

Title	Simulated gastrointestinal digestion of nisin and interaction between nisin and bile
Authors	Gough, Ronan;O'Connor, Paula M.;Rea, Mary C.;Gómez-Sala, Beatriz;Miao, Song;Hill, Colin;Brodkorb, André
Publication date	2017-08-14
Original Citation	Gough, R., O'Connor, P. M., Rea, M. C., Gómez-Sala , B., Miao, S., Hill, C. and Brodkorb, A (2017) 'Simulated gastrointestinal digestion of nisin and interaction between nisin and bile', LWT - Food Science and Technology, 86, pp. 530-537. doi:10.1016/ j.lwt.2017.08.031
Type of publication	Article (peer-reviewed)
Link to publisher's version	10.1016/j.lwt.2017.08.031
Rights	© 2017, Elsevier Ltd. All rights reserved. This manuscript version is made available under the CC-BY-NC-ND 4.0 license http:// creativecommons.org/licenses/by-nc-nd/4.0/
Download date	2025-04-04 04:44:37
Item downloaded from	https://hdl.handle.net/10468/4774



University College Cork, Ireland Coláiste na hOllscoile Corcaigh

Accepted Manuscript

Simulated gastrointestinal digestion of nisin and interaction between nisin and bile

Ronan Gough, Paula M. O'Connor, Mary C. Rea, Beatriz Gómez-Sala, Song Miao, Colin Hill, André Brodkorb

PII: S0023-6438(17)30601-1

DOI: 10.1016/j.lwt.2017.08.031

Reference: YFSTL 6453

To appear in: LWT - Food Science and Technology

Received Date: 22 June 2017

Revised Date: 9 August 2017

Accepted Date: 11 August 2017

Please cite this article as: Gough, R., O'Connor, P.M., Rea, M.C., Gómez-Sala, B., Miao, S., Hill, C., Brodkorb, André., Simulated gastrointestinal digestion of nisin and interaction between nisin and bile, *LWT - Food Science and Technology* (2017), doi: 10.1016/j.lwt.2017.08.031.

This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.





1	Simulated gastrointestinal digestion of nisin and interaction between nisin and bile
2	
3	Ronan Gough ^{a, b} , Paula M. O'Connor ^{a, c} , Mary C. Rea ^{a, c} , Beatriz Gómez-Sala ^a , Song Miao ^a , Colin Hill ^{b, c} ,
4	André Brodkorb ^{a,*}
5	^a Teagasc Food Research Centre, Moorepark, Fermoy, Co. Cork, Ireland
6	^b School of Microbiology, University College Cork, College Road, Cork, Ireland
7	^c APC Microbiome Institute, University College Cork, College Road, Cork, Ireland
8	
9	* Corresponding author. Teagasc Food Research Centre, Moorepark, Fermoy, Co. Cork, Ireland.
10	<i>E-mail address:</i> andre.brodkorb@teagasc.ie (A. Brodkorb)
11	
12	Abstract
13	Nisin, an antimicrobial peptide showing activity against many Gram positive bacteria, is widely used as a food
14	preservative. The simulated gastrointestinal digestion of nisin (variant A) was studied using the in vitro
15	INFOGEST digestion method. Following oral, gastric and small intestinal digestion, there was no intact nisin in
16	the system and the nisin was primarily digested by pancreatin. After digestion, six nisin fragments (1-11, 1-12,
17	1-20, 1-21, 1-29 and 1-32) were identified by reversed phase high performance liquid chromatography and mass
18	spectroscopy and four of these nisin fragments (1-20, 1-21, 1-29 and 1-32) demonstrated low antibacterial
19	activity against Lactococcus lactis HP in agar diffusion activity assays. Additionally, it was observed that bile
20	salts form a complex with nisin. This was examined by atomic force microscopy, turbidity and dynamic light
21	scattering, which showed that this interaction resulted in significantly larger bile salt micelles. The presence of
22	bile salts at physiological levels significantly altered the relative amounts of the nisin fragments 1-12, 1-20 and
23	1-29 produced during an <i>in vitro</i> digestion. This study highlights the importance of including bile in simulated
24	digestions of antimicrobial peptides in order to obtain a more accurate simulation of the in vivo digestion
25	products and their activity.
26	
27	Keywords:
28	Nisin; In vitro digestion; Bile; Antimicrobial peptide; Surfactant
29	

31 1. Introduction

32

Nisin is a 34 amino acid antimicrobial peptide produced by strains of *Lactococcus lactis* subsp. *lactis*that is active against many Gram-positive bacteria and is widely used as a food preservative (Gharsallaoui et al.,
2016). Nisin is extremely stable at pH 3 and can be autoclaved at this pH with < 5% loss of activity (Davies et
al., 1998), whereas above pH 6 it is unstable even at room temperature (Kelly et al., 2000).

37 The discovery that nisin is inactivated by pancreatin (Heinemann and Williams, 1966), primarily due to 38 its chymotrypsin component (Jarvis and Mahoney, 1969), was a factor in nisin being awarded GRAS status by 39 the FDA (U. S. Food and Drug Administration, 1988) and the European Food Safety Authority declaring that 40 nisin is safe for use in food (European Food Safety Authority, 2006) with its assigned E number being E 234 41 (European Commission, 2011). It has been demonstrated more recently that nisin is also cleaved by the trypsin 42 component of pancreatin (Chan et al., 1996). However these studies focused on pancreatic enzymes and did not 43 take into account the other components of the digestive system such as bile.

