial strains associated with foodborne 70 illnesses (Abler, et al. 1995). Elsewhere, the activity of a synthetic peptide bearing six leucine 71 and eight lysine residues was studied against a range of foodborne microorganisms including 72 *Listeria monocytogenes* (Aiyegoro, 2014; Appendini, and Hotchkiss, 2000) 73

Despite the promise of AMPs as safer meat preservatives, there are still several challenges 74 that must be addressed: (i) they must be effective against a diverse array of microorganisms; 75 (ii) phospholipids or proteins can potentially suppress their antimicrobial activity; (iii) AMPs 76 can be degraded rapidly by proteases (Anderson, et al. 2004; Malheiros, et al. 2010); (iv) 77 rapid development of antimicrobial resistance can occur (Mayrhofer, et al. 2004; Dobson, et 78 al. 2014; Perron, et al. 2006; Pränting, et al. 2008; Habets et al. 2012; Dobson, et al. 2013); 79 and (v) cost of manufacture (Wimley and Hristova, 2011). Although these challenges are 80 daunting, there is already one AMP preservative on the market which indicates feasibility. 81 The antimicrobial peptide-based preservative nisin (produced by certain strains of 82 Lactococcus lactis) has been approved by the FDA (Cleveland, et al. 2001) and is effective 83 against Gram-positive bacteria, including spores, but shows very low activity against Gram-84 negative bacteria, yeasts and molds²⁸. Nisin has been widely used as an exogenous addition to 85 a variety of food products around the world and is also naturally present in many dairy 86 products (Rydlo, et al. 2008; Müller-Auffermann, et al. 2015). Nisin has a dual mechanism of 87 action, which is facilitated by binding to the peptidoglycan precursor, lipid II. At lower 88 concentrations, nisin interferes with cell wall synthesis and at higher concentrations it forms 89 pores that disrupt the proton motive force in bacterial membranes (Müller-Auffermann, et al. 90 2015). When examined as a meat preservative, nisin displayed strong antimicrobial activity in 91 inoculated minced beef against Gram-positive L. monocytogenes; conversely, application of 92 nisin in minced sheep meat showed no antimicrobial activity against Salmonella Enteritidis 93 (Solomakos, et al. 2008; Govaris, et al. 2010). 94

The structural diversity of AMPs suggests that their activity is not tightly linked to a specific 95 amino acid sequence (Rathinakumar, *et al.* 2009). This observation led to the development of 96 random peptide mixtures (RPMs) as antimicrobial agents (Hayouka, *et al.* 2013). During 97 peptide synthesis, instead of using one amino acid at each coupling step, a mixture of two or 98

more amino acids (at a known stoichiometry) are used. The result is $2^{n}/3^{n}$ (where n represents 99 the peptide chain length equal to the number of coupling steps) sequences of random peptides 100 composed of hydrophobic and cationic amino acids but with controlled chain length and 101 stereochemistry. This novel AMP synthesis strategy may overcome some difficulties 102 associated with specific sequence of AMPS (Hayouka, et al. 2013; Topman, et al. 2018; 103 Stern, et al. 2016; Amso and Hayouka 2019), as this approach is cheaper and may confound 104 bacterial attempts to develop resistance. The aim of the current study was to investigate the 105 antimicrobial activity of RPMs in food. We have used minced turkey meat as a food model 106 and have coupled cultivation approaches with cultivation-independent molecular 107 characterization of microbial community structure to gain insights into the activity of AMPs 108 in meat. 109

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2. Material and Methods:

2.1 Synthesis of random peptide mixtures

RPMs were synthesized using the traditional solid phase peptide synthesis (SPPS). Synthesis 112 of random peptide mixtures (RPMs) was carried out according to Hayouka et al. (2013). 113 RPMs were synthesized using microwave irradiation on Rink Amide resin (Substitution 114 0.53 mmol g-1, 25 µmol) in Alltech filter tubes. Coupling reactions were conducted with 115 binary combinations of protected amino acids, with a freshly prepared stock solution that 116 contained the protected amino acids in 1:1 molar ratio of L Phenylalanine, L-leucine, and L 117 -lysine (25 µmol) of each amino acid, which were used for each coupling step. Upon 118 completion of the synthesis (20 cycles for 20 mer peptide chain length), the RPMs were 119 cleaved from the resin, resuspended in double distilled water (DDW), frozen on dry ice and 120 lyophilized. RPMs were analyzed by MALDI TOF to evaluate molecular weight and quality 121 and by amino acid analysis. 122

2.2 Assessment of minimal inhibitory concentration (MIC) values:

To determine the antimicrobial activity of FLK (L Phenylalanine, L-leucine, and L -lysine), 124 FK (L \square Phenylalanine and L -lysine) and ${}^{D}F^{D}L^{D}K$ (D \square Phenylalanine, D-leucine, and D -125 lysine) RPMs, MIC values were measured for B.subtilis NCIB 3610, L. monocytogenes 126 10403S, P. putida KT2440 and E. coli rp MG1655 strains (Table 1). MICs were determined 127 by growth in sterile 96 well plates (Corning 3650) by a broth microdilution method as 128 described by Hayouka et al. (2013). Bacteria were grown for 24 h in brain heart infusion 129 broth (BHI, HiMedia Laboratories, India) or Lysogeny broth (LB; BD, USA)) at 30°C or 130 37°C depended on the bacteria type with shaking (200 rpm). Then, the bacterial cultures were 131 diluted in growth medium to an optical density at 600 nm (OD600) of 0.1 using a 132 ThermoSpectronic (Genesys 10uv) spectrophotometer. 100 µl aliquots were added to 100 µl 133 of growth medium containing RPMs at various concentrations in each well. The plates were 134 then incubated at 30°C or 37°C for 24 h. Bacterial growth was determined by measuring the 135 OD at 595 nm using a Tecan Infinite Pro Plate reader. The MIC values were the lowest 136 concentrations of the peptide mixtures that caused inhibition of bacterial growth (Hayouka, et 137 al. 2013). MIC values were determined as the average obtained from three independent 138 experiments. The highest concentrations tested were 200 µg/ml for RPMs, 1 mg/ml (14.49 139 mM) for sodium nitrite and 0.25 mg/ml (0.074 mM) for Nisin. 140