44 Bile salts, the major functional component of bile, are biological surfactants which are involved in the 45 digestion and absorption of lipids in the small intestine; in particular they transport the products of lipolysis in 46 bile salt micelles to the sites of absorption (Bauer et al., 2005). For the most common human and porcine bile 47 salts, micelle formation takes place in two stages; hydrophobic interactions between bile salts results in primary 48 micelles, which then interact via hydrogen bonding to form secondary micelles (Kandrac et al., 2006; Partay et 49 al., 2007; Small, 1968). The minimum bile salt concentration required for micelle formation is termed the 50 critical micelle concentration (CMC). As the concentration of sodium ions affects the CMC, experiments with 51 bile salts are commonly performed in 0.15 moles/L Na⁺ solutions to simulate physiological conditions 52 (Hofmann and Hagey, 2008). In a 0.15 moles/L Na⁺ solution, most bile salts have a CMC below 10 53 millimoles/L (Hofmann and Roda, 1984); 10 millimoles/L is also the bile salt concentration recommended for 54 simulating physiological conditions during in vitro digestion (Minekus et al., 2014). 55 Previous digestion studies on nisin have focussed on pancreatic enzymes from the small intestine and 56 those that investigated the nisin fragments produced by digestion used enzymes individually and often used 57 digestions in excess of 20 h (Chan et al., 1996; Heinemann and Williams, 1966; Jarvis and Mahoney, 1969; 58 Slootweg et al., 2013). In order to study how nisin is digested under more physiologically relevant conditions, 59 the INFOGEST method, a recently developed standardized static method for the digestion of food (Minekus et

60 al., 2014) was utilised. This method is the consensus of an international network of scientists and is based on

61	physiological conditions with each digestion comprising an oral, gastric and intestinal stage (Minekus et al.,
62	2014). This approach would establish which nisin fragments are produced under physiological conditions and
63	also their biological activity. In addition, by performing versions of the digestion without individual digestion
64	components, the importance of non-proteolytic components such as bile on the digestion profile of nisin could
65	be established.
66	
67	2. Materials and methods
68	
69	2.1. Materials
70	
71	All reagents were obtained from Sigma-Aldrich (Arklow, Ireland) unless otherwise stated. For the
72	simulated digestions the specific Sigma-Aldrich products used were: salivary amylase (A1031), pepsin (P6887),
73	bile (B8631) and pancreatin (P7545). Tween [®] 80 was obtained from Merck Millipore (Darmstadt, Germany).
74	The nisin preparation used was Nisaplin [®] (DuPont, Beaminster, UK) (nisin variant A; referred to as 'nisin'
75	throughout this text). This was enriched by salting out as previously described (Gough et al., in press).
76	
77	2.2. Digestion
78	
79	Simulated oral, gastric and small intestinal digestions were performed as described in the INFOGEST
80	method (Minekus et al., 2014). Five variations of the digestion were performed: (i) nisin with all digestion
81	components, (ii) nisin with all digestion components except bile, (iii) nisin with all digestion components except
82	pancreatin, (iv) nisin with all digestion components except pepsin, bile and pancreatin, (v) all digestion
83	components but no nisin. A minimum of three replicates were performed of each of these five digestion setups.
84	The initial nisin concentration was chosen so that the nisin concentration in the digestion product would be
85	sufficient for quantification by reversed phase - high performance liquid chromatography (RP-HPLC). The
86	digestion containing nisin and all digestion components was performed as follows: for the oral stage 5 mL of an
87	8.7 mg/mL nisin solution was combined with simulated salivary fluid (SSF) and salivary amylase (75 U/mL in
88	final oral solution) to a final total volume of 10 mL; this was incubated at 37 °C for 2 minutes. For the gastric
89	stage, the sample pH was adjusted to 3 using dilute HCl and combined with simulated gastric fluid (SGF) and
90	pepsin (2,000 U/mL in final gastric solution) to a final total volume of 20 mL; this was incubated at 37 °C for 2

91	hours. For the small intestinal stage the pH was adjusted to 7 using dilute NaOH and combined with simulated
92	intestinal fluid (SIF) and bile (10 millimoles/L bile salts in final intestinal solution) and pancreatin (100 TAME
93	U/mL in final intestinal volume) to a final total volume of 40 mL, this was incubated at 37 °C for 2 hours. The
94	digestion products were snap-frozen in liquid nitrogen.
95	
96	2.3. Determination of the effect of the presence of bile during digestion on the activity of the digestion products
97	
98	To determine the effect of the presence of bile during digestion on the activity of the digestion products
99	a simplified digestion method based on Minekus et al. (2014) was used; nisin was incubated with pancreatin in a
100	MOPS buffer at pH 7 and 37 °C for 2 h with bile added either before or after digestion, with an equivalent
101	volume of water added to samples that did not receive bile. The final constituents in each sample, in a total
102	volume of 0.5 mL, were 100 µg/mL nisin, bile at a bile salt concentration of 0.3 millimoles/L, pancreatin at a
103	concentration such that its trypsin activity was 100 TAME units per mL, 50 millimoles/L MOPS, 0.15 moles/L
104	NaCl and the pH was 7. The digestion products were analysed by activity assay as described in section 2.6.
105	
106	2.4. Reversed Phase - High Performance Liquid Chromatography (RP-HPLC)
107	
108	RP-HPLC was carried out on a Jupiter, 5 μ m, C18, 300 Å, 250 mm \times 4.6 mm column from
109	Phenomenex (Macclesfield, UK) with an acetonitrile (Thermo Fisher Scientific, Dublin, Ireland) gradient as
110	described previously (Gough et al., in press). In the case of digested nisin, fractions were collected throughout
111	the gradient to determine the nisin fragments produced by digestion.
112	
113	2.5. Matrix-assisted laser desorption/ionization time of flight mass spectrometry (MALDI TOF MS)
114	
115	The molecular mass of the RP-HPLC peaks were determined using MALDI TOF MS using an Axima
116	TOF ² mass spectrometer (Shimadzu Biotech, Kyoto, Japan) as previously described (Field et al., 2012).
117	
118	2.6. Activity Assay
119	

120	Biological activity was estimated by agar diffusion activity assays (Ryan et al., 1996) in agar plates
121	seeded with Lactococcus lactis subsp. cremoris HP which were prepared as described previously (Gough et al.,
122	in press). Serial two-fold dilutions of the samples were performed in 0.15 moles/L NaCl, 50 millimoles/L
123	MOPS, pH 7. In specific cases a surfactant (0.3 millimoles/L bile salts, 8 millimoles/L Tween [®] 80 or 0.2
124	millimoles/L Triton TM X-100) was included in the diluent. The samples (50 µL) were dispensed into the wells
125	and the plates incubated overnight at 30 °C. The activity of nisin resulted in zones of inhibition surrounding the
126	wells. Activity is expressed as minimum inhibitory concentration (MIC) in terms of μ g/mL (Chan et al., 1996).
127	MIC was calculated by plotting the area of the zone of inhibition at each dilution stage against the log of the
128	nisin concentration (Bernbom et al., 2006); these had a linear relationship and the MIC was calculated from the
129	equation of the line.
130	
131	2.7. Atomic Force Microscopy (AFM)
132	
133	For AFM, samples comprised 10 millimoles/L bile salts, 0.15 moles/L NaCl, and 50 millimoles/L
134	MOPS at pH 7, with or without 0.5 μ g/mL nisin. The nisin concentration of 0.5 μ g/mL was chosen as this is
135	within the range that could occur in the small intestine after consumption of a nisin containing foodstuff
136	(Delves-Broughton, 2005; Minekus et al., 2014). Aliquots (5 µL) were deposited onto freshly cleaved mica
137	surfaces, dried in a desiccator and subsequently stored at ambient conditions to ensure equilibrated hydration.
138	AFM images were obtained with an Asylum Research MFP-3D-AFM (Asylum Research UK Ltd., Oxford, UK)

using AC-mode in ambient air. An aluminium reflex coated cantilever with a tetrahedral tip (AC 240), spring
constant of 1.8 N/m (Olympus Optical Co. Ltd., Tokyo Japan), working frequency of 50-90 kHz, and scan rate

- 141 of 0.5-1 Hz was used at a 512×512 resolution. The radius of curvature of the tetrahedral tip was 10 (± 3) nm.
- 142

143 2.8. *Turbidity*

144

Turbidity was measured at 600 nm as per (Dahmane et al., 2008) using a Cary 100 Bio
Spectrophotometer with temperature control (Agilent Technologies Ireland Ltd., Little Island, Ireland). The
samples were prepared at pH 7 in a 50 millimoles/L MOPS buffer containing 0.15 moles/L NaCl and analysed
at 37 °C.

150	2.9. Dynamic Light Scattering
151	
152	Z-average was measured by Dynamic Light Scattering (DLS) using a Zetasizer Nano ZS (Malvern
153	Instruments, Malvern, UK). The samples were prepared in 0.15 moles/L NaCl, adjusted to pH 7 using NaOH,
154	filtered through a 0.22 µM PVDF filter (Gilson Scientific, Luton, UK) and analysed at 20 °C.
155	
156	3. Results and discussion
157	
158	3.1. Simulated digestion
159	
160	Oral and gastric digestion of nisin without pepsin resulted in a 6% (\pm 0.6, n = 3) reduction in intact
161	nisin when measured by RP-HPLC and the inclusion of pepsin brought the total reduction to 16% (\pm 2.2, n = 5);
162	this limited digestion in the oral and gastric stages correlates with published results which show that nisin is
163	primarily digested in the small intestine (Jarvis and Mahoney, 1969).
164	Gastrointestinal digestion without proteases or bile resulted in > 50% loss in intact nisin (Table 1). As
165	the oral and gastric stages caused limited reduction in nisin, this reduction can primarily be attributed to the
166	small intestinal pH of 7 and temperature of 37 °C, as above pH 6 nisin is unstable with a temperature dependent
167	decomposition rate (Kelly et al., 2000). It was noted that pH and temperature were not entirely responsible for
168	the reduction in detectable nisin and that the simulated intestinal fluid, in particular its sodium bicarbonate
169	component, played a minor role (data not shown).
170	Performing the small intestinal stage of digestion with bile and/or pancreatin resulted in no intact nisin
171	being detectable by RP-HPLC (Table 1) and the products of digestions that included bile had greater
172	antibacterial activity than similar digestions without bile.
173	The highest antibacterial activity was in digestions without pancreatin; this correlates with previous
174	reports that pancreatin is primarily responsible for nisin digestion (Heinemann and Williams, 1966). However in
175	digestions with all components except pancreatin, there was no intact nisin detected by RP-HPLC. The high
176	antibacterial activity implies that intact nisin was present and suggests another digestion component may be
177	affecting the behaviour of nisin on the RP-HPLC column, thus interfering with its detection. This component
178	appeared to be bile, as digestions without bile or proteases had detectable nisin in their products.
179	