2.3 Meat preparation:

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Fresh minced turkey meat was purchased at a local super-market and immediately transferred 142 to the lab. The meat was ground for a second time in an ethanol cleaned grinder and divided 143 into 40 gr portions. Samples were stored at -20°C. At the beginning of each experiment, a 40 144 gr portion was defrosted at 4 °C. The portion was ground and divided into 10 gr or 1 gr meat 145 balls, and each meatball was placed in a sterile test tube. 150 μ l of double distilled water 146

Name	Growth conditions: Media/ Temperature and antibiotic	

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ol sample test tubes. For treatment samples, 150 µl of DDW containing each of the different 153 treatment compounds was added to each test tube. For treatments with sodium nitrite, final 154 concentrations of 2.17 /1.08 /0.5 mM were used. For treatments with RPMs of FLK (L 155 Phenylalanine, L-leucine, and L -lysine) or ${}^{D}F^{D}L^{D}K$ (D Phenylalanine, D-leucine, and D -156 lysine), RPMs were dissolved in double distilled water (DDW) to final concentration of 157 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 158 concentration of 0.074 mM. For the combination of sodium nitrite and FLK, the 150 µl 159 solution added to test tubes contained FLK random peptide mixture dissolved in DDW at a 160 final concentration of 1.92 mM and 5 µl of sodium nitrite dissolved in DDW to arrive at a 161 final concentration of 1.08 mM or 0.5 mM. Samples were mixed and stored under 162 refrigerated conditions at 4 °C for the length of the experiment. Microbiological analyses 163 were performed at 0, 1, 3 and 5 days of storage. 164

Table S1. Bacterial strains and growth conditions used in this study.All strains were165maintained at -80°C in glycerol stock (25% v/v) until use.166

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L. monocytogenes	BHI, 37°C, overnight, 100 µg/ml streptomycin	170
10403S		171
P. putida KT2440	LB, 30°C, overnight, 100 µg/ml ampicillin	172
<i>B. subtilis</i> NCIB 3610	BHI, 37°C, overnight	
E. coli rp	BHI, 37°C, overnight	173

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The L. monocytogenes 10403S culture was a generous gift from Prof. Anat Herskovits from175Tel Aviv University. The strain (10403S) was modified by deleting the hly gene that codes176the listeriolysin O toxin responsible for the species' virulence and has a streptomycin177resistance.178

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2.4 Inoculated meat samples:

1 g samples of minced turkey meat were placed in sterile test tubes and inoculated with single 181 strain of L. monocytogenes or P. putida separately (ca. 10^4 CFU/g). The bacterial cultures 182 were diluted in the appropriate medium to an optical density at 600 nm (OD600) of 0.1 using 183 a ThermoSpectronic (Genesys 10uv) spectrophotometer. Cultures were diluted to 10^5 184 CFU/ml in saline solution 0.9% for inoculation of turkey meat samples. Microbial load was 185 determined by serial dilution and plating on BHI agar or LB agar plates. 100 µl/g of the 10^5 186 CFU/ml bacterial stock was used to inoculate the meat. To ensure proper distribution of the 187 bacteria, the samples were properly mixed before addition of the treatment. Subsequently, 188 sodium nitrite (1.08 mM), FLK (1.92 mM) and their combination were added to the 189 inoculated samples. 190

2.5 Microbiological analysis:

To monitor the microbial load, we evaluated the samples at different time points 0, 1, 3 and 5 192 days. After the treatment, a 9 ml saline solution 0.9% was added to each 1 gr minced turkey 193 sample. Samples were vigorously vortexed for 60 seconds at room temperature, and then 194 serially diluted 1:10 in 0.9% saline solution. 100 µl from each sample were spread plated by 195 duplicates on LB agar or BHI agar plate and held at 30 °C for 24 h. Each sample was 196 analyzed with at least three independent repetitions. After 24 h, the microbial load was 197 determined and the average number of CFU per gram was calculated by counting plates 198 containing 20-200 colonies. 199

2.6 Statistical analysis

The results are presented as the mean \pm SEM. One-way analysis ANOVA of variance 201 followed by Tukey post-hoc analysis was used for statistical analysis. An independent T test 202 analysis which compares the means of the treatments was performed. The results were 203 considered to be statistically significant if p < 0.05 or p < 0.01 as mentioned for each 204 experiment. 205

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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 207 portions, one was used for DNA extraction and the other was used for cultivation-based 208 approaches. Samples were amended with the following compounds: double-distilled water 209 (DDW; 'Control'), 1.92 mM (5 mg/ml) of RPMs FLK ('FLK'), 1.08 mM sodium nitrite (75 210 ppm), and a combination of sodium nitrite (1.92 mM) and FLK (1.08 mM) ('FLK+Nitrite). 211 Samples were stored at 4°C. Samples were taken at day 0 (prior to amendment), after 3 days, 212 and after 5 days. Total genomic DNA (gDNA) was extracted using an Exgene[™] Soil DNA 213 Prep Kit (Songpa-gu, Korea), following the manufacturer's standard protocol. Genomic DNA 214 was PCR amplified with primers CS1_515Fb and CS2_806Rb (modified from the primer set 215

employed by the Earth Microbiome Project (EMP; GTGYCAGCMGCCGCGGTAA and 216 GGACTACNVGGGTWTCTAAT) targeting the V4 regions of microbial small subunit 217 ribosomal RNA genes. Amplicons were generated using a two-stage "targeted amplicon 218 sequencing (TAS)" protocol (Naqib, et al. 2018; Bybee, et al. 2011). The primers contained 219 5' common sequence tags (known as common sequence 1 and 2, CS1 and CS2) as described 220 previously (Moonsamy, et al. 2013; Green, et al. 2015). First stage PCR amplifications were 221 performed in 10 microliter reactions in 96-well plates, using the MyTaq HS 2X mastermix. 222 PCR conditions were 95°C for 5 minutes, followed by 28 cycles of 95°C for 30", 55°C for 223 45" and 72°C for 60." 224