180 3.2. Analyses of nisin fragments

181

182 As the products of digestions involving pancreatin demonstrated antibacterial activity that could not be 183 accounted for by the bile or digestive enzymes, the activity was likely due to fragments of nisin. To determine 184 which nisin fragments were produced and which of these were bioactive, the digestion products were separated by RP-HPLC and the fractions collected (Fig. 1B). The fractions were analysed using MALDI-TOF mass 185 186 spectrometry and by activity assay (Fig. 2). Digestions with pancreatin produced peptides with molecular 187 masses corresponding to the theoretical and published molecular masses of nisin fragments 1-12, 1-20, 1-21, 1-29 and 1-32 and also a peptide with a molecular mass within two daltons of the theoretical mass of nisin 188 189 fragment 1-11 (Table 2). 190 Major peaks on the RP-HPLC traces (Fig. 1A and B) corresponded to nisin 1-12, 1-20, 1-29 and intact 191 nisin, whereas nisin 1-21 gave a minor peak. Intact nisin had a shoulder region corresponding to the nisin 192 variant [Ser³³]-nisin in which the serine residue at position 33 did not undergo post-translational modification to 193 dehydroalanine (Chan et al., 1996). The peaks corresponding to nisin fragments 1-11 and nisin 1-32 were 194 obscured by the co-eluting bile and pancreatic components. Although the RP-HPLC peak of nisin 1-32 was 195 completely obscured by the background, its elution point was identified by activity assay. 196 Nisin 1-29 eluted at slightly different time points depending on whether the digestion was performed 197 without bile (25.1 min) or with bile included (25.4 min). The peaks at 22.8 min and 26.9 min and the four major 198 peaks between 25.5 min and 26.1 min were primarily due to bile and pancreatin and occurred in the control 199 digestions that did not have nisin. The presence of bile in the digestion affected the peak heights of nisin 200 fragments 1-12, 1-20 and 1-29 (Fig. 1B). 201 RP-HPLC fractionation did not lead to pure peptide fractions due to overlap between the elution of the 202 fragments, for example in Fig. 2 nisin fragment 1-29 was detected in the mass spectrometry analysis of the 203 elution peak of nisin fragment 1-11 and was most likely the source of the antimicrobial activity in the activity 204 assay of nisin fragment 1-11, also nisin fragment 1-20 was visible in the mass spectrometry analysis of the 205 elution peak of nisin fragment 1-32.

Nisin fragment 1-32 was not detected in the products of digestion in the presence of bile; as bile
interfered with the detection of intact nisin (1-34) by RP-HPLC, it is proposed this also occurs with nisin
fragment 1-32.

209 Nisin fragments 1-12, 1-20 and 1-21 have previously been produced by digests with trypsin or 210 chymotrypsin for a minimum of 16 hours (Chan et al., 1996; Slootweg et al., 2013). Nisin fragment 1-29 has 211 been produced by an 8 hour digestion with the bacterial protease thermolysin and by a 4 hour digestion of the 212 nisin variant ([Ser³³]-nisin) with carboxypeptidase Y (Chan et al., 1996). Nisin fragment 1-32 has been produced 213 by a 6 day acid treatment (Chan et al., 1989; Chan et al., 1996). To the authors knowledge it has not been previously demonstrated that these fragments can be produced under physiological conditions. 214 215 In Table 2 it is shown that the molecular masses observed in Fig. 2 are within two daltons of the 216 predicted masses and that the inclusion of bile in a digestion altered the proportions of the nisin fragments produced when compared by peak height in a RP-HPLC chromatogram. In this study all the nisin fragments 217 218 corresponding to amino acids 1-20 or longer demonstrated antibacterial activity against Lactococcus lactis (Fig. 2), which is in agreement with Chan et al. (1996). The decrease in nisin 1-12 and an increase in nisin 1-20 and 219 220 1-29, which are observed in Fig. 1B are shown to be significant (Table 2). This implies that the bile reduces the 221 cleavage of nisin during digestion.

222

223 3.3. Nisin interaction with bile and other surfactants

224

225 As bile had been shown to increase nisin activity (Table 1) and alter its digestion (Table 2) the bile-226 nisin interaction was further examined and compared to nisin's interaction with other surfactants. The 227 surfactants Tween[®] 80 and TritonTM X-114 were chosen for comparison as they increase the activity of nisin (Joosten and Nunez, 1995; Jozala et al., 2008). Triton[™] X-100 was substituted for Triton[™] X-114, as Triton[™] 228 229 X-114 phase separates at the incubation temperature of the activity assay (30 °C) (Bordier, 1981). Regarding the 230 concentrations used; 10 millimoles/L bile salts is physiological concentration (Minekus et al., 2014), while 0.3 millimoles/L bile salts and 0.2 millimoles/L Triton™ X-100 were the highest concentrations that did not cause 231 232 antibacterial activity in activity assays. While Tween[®] 80 did not have an antibacterial affect at concentrations > 8 millimoles/L, 8 millimoles/L was chosen because higher concentrations had a noticeable effect on viscosity. 233 234 All these surfactants were at a molar excess over the nisin component (100 μ g/mL nisin \approx 0.03 millimoles/L 235 nisin).

To investigate how surfactants affected the MIC of nisin when determined by activity assays; serial
dilutions were performed in a MOPS/NaCl diluent on its own and with each of the surfactants, with a starting
nisin concentration of 100 µg/mL (Table 3). Bile caused a reduction in MIC compared to the control, however a

greater reduction was caused by Tween[®] 80 and Triton[™] X-100 and both of these reduced the MIC by the same
amount (Table 3). This implies that nisin interacts differently with bile compared to the other surfactants.

- Surfactants can increase the activity of bioactive peptides in activity assays by reducing or preventing binding to glass or polypropylene assay containers through competition with the peptides for binding sites on the container or interacting with the binding sites on the peptides, primarily through hydrophobic interactions (Duncan et al., 1995; Joosten and Nunez, 1995). If nisin activity was increased by bile, through the formation of a peptide-surfactant complex, there would also be an increase in the particle size of the bile salt micelle.
- The effect of nisin-surfactant interaction on particle size was examined by AFM, turbidity and DLS. AFM analysis of bile with and without nisin (Fig. 3A and B) showed individual particles whose cross-sections had z-heights ranging from 20 to 190 nm, which was similar to the z-average means obtained by DLS for bile (100 nm \pm 5) and bile with nisin (118 nm \pm 9) (Fig. 3D) and similar to the published values for bile salt secondary micelles (50 to 200 nm) (Hildebrand et al., 2004).

The turbidity of a system relates to both the size and density of particles that scatter light. In Fig. 3C, nisin was solubilised in a range of surfactants and turbidity increased with increasing nisin concentration, with the greatest turbidity increases occurring in the presence of bile. DLS (Fig. 3D) found that the z-average diameter of 10 millimoles/L bile salts with 100 μ g/mL nisin was significantly larger than that without nisin (p = 0.0123, n = 4). There was no significant change in particle size when nisin was in solution with TritonTM X-100 or Tween[®] 80 (data not shown). To produce results of suitable quality by DLS, the samples were filtered and measured at 20 °C and pH 7 without a buffer.

The particle size analysis (Fig. 3) suggests that nisin formed a complex with bile and this was different to its interaction with other surfactants. In the activity analysis (Table 3) bile caused less of an increase in nisin activity than the other surfactants; this could be explained by bile forming a complex with nisin whereas the other surfactants bound to the assay container and thus reduced non-specific nisin binding in a different way. In the products of digestions which contained all digestion components except pancreatin (Table 1), nisin was detected by activity assay but not detected by RP-HPLC; nisin could be favouring hydrophobic interaction with bile over hydrophobic interactions with the RP-HPLC column.

With respect to the mechanism for bile-nisin interaction, both hydrophobic and ionic interactions are possible. Bile salts are anionic with a negative charge on an amino acid that is attached to one end of the main body of the bile salt via a short hydrocarbon chain (Fig. 4) (Hofmann and Hagey, 2008; Small, 1968). Nisin has a pI of 8.5 and thus is cationic under physiological conditions with the bulk of the positive charge being in the

269 C-terminal domain (Fig. 4) and this is responsible for its initial interaction with its negatively charged targets 270 (Breukink et al., 1997). The oppositely charged terminal regions of bile salts and nisin make ionic interactions 271 likely. Bile salts are planar amphipathic molecules and thus have a hydrophobic and a hydrophilic side (Fig. 4); 272 when forming a primary micelle they orientate their hydrophobic sides towards each other, giving the micelle a 273 hydrophobic core (Fig. 4) (Hofmann and Hagey, 2008). Nisin is also amphipathic with the C-terminal being 274 hydrophilic while the N-terminal is hydrophobic (Fig. 4) (Gharsallaoui et al., 2016). The hydrophobic N-275 terminal region of nisin has been reported to interact with the hydrophobic cores of surfactants such as 276 dodecylphosphocholine and sodium dodecyl sulphate micelles (van den Hooven et al., 1996). It is therefore 277 possible that the hydrophobic N-terminal region of nisin also interacts with the hydrophobic core of bile salt micelles. The reduced cleavage in the N-terminal region of nisin when a simulated gastrointestinal digestion is 278 279 performed in the presence of bile (Table 2) may be due to the bile salts interacting with and surrounding the N-280 terminal region of the nisin and limiting the capacity of proteolytic enzymes to interact with the N-terminal 281 region.