Subsequently, a second PCR amplification was performed in 10 microliter reactions in 96-225 well plates. A mastermix for the entire plate was made using the MyTaq HS 2X mastermix. 226 Each well received a separate primer pair with a unique 10-base barcode, obtained from the 227 Access Array Barcode Library for Illumina (Fluidigm, South San Francisco, CA; Item# 100-228 4876). These Access Array primers contained the CS1 and CS2 linkers at the 3' ends of the 229 oligonucleotides. Cycling conditions were as follows: 95°C for 5 minutes, followed by 8 230 cycles of 95°C for 30", 60°C for 30" and 72°C for 30". A final, 7-minute elongation step was 231 performed at 72°C. Samples were pooled in equal volume using an EpMotion5075 liquid 232 handling robot (Eppendorf, Hamburg, Germany). The pooled library was purified using an 233 AMPure XP cleanup protocol (0.6X, vol/vol; Agencourt, Beckmann-Coulter) to remove 234 fragments smaller than 300 bp. The pooled libraries, with a 20% phiX spike-in, were loaded 235 onto an Illumina MiniSeq mid-output flow cell (2x153 paired-end reads). Fluidigm 236 sequencing primers, targeting the CS1 and CS2 linker regions, were used to initiate 237 sequencing. De-multiplexing of reads was performed on instrument. Library preparation, 238 pooling, and sequencing were performed at the University of Illinois at Chicago Sequencing 239 Core (UICSQC). 240

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 243 spoilage bacteria in minced turkey meat. We previously described the antimicrobial activity 244 of different RPMs composed from a binary combination of hydrophobic and cationic residues 245 where the most active mixtures were 20-mers containing L-leucine (L) and L-phenylalanine 246 (F) as their hydrophobic residue with L-lysine (K) as the cationic amino acid (Hayouka, et al. 247 2013). Here, we designed and synthesized for the first time a ternary random peptide mixture 248 by combining the most active cationic amino acid residue (Lysine) with the two most active 249 hydrophobic amino acids residues (Leucine and Phenylalanine). These ternary peptide 250 mixtures FLK was composed of 25% F, 25% L, and 50% K to preserve the optimal 1:1 251 proportion between cationic and hydrophobic amino acids. To verify the subunit proportion 252 after synthesis, we performed amino acid analysis and determined the molecular weight range 253 of the mixture using MALDI-TOF mass spectrometry (Figure S1). In addition, we 254 synthesized a ternary enantiomer consisting of a D-homochiral random peptide mixture of D-255 phenylalanine (^DF), D-leucine (^DL), and D-lysine (^DK) to evaluate the effect of 256 stereochemistry on bioactivity. 257

To determine the antimicrobial activity of the ternary RPMs, we performed minimal 258 inhibition concentration (MIC) assays using *Bacillus. subtilis* and *Listeria. monocytogenes* as 259 model Gram-positive bacteria, and *Pseudomonas*. *putida* and *E. coli* as model Gram-260 negative bacteria (**Table 1**). The new FLK RPM showed broad antimicrobial activity toward 261 all tested bacteria. The MIC values for *B. subtilis, L. monocytogene* and *P. putida* were 13 262 μ g/mL; for *E. coli* the MIC value was 25 μ g/mL. Both FLK and ^DF^DL^DK possessed strong 263

bacteriostatic activity against the tested bacteria as compared to FK peptide mixtures and264similar activity to LK peptide mixtures.265

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Table 1. Minimum inhibitory concentration (MIC) values for LK, FK, FLK ${}^{D}F^{D}L^{D}K$ RPMs, nisin, and270sodium nitrite. Values, in the units of μ g/ml, represent the median value obtained from at least three independent271repetitions for each bacterial strain with tests performed *in vitro*.272

Treatment	B. subtilis	L. monocytogenes	P. putida	<i>E. coli</i> 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

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We also compared the activity of our RPMs with nisin (Table 1), and our findings confirm 274 previous results showing that Nisin has no antimicrobial activity against Gram-negative 275 bacteria. Prior studies have shown that RPMs such as LK 20-mer and FK 20-mer can be 276 active against both Gram-positive and Gram-negative bacteria and towards mature biofilms 277 (Hayouka, et al. 2013; Topman, et al. 2018; Stern, et al. 2016; Amso and Hayouka 2019), 278 and we observed that our RPMs were indeed active against both Gram-negative and -positive 279 bacteria. No inhibition of bacterial growth was observed in vitro when sodium nitrite was 280 added (14.49 mM, 1 mg/mL), despite this concentration being significantly higher than 281

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. 284

Measuring the efficacy of antimicrobial agents in a food system holds several challenges. A 285 decrease in antimicrobial activity is usually observed, due to the interaction of the agent with 286 other components in the food matrix such as lipids, proteins, and sugars (Rydlo, et al. 2006). 287 To determine the effective concentration of FLK in meat, various concentrations were added 288 and the microbial load was quantified. A dose-dependent relationship was observed (Figure 289 1), whereby FLK RPM concentrations of 1.92 mM and 2.69 mM exhibited the greatest 290 antimicrobial activity. FLK at 1.92 mM concentration was therefore used for subsequent 291 experiments. 292



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Figure 1: The effect of varying FLK concentration (mg/ml) on total aerobic heterotrophic bacterial abundance294in minced turkey meat during storage at 4°C. The y-axis represents the decrease in cfu/g between the control295and treatment samples (mean \pm SEM, n = 9). Samples were diluted, plated, and counted on after 0, 1, and 3 days296of storage. a,b,c p < 0.01 indicates a statistically significant difference between the treatments at the day of</td>297treatment.298

The antimicrobial activity of both FLK and ^DF^DL^DK (1.92 mM) were compared to nisin 300 (0.074 mM, Figure 2) and sodium nitrite (2.17 mM, Figure 3) in minced turkey meat. The 301 microbial load of the minced turkey meat was assessed at 1, 3 and 5 days post-inoculation, 302 and compared to the microbial loads at day 0 (Figure 3). FLK displayed strong antimicrobial 303 activity and stability even after 5 days of storage, and there was no substantial advantage in 304 using the ^DF^DL^DK (Figure S2). After 3 days of incubation the antimicrobial activity of FLK 305 and nitrite was similar (~1 log CFU/g reduction). After 5 days FLK displayed strong 306 antimicrobial activity, representing an average log reduction of ~1.55 CFU/g, while sodium 307 nitrite displayed stronger antimicrobial activity of ~3 log CFU/g reduction. Nisin at this level 308 showed weak antimicrobial activity toward meat bacterial population with a log reduction of 309 maximum 0.5 log CFU/g during the entire storage period (Figure S3). This poor 310 antimicrobial activity of nisin in meat has been reported previously (Cleveland, et al. 2001; 311 Solomakos, et al. 2008; Govaris, et al. 2010). 312



Figure 2: Comparing the antimicrobial activity of RPMs with Nisin. The antimicrobial activity of Phe-Leu-314Lys (FLK, 1.92 mM) and Nisin (0.074 mM) against meat bacterial population in minced turkey meat during315storage at 4 °C. Samples were diluted, plated, and counted at different time points; 0, 1, 3 and 5 days of storage316(mean \pm SEM, n = 17,7,8). *p < 0.01 indicates a statistically significant difference between the RPMs and Nisin</td>317treatments at the day of treatment.318

3.2 The effect of combining FLK and sodium nitrite

Despite the health concerns regarding sodium nitrite usage, it remains one of the most 320 common meat preservatives. The use of nitrite is primarily due to its strong antimicrobial 321 activity against C. botulinum, a heat-resistant, spore-forming, toxin producer that causes 322 botulism (Lamas, et al. 2016; Cammack, et al. 1999). The efficacy of antimicrobial 323 compounds can sometimes be potentiated by utilizing them in combination (Marquette and 324 Bechinger 2018). Mixtures of AMPs and conventional antibiotics have shown synergistic 325 activity (Rank, et al. 2017; Kim, et al. 2017; Chou, et al. 2016), typically due to two different 326 modes of action (Marquette and Bechinger 2018). For example, AMPs that cause damage to 327 bacterial cell membranes (which does not necessarily result in cell death) will increase 328 membrane permeability, which could lead to improved efficacy of sodium nitrite. For this 329 reason, the combination of sodium nitrite and RPMs could lead to an improvement in 330 antimicrobial activity whilst reducing the amount of sodium nitrite required to suppress 331 bacterial growth, and hence its associated health risks. Therefore, we monitored microbial 332 growth in turkey meat with a combination of FLK (1.92 mM) and sodium nitrite at 333 concentrations of 1.08 mM (half dose) and 0.5 mM (quarter dose, Figure 4). The treatment 334 regimen with the half dose of sodium nitrite resulted in significantly lower microbial load 335 after both 3 and 5 days, compared to the individual treatments. By combining RPM with 336 nitrite, we were able to reduce the effective concentration of nitrite by 50%. We further 337 reduced the nitrite concentration to a quarter dose and still observed a synergistic effect. After 338 5 days of storage at 4°C the antimicrobial effect was maintained, with a significant log 339 reduction of ~2.5 log CFU/g or greater at both nitrite concentrations. 340



Figure 3: Antimicrobial activity of FLK (1.92 mM) and nitrite (2.17 mM / 150 ppm) in minced turkey342meat stored at 4°C. The y-axis represents the decrease in total aerobic heterotrophic bacterial CFU/g between343control and treatment samples (mean \pm SEM, n = 9). Meat samples were diluted, plated, and counted after 1, 3344and5 days of storage. *p < 0.01 indicates a statistically significant difference between the RPMs treatments and</td>345sodium nitrite treatments at the day of treatment.346

3.3 The effect of treatment on meat microbial community structure

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Rapid microbial growth in meat contributes to the development of an off flavor and/or color, 348 leading to meat that is unappealing and unsuitable for human consumption. The diversity and 349 composition of meat microbial communities is dependent on the storage conditions and 350 competition between organisms present in the meat (Hyldgaard, et al. 2015; Doulgeraki, et 351 al. 2012). Therefore, the effect of treatment on the microbial community structure in turkey 352 meat was determined, via a combination of cultivation-dependent and cultivation-353 independent analyses. Cultivation-independent analyses were performed by DNA extraction 354 and 16S rRNA gene amplicon sequencing, and these analyses generated information 355 regarding the relative abundance of microbial taxa in samples. Prior studies have shown that 356

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 at361different concentrations in minced turkey meat stored at 4°C. The y-axis represents the decrease in total362aerobic heterotrophic bacterial CFU/g between control and treatment samples (mean \pm SEM, n = 9). (A) Sodium363nitrite concentration of 1.08 mM (75 ppm); and (B) sodium nitrite concentration of 0.5 mM (35 ppm). Samples364were diluted, plated, and counted at 0, 1, 3 and 5 days (mean \pm SEM, n = 17, 10, 14). *,**p < 0.01 indicates a</td>365statistically significant difference between treatments and the control at the same day.366

Microbial communities changed in all treatments during the five days of incubation, though 367 the magnitude of the effect differed between treatments (Figures 5 and 6). Alpha diversity 368 (within-sample diversity) was calculated using the Shannon index (a measure of microbial 369 richness and evenness) at the taxonomic level of genus (Figure 5). Microbial diversity in the 370 control treatment at day 5 was significantly lower than the baseline diversity (Mean Shannon 371 Index, 1.78 vs 2.37; Tukey's test p=0.029). In addition, microbial diversity (Shannon Index) 372 in the control treatment at day 5 was significantly lower than the diversity of sodium nitrite 373 treatment and the sodium nitrite/FLK combined treatment at day 5 (Tukey's test p<0.015). 374 The microbial diversity of the sodium nitrite and nitrite/FLK combined treatment was not 375 significantly different from that of the baseline microbial diversity. 376