282 Having shown that nisin formed a complex with bile salts, which altered its digestion products, it was 283 investigated how much this affected its antibacterial activity after digestion. The loss of nisin activity during 284 digestion is primarily due to pancreatic enzymes (Section 3.1). To look specifically at whether bile could 285 attenuate the loss of nisin activity due to pancreatin, a simplified digestion was performed with pancreatin in 286 buffer and bile added at the beginning or end of the digestion and the activity of the product determined by 287 activity assay. The final concentration of bile salts was 0.3 millimoles/L so that the antibacterial activity of bile 288 salts would not distort the results of the activity assay. The MOPS/NaCl buffer (pH 7) that was used as a diluent 289 for the activity assay contained 0.3 millimoles/L bile salts, so that the surfactant effect would be consistent at all 290 stages of the assay. The addition of bile before or after a 2 h digestion resulted in MICs of 11.8 μ g/mL (± 0.3, n = 3) and 12.7 μ g/mL (± 0.2, n = 3) respectively. Although statistically significant (p = 0.01), the difference in 291 292 activity was slight.

Although the presences or absence of bile in a static *in vitro* digestion had a significant effect on the nisin fragments produced, this in turn had a negligible effect on antibacterial activity. As nisin fragments 1-12, 1-20 and 1-29 have low antibacterial activity (< 6% the activity of intact nisin against *L. lactis* (Chan et al., 1996)) it is assumed that increases or decreases in their amount had a minimal effect on overall antibacterial activity.

299	4. Conclusions
300	
301	Nisin was digested by intestinal proteases as has been previously highlighted (Heinemann and
302	Williams, 1966; Jarvis and Mahoney, 1969) although intestinal pH and temperature by themselves also caused
303	significant loss in nisin. The digestion products include six nisin fragments, four of which have limited
304	antibacterial activity. Although nisin fragments have been previously identified (Chan et al., 1996; Slootweg et
305	al., 2013), this is the first time that fragments generated during an in vitro digestion under physiological
306	conditions have been identified. Nisin interacts with bile, forming a complex that alters the relative amounts of
307	the nisin fragments produced by digestion. This study highlights the importance of including bile in simulated
308	digestions of antimicrobial peptides regardless of the presence or absence of a lipid component in the test
309	samples.
310	
311	Acknowledgements
312	
313	This work was funded by the Irish Department of Agriculture, Food and the Marine under the FIRM
314	programme (grant number 10/RD/TMFRC/701). R. Gough was funded under the Teagasc Walsh Fellowship
315	scheme.
316	
317	References
318	
319	Bauer, E., Jakob, S., Mosenthin, R., (2005). Principles of physiology of lipid digestion. Asian-Australasian
320	Journal of Animal Sciences 18(2), 282-295.
321	Bernbom, N., Licht, T.R., Brogren, C.H., Jelle, B., Johansen, A.H., Badiola, I., Vogensen, F.K., Norrung, B.,
322	(2006). Effects of Lactococcus lactis on composition of intestinal microbiota: role of nisin. Applied and
323	Environmental Microbiology 72(1), 239-244.
324	Bordier, C., (1981). Phase separation of integral membrane proteins in Triton X-114 solution. Journal of
325	Biological Chemistry 256(4), 1604-1607.
326	Breukink, E., van Kraaij, C., Demel, R.A., Siezen, R.J., Kuipers, O.P., de Kruijff, B., (1997). The C-terminal
327	region of nisin is responsible for the initial interaction of nisin with the target membrane. Biochemistry
328	36(23), 6968-6976.

- 329 Chan, W.C., Bycroft, B.W., Lian, L., Roberts, G.C.K., (1989). Isolation and characterisation of two degradation
- products derived from the peptide antibiotic nisin. FEBS Letters 252(1-2), 29-36.
- 331 Chan, W.C., Leyland, M., Clark, J., Dodd, H.M., Lian, L.Y., Gasson, M.J., Bycroft, B.W., Roberts, G.C.K.,
- 332 (1996). Structure-activity relationships in the peptide antibiotic nisin: antibacterial activity of fragments of
- 333 nisin. FEBS Letters 390(2), 129-132.
- 334 Dahmane, S., Lasia, A., Zhao, Y., (2008). Electrochemically active block copolymer micelles containing
- coumarin moieties. Macromolecular Chemistry and Physics 209(10), 1065-1072.
- 336 Davies, E.A., Bevis, H.E., Potter, R., Harris, J., Williams, G.C., Delves-Broughton, J., (1998). Research note:
- The effect of pH on the stability of nisin solution during autoclaving. Letters in Applied Microbiology 27(3),
- **338** 186-187.
- 339 Delves-Broughton, J., (2005). Nisin as a food preservative. Food Australia 57(12), 525-527.
- 340 Duncan, M.R., Lee, J.M., Warchol, M.P., (1995). Influence of surfactants upon protein/peptide adsorption to
- 341 glass and polypropylene. International Journal of Pharmaceutics 120(2), 179-188.
- 342 European Commission, (2011). Commission regulation (EU) no 1129/2011 of 11 November 2011 amending
- annex II to regulation (EC) no 1333/2008 of the European Parliament and of the Council by establishing a
- 344 Union list of food additives. Official Journal of the European Union L295, 1-177.
- 345 European Food Safety Authority, (2006). Opinion of the Scientific Panel on Food Additives, Flavourings,
- 346 Processing Aids and Materials in Contact with Food on a request from the Commission related to the use of
- nisin (E 234) as a food additive. The EFSA Journal 4(3), 1-16.
- 348 Field, D., Begley, M., O'Connor, P.M., Daly, K.M., Hugenholtz, F., Cotter, P.D., Hill, C., Ross, R.P., (2012).
- 349 Bioengineered nisin A derivatives with enhanced activity against both gram positive and gram negative
- 350pathogens. PloS One 7(10).
- 351 Gharsallaoui, A., Oulahal, N., Joly, C., Degraeve, P., (2016). Nisin as a food preservative: Part 1:
- 352 Physicochemical properties, antimicrobial activity, and main uses. Critical Reviews in Food Science and
- **353** Nutrition 56(8), 1262-1274.
- Gough, R., Gómez-Sala, B., O'Connor, P.M., Rea, M.C., Miao, S., Hill, C., Brodkorb, A., (in press). A simple
 method for the purification of nisin. Probiotics and Antimicrobial Proteins, DOI: 10.1007/s12602-1201719287-12605.
- Heinemann, B., Williams, R., (1966). Inactivation of nisin by pancreatin. Journal of Dairy Science 49(3), 312314.