Figure 5: Shade plot of 20 most abundant microbial taxa based on genus-level annotations. Sample 379 grouping was retained, and the scale is the square root of relative abundance. Bactria from the genus 380 Pseudomonas were most abundant in FLK (day 5 or d5) and control (d5) samples, while Escherichia-Shigella 381 were only present substantially in the control (day 0, d0) samples. Bacteria from the genera Pseudoalteromonas, 382 Psychromonas and Psychrobacteria were abundant in samples treated with nitrite at d5. Above the shade plot is 383 a box plot of Shannon index values (genus-level) for five replicates of each group. By ANOVA, group means 384 were significantly different at the 0.05 level. Tukey's test indicated that the diversity of the day 5 control 385 samples was significantly different than that of the d0 control, d5 nitrite treatment, and d5 nitrite + FLK 386 treatment samples, but not the d5 FLK treatment samples. Diversity indices were generated from datasets 387 rarefied to 17,000 sequences/sample. 388

The change, or lack thereof, in microbial alpha diversity by these treatments was consistent 389 with the observed microbial community structure. Baseline microbial communities were 390 largely comprised of bacteria from the genera Escherichia-Shigella, Psychromonas, 391 Pseudoalteromonas, Psychrobacter and Pseudomonas (Figure 5). Bacteria from the genera 392 Escherichia-Shigella (order Enterobacteriales) were abundant in the baseline samples 393 (average relative abundance of 17.6%) and were much lower in all treatments at day 5 394 (average relative abundance of 1.0%). Although the microbial structure of each treatment was 395 significantly different from all other treatments using analysis of similarity (ANOSIM 396 R>0.448; p<0.032), day 5 microbial communities in treatments containing nitrite (Nitrite, 397 Nitrite+FLK) were the most similar to each other. They were dominated by bacteria from the 398 genera Psychromonas, Pseudoalteromonas and Psychrobacter, with low relative abundance 399 of bacteria from the genus Pseudomonas (order Pseudomonadales; Figures 5 and 6). 400 Conversely, the relative abundance of bacteria from the genera Pseudomonas and 401 Brochothrix was high in samples from the control treatment at day 5. At day 5, the FLK 402 treatment was intermediate between the control and treatments with nitrite; three replicates 403 were comparable to treatments with nitrite, while two replicates were more similar to control 404 treatment samples (Figure 6). 405



Figure 6: (Left panel) Non-metric multi-dimensional (nMDS) plot of microbial communities in meat samples.407Data were rarefied to 17,000 sequences per sample, and analysis was performed at the taxonomic level of genus.408Data were square-root transformed. The 2D stress of the nMDS plot is 0.07. Analysis of similarity (ANOSIM)409analyses demonstrated that all groups were significantly different than each other (P=0.008 to 0.032; R values410ranged from 0.448 to 1). (Right panel) Average abundance of the ten most abundant bacterial orders in the data411set, 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 431 bacteria from the phyla Firmicutes, Proteobacteria and Bacteroidetes. These organisms have 432 been shown to be responsible for the effects on sensorial properties (Hyldgaard, et al. 2015; 433 Raimondi, et al. 2018; Thomas, et al. 2011; Benson, et al. 2011). In the control treatment, 434 the relative abundance of both Pseudomonas and Brochothrix increased significantly 435 compared to the baseline and other treatments at day 5 (Figure S3). Brochothrix was partially 436 inhibited by FLK RPM treatment, with significantly lower relative abundance in the day 5 437 FLK treatment relative to the day 5 control (Figure S4). Brochothrix is a significant 438 contributor to the spoilage of cooked meat products (Nychas, et al. 2008) and aerobically 439 spoiled meat that has been stored under refrigerated conditions (Kilcher, et al. 2010; Russo, 440 et al. 2006). We observed that the relative abundance of Brochothrix was significantly lower 441 in the presence of nitrite and moderately so with FLK alone (Figure S5). However, the shift 442 in relative abundance of bacteria from the genus Pseudomonas was most strongly associated 443 with spoilage (i.e., control treatment, day 5) in this study and in prior studies (Nychas, et al. 444 2008). The growth of *Pseudomonas* was inhibited in treatments containing nitrite, and with 445 FLK alone a moderate reduction was observed compared to the control treatments at day 5. 446 Other bacteria, such as those from the genera Carnobacterium and Lactobacillus, are 447 potential spoilers of poultry meat (Rouger, et al. 2017) but the relative abundance of these 448 two taxa was extremely low in this study, never exceeding 0.5% of the observed microbial 449 community in any sample. 450

3.4 Antimicrobial activity in inoculated minced turkey meat

Cultivation-independent microbiome analyses demonstrated that one of the main contributors 453 to meat spoilage was Pseudomonas spp. Bacteria from the species P. putida are also often 454 isolated from aerobically spoiled meat (Hyldgaard, et al. 2015; Doulgeraki, et al. 2012), even 455 when stored at 4°C. Previous studies have shown that ɛ-Poly-L-lysine, a cationic peptide 456 produced by Streptomyces albulus, has weak antimicrobial activity against P. putida when 457 added to the meat alone. In our study we evaluated the antimicrobial activity of FLK (1.92 458 mM) against a representative food spoilage bacterial strain, P. putida KT2440, as part of a 459 combined treatment with sodium nitrite (1.08mM) (Figure 7). 460



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Figure 7: Antimicrobial activity of FLK (1.92 mM) and sodium nitrite (1.08 mM) alone and in462combination against *P. putida* and *L. monocytogenes* in minced turkey meat stored at 4°C. The y-axis463represents the decrease in total aerobic heterotrophic bacterial CFU/g between control and treatment samples464(mean \pm SEM, n = 9). Initial microbial loads of (A) *P. putida* (10⁴ CFU/mL) and (B) *L. monocytogenes* (10⁴465CFU/mL) were used. Samples was diluted, plated and counted at time point 0, 1 and 3 days of storage (mean \pm 466SEM, n = 9 for both bacteria inoculation). *,**p < 0.01 indicates a statistically significant difference between</td>467treatments at the day of measurement.468

Due to their ability to proliferate at low temperature, bacterial strains in the control samples 469 increased significantly during the storage period. Initial populations of $\sim 10^4$ CFU/g increased 470 to approximately 10^6 CFU/g by the end of 3 days at 4°C in control treatments. When FLK 471 was added to the inoculated meat, an approximate 0.9 log CFU/g reduction of P. putida was 472 observed relative to the control after 3 days. A combination of FLK and sodium nitrite (1.08 473 mM) was found to be most effective for inhibition of bacterial growth, and compared to the 474 control treatment, a combination of FLK and nitrite led to an approximately 1.9 log CFU/g 475 reduction in cell numbers after 3 days of incubation, and this was significantly greater than 476 for FLK or nitrite treatment alone (Figure 7A). Similar findings were observed for the 477 foodborne pathogen Listeria monocytogenes, a Gram-positive pathogenic bacterium that has 478 been associated with several outbreaks of foodborne disease over the past decade due in part 479 to a wide temperature range for survival and growth (1-44°C) (Solomakos, et al. 2008; 480 Farber and Peterkin 1991). This non-spore forming intracellular bacteria causes listeriosis, 481 which can lead to septicemia, meningitis, gastroenteritis and fetal death. In meat incubated 482 with L. monocytogenes, FLK alone led to a significant reduction in microbial growth, by a 483 decrease of 1.85 CFU/g and was superior to nitrite alone (Figure 7B). However, a 484 combination of FLK and sodium nitrite improved the antimicrobial effect against L. 485 monocytogenes even after 5 days of storage (Figure 7B). 486

4. Conclusion

Bacteria in food can lead to its spoilage and is therefore a significant economic burden and a 490 major public health concern to society. The use of preservatives is essential to mitigate 491 spoilage but there are toxicity issues associated with current antimicrobial agents such as 492 sodium nitrite. Therefore, there is a need to develop new bactericidal agents and food 493 preservative regimens that are less toxic to humans. In this study, we evaluated the potential 494 of antimicrobial RPMs as effective and less toxic food preservatives. Our findings reveal 495 strong antimicrobial activity for 20-mer RPMs that consist of randomized combinations of 496 the amino acids phenylalanine, leucine and lysine against multiple strains of spoilage 497 bacteria. We also evaluated the antimicrobial activity of RPMs in combination with sodium 498 nitrite. By using this approach, we were able to lower the concentration of nitrite required to 499 suppress the spoilage of the meat and the growth of selected bacteria such as P. putida and L. 500 monocytogenes. 501

Treating food products with antimicrobial agents can trigger microbiota shifts, as they have 502 the potential to inhibit or even eliminate certain populations which can create new 503 opportunities for the growth of other spoilage organisms or even pathogens. To address this 504 concern, we performed non-targeted analysis of microbial community structure in meat 505 samples incubated with and without antimicrobial compounds. We observed that microbial 506 diversity in the control treatment after 5 days was significantly lower than the baseline 507 diversity due to the growth of bacteria from the genera Pseudomonas and Brochothrix. 508 However, the microbial diversity of the nitrite and nitrite/FLK treated meat was not 509 significantly different from that of the baseline microbial diversity. In addition, community 510 structure of nitrite and nitrite/FLK samples was comparable after five days of incubation, and 511 most similar to the baseline community structure. Cultivation-based analyses determined that 512

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a significant reduction in the absolute abundance of bacteria in meat treated with sodium	513
nitrite and/or RPMs had occurred. Our findings demonstrate the great potential of RPMs as	514
safe and effective food preservatives. Ultimately, their usage could lead to a significant	515
improvement in the economics of food production and better outcomes for human health.	516
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References	520
Abler, L. A., Klapes, I. N. A., Sheldon, I. B. W. and Klaenhammer, T. R., 1995. Inactivation	521
of Food-borne Pathogens with Magainin Peptides. J Food Prot. 58, 381-388.	522
Amso Z, Hayouka Z., 2019. Antimicrobial random peptide cocktails: a new approach to fight	523
pathogenic bacteria. Chem Commun. 55, 2007-2014.	524
Anderson, R.C., Wilkinson, B., Yu, P.M., 2004. Ovine antimicrobial peptides : New products	525
from an age-old industry Ovine antimicrobial peptides: new products from an age-old	526
industry. Australian Journal of Agricultural Research. 55, 69–75.	527
Aiyegoro, O. A., 2004. Microbial Contamination of Processed Meat. Encyclopedia of Meat	528
Sciences. 2, (Elsevier Ltd.).	529
Appendini, P. & Hotchkiss, J. H., 2000. Antimicrobial activity of a 14-residue synthetic	530
peptide against foodborne microorganisms. J. Food Prot. 63, 889–893.	531
Benson, A. K., David J.R.D., Gilbreth S.E., Smith G., Nietfeldt J., Legge R., Kim J., Sinha	532
R., Duncan C.E., Ma J., Singh I. 2014. Microbial successions are associated with changes in	533
chemical profiles of a model refrigerated fresh pork sausage during an 80-day shelf life study.	534
Appl. Environ. Microbiol. 80, 5178–5194, (2014).	535