- 359 Hildebrand, A., Garidel, P., Neubert, R., Blume, A., (2004). Thermodynamics of demicellization of mixed
- 360 micelles composed of sodium oleate and bile salts. Langmuir 20(2), 320-328.
- Hofmann, A.F., Hagey, L.R., (2008). Bile acids: chemistry, pathochemistry, biology, pathobiology, and
 therapeutics. Cellular and Molecular Life Sciences 65(16), 2461-2483.
- 363 Hofmann, A.F., Roda, A., (1984). Physicochemical properties of bile acids and their relationship to biological
- 364 properties: an overview of the problem. Journal of Lipid Research 25(13), 1477-1489.
- Hsu, S.-T.D., Breukink, E., Tischenko, E., Lutters, M.A.G., de Kruijff, B., Kaptein, R., Bonvin, A.M.J.J., van
- 366 Nuland, N.A.J., (2004). The nisin–lipid II complex reveals a pyrophosphate cage that provides a blueprint
- for novel antibiotics. Nature Structural & Molecular Biology 11(10), 963-967.
- Jarvis, B., Mahoney, R.R., (1969). Inactivation of nisin by alpha-chymotrypsin. Journal of Dairy Science 52(9),
 1448-1450.
- Joosten, H.M.L.J., Nunez, M., (1995). Adsorption of nisin and enterocin-4 to polypropylene and glass surfaces
 and its prevention by Tween-80. Letters in Applied Microbiology 21(6), 389-392.
- Jozala, A.F., Lopes, A.M., Mazzola, P.G., Magalhdes, P.O., Penna, T.C.V., Pessoa, A., (2008). Liquid-liquid
 extraction of commercial and biosynthesized nisin by aqueous two-phase micellar systems. Enzyme and

374 Microbial Technology 42(2), 107-112.

- Kandrac, J., Kevresan, S., Gu, J.K., Mikov, M., Fawcett, J.P., Kuhajda, K., (2006). Isolation and determination
 of bile acids. European Journal of Drug Metabolism and Pharmacokinetics 31(3), 157-177.
- 377 Kelly, N.A., Reuben, B.G., Rhoades, J., Roller, S., (2000). Solvent extraction of bacteriocins from model
- 378 solutions and fermentation broths. Journal of Chemical Technology and Biotechnology 75(9), 777-784.
- 379 Li, R., Carpentier, E., Newell, E.D., Olague, L.M., Heafey, E., Yihwa, C., Bohne, C., (2009). Effect of the
- structure of bile salt aggregates on the binding of aromatic guests and the accessibility of anions. Langmuir
 25(24), 13800-13808.
- 382 Minekus, M., Alminger, M., Alvito, P., Ballance, S., Bohn, T., Bourlieu, C., Carriere, F., Boutrou, R., Corredig,
- 383 M., Dupont, D., Dufour, C., Egger, L., Golding, M., Karakaya, S., Kirkhus, B., Le Feunteun, S., Lesmes, U.,
- 384 Macierzanka, A., Mackie, A., Marze, S., McClements, D.J., Menard, O., Recio, I., Santos, C.N., Singh, R.P.,
- 385 Vegarud, G.E., Wickham, M.S., Weitschies, W., Brodkorb, A., (2014). A standardised static in vitro
- digestion method suitable for food an international consensus. Food & Function 5(6), 1113-1124.
- 387 Partay, L.B., Jedlovszky, P., Sega, M., (2007). Molecular aggregates in aqueous solutions of bile acid salts.
- 388 Molecular dynamics simulation study. Journal of Physical Chemistry B 111(33), 9886-9896.

Rose, A.S., Hildebrand, P.W., (2015). NGL Viewer: a web application for molecular visualization. Nucleic