Böhme, K., Fernández-No1, I.C., Barros-Velázquez, j., Gallardo J.M., Cañas, B., and Calo-	536
Mata, P, 2012. Species Identification of Food Spoilage and Pathogenic Bacteria by MALDI-	537
TOF Mass Fingerprinting. Food Qual. 32, 29–46.	538
Borch, E., Kant-Muermans, M. L. & Blixt, Y, 1996. Bacterial spoilage of meat and cured	539
meat products. Int. J. Food Microbiol. 33, 103-120.	540
Brogden, K. A, 2005. Antimicrobial peptides: Pore formers or metabolic inhibitors in	541
bacteria? Nature Reviews Microbiology. 3, 238–250.	542
Bybee, S. M., Bracken-Grissom, H., Haynes, B. D., Hermansen, R. A., Byers, R.L., Clement,	543
M.J., Udall, J.A., Wilcox, E. R., Crandall, K.A, 2011. Targeted amplicon sequencing (TAS):	544
A scalable next-gen approach to multilocus, multitaxa phylogenetics. Genome Biol. Evol. 3,	545
1312–1323.	546
Cammack, R., Joannou, C.L., Cui, x.y., Torres Martinez, C., Maraj, S.R., Hughes M.N, 1999.	547
Nitrite and nitrosyl compounds in food preservation. Biochimica et Biophysica Acta (BBA) -	548
Bioenergetics, 1411, 475-488.	549
Chou, S., Shao, C., Wang, J., Shan, A., Xu, L., Dong, N., Li Z. 2016. Short, multiple-	550
stranded β -hairpin peptides have antimicrobial potency with high selectivity and salt	551
resistance. Acta Biomater. 30, 78–93.	552
Cleveland, J., Montville, T. J., Nes, I. F. & Chikindas, M. L. 2001. Bacteriocins : safe, natural	553
antimicrobials for food preservation. International Journal of Food Microbiology. 71, 1–20.	554
Crosby, N. T. & Sawyer, R. 1976. N-Nitrosamines: A review of chemical and biological	555
properties and their estimation in foodstuffs. Adv. Food Res. 22, 1–71.	556
Diamond, G., Beckloff, N., Weinberg, A. & Kisich, K. O. 2009. NIH Public Access. 15,	557
2377–2392.	558

Dobson, A. J., Purves, J. & Rolff, J. 2014. Increased survival of experimentally evolved	559
antimicrobial peptide-resistant Staphylococcus aureus in an animal host. Evol. Appl. 7, 905-	560
912.	561
Dobson, A. J., Purves, J., Kamysz, W. & Rolff, J. 2013. Comparing Selection on S. aureus	562
between Antimicrobial Peptides and Common Antibiotics. PLoS One. 8, e76521.	563
Doulgeraki, A. I., Ercolini, D., Villani, F. & Nychas, G. E. 2012. Spoilage microbiota	564
associated to the storage of raw meat in different conditions. Int. J. Food Microbiol. 157,	565
130–141.	566
Farber J.M and Peterkin P.I. 1991. Listeria monocytogenes, a Food-Borne Pathogen.	567
Microbiol Rev. 55, 476–511.	568
Filippis, F. De, Parente, E. & Ercolini, D. 2018. Recent Past, Present, and Future of the Food	569
Microbiome. Annu Rev Food Sci Techno. 9, 589-608.	570
Govaris, A., Solomakos, N., Pexara, A. & Chatzopoulou, P. S. 2010. The antimicrobial effect	571
of oregano essential oil, nisin and their combination against Salmonella Enteritidis in minced	572
sheep meat during refrigerated storage. Int. J. Food Microbiol. 137, 175–180.	573
Green, S. J., Venkatramanan, R. & Naqib, A. 2015. Deconstructing the polymerase chain	574
reaction: Understanding and correcting bias associated with primer degeneracies and primer-	575
template mismatches. PLoS One. 10, 1–21.	576
Hancock R.E.W. 2001. Cationic peptides: effectors in innate immunity and novel	577
antimicrobials. The Lancet infectious diseases. 1, 156-164.	578
Hayouka, Z., Chakraborty, S., Liu R, Weisblum B, Gellman S. 2013. Interplay among	579
subunit identity, subunit proportion, chain length, and stereochemistry in the activity profile	580
of sequence-random peptide mixtures. J. Am. Chem. Soc. 135, 11748-11751.	581

Honikel, K. O. 2008. The use and control of nitrite and nitrite for the processing of meat582products. Meat Sci. 78, 68–76.583

Hyldgaard, M. Hyldgaard, M., Meyer, R. L., Peng, M., Hibberd, A. A., Fischer, 584
Sigmundsson, A. J., Mygind, T. 2015. Binary combination of epsilon-poly-l-lysine and 585
isoeugenol affect progression of spoilage microbiota in fresh turkey meat, and delay onset of 586
spoilage in Pseudomonas putida challenged meat. Int. J. Food Microbiol. 215, 131–142.

Kilcher, S., Loessner, M. J. & Klumpp, J. 2010. *Brochothrix thermosphacta* bacteriophages
feature heterogeneous and highly mosaic genomes and utilize unique prophage insertion sites.
J. Bacteriol. 192, 5441–5453.
590

Kim, E. Y., Rajasekaran, G. & Shin, S. Y. 2017. LL-37-derived short antimicrobial peptide 591
KR-12-a5 and its D-amino acid substituted analogs with cell selectivity, anti-biofilm activity, 592
synergistic effect with conventional antibiotics, and anti-inflammatory activity. Eur. J. Med. 593
Chem. 136, 428–441. 594

Lamas, A., Miranda, J., Vázquez, B., Cepeda, A. & Franco, C. 2016. An Evaluation of
Alternatives to Nitrites and Sulfites to Inhibit the Growth of *Salmonella enterica* and *Listeria monocytogenes* in Meat Products. Foods. 5, 74.

Malheiros, S., Daroit, D. J. & Brandelli, A. 2010. Food applications of liposomeencapsulated antimicrobial peptides. Trends in Food Science & Technology. 21, 284-292. 599

Marquette, A. & Bechinger, B. 2018. Biophysical Investigations Elucidating the Mechanisms 600 of Action of Antimicrobial Peptides and Their Synergism. Biomolecules. 8, 18. 601

Mayrhofer, S., Paulsen, P., Smulders, F. J. M. & Hilbert, F. 2004. Antimicrobial resistance 602 profile of five major food-borne pathogens isolated from beef, pork and poultry. Int J Food 603 Microbiol. 97, 23–29. 604

McMindes, M. K. 1988. Nitrite mode of action: the inhibition of yeast pyruvate	605
decarboxylase and clostridial pyruvate: ferredoxin oxidoreductase by nitric oxide and	606
menadione. Diss. Abstr. Int. B 49, 2011.	607
Habets. M.G, Rozen, D. E. & Brockhurst, M. A. 2012. Variation in Streptococcus	608
pneumoniae susceptibility to human antimicrobial peptides may mediate intraspecific	609
competition. Proc Biol Sci. 279, 3803–3811.	610
Moonsamy, P. V., Williams, T., Bonella, P., Holcomb, C. L., Höglund, B. N., Hillman, G.,	611
Goodridge, D., Turenchalk, G. S., Blake, L. A., Daigle, D. A., Simen, B. B., Hamilton A.	612
May, A.P., Erlich, H. A. 2013. High throughput HLA genotyping using 454 sequencing and	613
the Fluidigm Access Array TM system for simplified amplicon library preparation. Tissue	614
Antigens. 81, 141–149.	615
Müller-Auffermann, K., Grijalva, F., Jacob, F. & Hutzler, M. 2015. Nisin and its usage in	616
breweries: A review and discussion. J. Inst. Brew. 121, 309–319.	617
Müller-Herbst, S., Wüstner, S., Kabisch, J., Pichner, R. & Scherer, S. 2016. Acidified nitrite	618
inhibits proliferation of Listeria monocytogenes - Transcriptional analysis of a preservation	619
method. Int. J. Food. Microbiol. 226, 33-41.	620
Nakatsuji, T. & Gallo, R. L. 2012. Antimicrobial peptides: old molecules with new ideas. J.	621
Invest. Dermatol. 132, 887–95.	
Naqib, A., Poggi, S., Wang, W., Hyde, M., Kunstman, K., Green, S. J. 2018. Making and	623

sequencing heavily multiplexed, high-throughput 16S ribosomal RNA gene amplicon 624 libraries using a flexible, two-stage PCR protocol. Methods in Molecular Biology. 1783, 625 149–169. 626

Nychas, G. J. E., Skandamis, P. N., Tassou, C. C. & Koutsoumanis, K. P. 2008. Meat	627
spoilage during distribution. Meat Sci. 78, 77–89.	628
Perez, R., Perez, M. T. & Elegado, F. 2015. Bacteriocins from Lactic Acid Bacteria: A	629
Review of Biosynthesis, Mode of Action, Fermentative Production, Uses, and Prospects. Int.	630
J. Philipp. Sci. Technol. 8, 61–67.	631
Perron, G. G., Zasloff, M. & Bell, G. 2006. Experimental evolution of resistance to an	632
antimicrobial peptide. Proc. Biol. Sci. 273, 251–256.	633
Pränting, M, Negrea, A., Rhen, M. and Andersson, D.I. 2008. Mechanism and Fitness Costs	634
of PR-39 Resistance in Salmonella Enterica Serovar Typhimurium LT2. Antimicrob Agents	635
Chemother. 52, 2734–2741.	636
Raimondi, S., Nappi, M. R., Sirangelo, T. M., Leonardi, A., Amaretti, A., Ulrici, A.,	637
Magnani, R., Montanari, C., Tabanelli, G., Gardini, F., Rossi, M. 2018. Bacterial community	638
of industrial raw sausage packaged in modified atmosphere throughout the shelf life. Int. J.	639
Food Microbiol. 280, 78–86.	640
Rank, L.A., Walsh, N.M., Liu, R., Lim, F.Y., Bok, J.W., Huang, M., Keller, N.P., Gellman,	641
S.H., Hull, C.M. 2017. A cationic polymer that shows high antifungal activity against diverse	642
human pathogens. Antimicrob. Agents Chemother. 61, e00204-17.	643
Rathinakumar, R., Walkenhorst, W. F. & Wimley, W. C. 2009. Broad-Spectrum	644
Antimicrobial Peptides by Rational Combinatorial Design and High-Throughput Screening :	645
The Importance of Interfacial Activity. J. Am. Chem. Soc. 131, 7609-17.	646
Rouger, A., Tresse, O. & Zagorec, M. 2017. Bacterial Contaminants of Poultry Meat:	647
Sources, Species, and Dynamics. Microorganisms. 5, 50.	648

Russo, F., Ercolini, D., Mauriello, G. & Villani, F. 2006. Behaviour of Brochothrix	649
thermosphacta in presence of other meat spoilage microbial groups. Food Microbiol. 23,	650
797–802.	651
Rydlo, T., Miltz, J. & Mor, A. 2006. Eukaryotic antimicrobial peptides: Promises and	652
premises in food safety. J. Food Sci. 71, R125 - R135.	653
Solomakos, N., Govaris, A., Koidis, P. & Botsoglou, N. 2008. The antimicrobial effect of	654
thyme essential oil, nisin, and their combination against Listeria monocytogenes in minced	655
beef during refrigerated storage. Food Microbiology. 25, 120–127.	656
Stieglmeier, M. Wirth, R., Kminek, G., Moissl-Eichinger, C. 2009. Cultivation of Anaerobic	657
and Facultatively Anaerobic Bacteria from Spacecraft-Associated Clean Rooms. Appl	658
Environ Microbiol. 75, 3484-91.	659
Stern, T., Zelinger, E. & Hayouka, Z. 2016. Random peptide mixtures inhibit and eradicate	660
methicillin-resistant Staphylococcus aureus biofilms. Chem. Commun. 52, 7102–7105.	661
Thomas, F., Hehemann, J. H., Rebuffet, E., Czjzek, M. & Michel, G. 2011. Environmental	662
and gut Bacteroidetes: The food connection. Front. Microbiol. 2, 1–16.	663
Tompkin R.B., Christiansen, L. N. & Shaparis, A. B. 1978. The effect of iron on botulinal	664
inhibition in perishable canned cured meat. Int. J. Food Sci. Technol. 13, 521–527.	665
Topman, S., Tamir-Ariel, D., Bochnic-Tamir, H., Stern Bauer, T., Shafir, S. Burdman, S.	666
Hayouka, Z. 2018. Random peptide mixtures as new crop protection agents. Microb.	667
Biotechnol. 11, 1027-1036.	668
William C. Wimley and Kalina Hristova. 2011. Antimicrobial Peptides: successes, challenges	669
and unanswered questions. J Membr Biol. 239, 27–34.	670

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.