390 Acids Research 43(W1), W576-W579.

- Ryan, M.P., Rea, M.C., Hill, C., Ross, R.P., (1996). An application in cheddar cheese manufacture for a strain
- 392 of *Lactococcus lactis* producing a novel broad-spectrum bacteriocin, lacticin 3147. Applied and
- **393** Environmental Microbiology 62(2), 612-619.
- 394 Sahl, H.G., (1994). Gene-encoded antibiotics made in bacteria, in: Marsh, J., Goode, J.A. (Eds.), Antimicrobial
- 395 *Peptides*. John Wiley & Sons Ltd, Chichester, pp. 27-42.
- 396 Slootweg, J.C., Liskamp, R.M.J., Rijkers, D.T.S., (2013). Scalable purification of the lantibiotic nisin and
- isolation of chemical/enzymatic cleavage fragments suitable for semi-synthesis. Journal of Peptide Science19(11), 692-699.
- Small, D.M., (1968). Size and structure of bile salt micelles, in: Gould, R.F. (Ed.), *Molecular association in biological and related systems*. American Chemical Society, Washington D.C., pp. 31-52.
- 401 Small, D.M., (1971). The physical chemistry of cholanic acids, in: Nair, P.P., Kritchevsky, D. (Eds.), *The bile*
- 402 *acids: chemistry, physiology, and metabolism.* Springer, Boston, pp. 249-356.
- 403 U. S. Food and Drug Administration, (1988). Nisin preparation; affirmation of GRAS status as a direct human
 404 food ingredient. Federal Register 53(66), 11247-11251.
- 405 van den Hooven, H.W., Spronk, C., van de Kamp, M., Konings, R.N.H., Hilbers, C.W., van de Ven, F.J.M.,
- 406 (1996). Surface location and orientation of the lantibiotic nisin bound to membrane-mimicking micelles of
- dodecylphosphocholine and of sodium dodecylsulphate. European Journal of Biochemistry 235(1-2), 394-
- 408 403.

Table 1

Products of in vitro gastrointestinal digestions of nisin.

Starting material	Oral, gastric and small intestinal digestion	Analysis of digestion products	
		% nisin (RP-HPLC)	MIC (or MIC equivalent)
Nisin solution	All components	0% (±0)	$22 \mu g/mL (\pm 5)$
Nisin solution	All components except bile	0% (±0)	41 μ g/mL (± 2)
Nisin solution	All components except pancreatin	$0\% (\pm 0)$	$0.9 \ \mu g/mL \ (\pm 0.2)$
Nisin solution	All components except pepsin, bile and pancreatin	48% (±2)	$2.9 \ \mu g/mL \ (\pm 0.5)$
H ₂ O (no nisin)	All components	n/a	$82 \mu g/mL (\pm 21)$
Nisin solution	Not digested	100%	$1.8 \mu g/mL (\pm 0.1)$

The digestion products were analysed by RP-HPLC and agar diffusion activity assay. The amount of nisin detected by RP-HPLC is expressed as a % of the total initial nisin. Activity is expressed as minimum inhibitory concentration (MIC, μ g/mL). An equivalent MIC is given for the products of digestions without nisin; SD in brackets, $n \ge 3$.

Nisin fragments detected in the products of digestio	ı.

U		6	
Nisin fragment	Observed / predicted molecular mass	Antibacterial activity	Effect of inclusion of bile in digestion on height of corresponding peak in RP-HPLC chromatogram
1-11	1023 / 1021	None detected	Peak height not determinable due to background interference from co-eluting bile and pancreatin
1-12	1151 / 1150 ^b	None detected	$1.9 (\pm 0.3)$ fold decrease (p = 0.0009)
1-20	1881 / 1881 ^a	Yes	$1.4 (\pm 0.1)$ fold increase (p = 0.02)
1-21	2013 / 2012 ^b	Yes	Not significant $(p = 0.06)$
1-29	2810 / 2809 ^a	Yes	$3.5 (\pm 0.3)$ fold increase (p < 0.0001)
1-32	3159 / 3157 ^a	Yes	Peak height not determinable due to background interference from co-eluting pancreatin

Mean fold increases and SD are derived from three sets of replicates; p values are in brackets. ^aChan et al. (1996). ^bSlootweg et al. (2013).

Table 3

Effect of surfactants on the minimum inhibitory concentration (MIC, μ g/mL) of nisin in agar diffusion activity assays (SD in brackets, n =3).

Surfactant	MIC (µg/mL)
No surfactant	1.81 (± 0.11)
0.3 millimoles/L bile salts	$0.05~(\pm 0.01)$
8 millimoles/L Tween® 80	0.008 (± 0.001)
0.2 millimoles/L Triton TM X-100	0.008 (± 0.001)



Fig. 1. RP-HPLC chromatograms of (A) undigested nisin and (B) the products of nisin digestion without bile (—) and with bile included (----). Regions where nisin fragments and intact nisin eluted are highlighted. The effect of including or excluding bile from a digestion with respect to the amount of nisin fragments 1-12, 1-29 and 1-20 produced is highlighted.



Fig. 2. Mass spectrometry analysis and well diffusion activity assay (inserts) of nisin fragments produced by the digestion of nisin. Analysis of intact nisin from the products of the control digestion is included for comparison in which 3354.70 Da correlates with the predicted molecular mass of intact nisin (3355.12 Da) (Chan et al., 1996) and 1678.14 Da correlates with the molecular mass of doubly charged intact nisin.



Fig. 3. Effect of nisin-surfactant interaction on particle size as examined by Atomic Force Microscopy AFM (A and B), Turbidity (C) and Dynamic Light Scattering (DLS) (D). AFM: AFM of bile (A) and AFM of bile with nisin (B). Turbidity (C): \blacklozenge 10 millimoles/L bile salts, \blacklozenge 0.3 millimoles/L bile salts, \bigstar 0.2 millimoles/L TritonTM X-100, \times 8 millimoles/L Tween[®] 80 and — No surfactant (\pm SD, n = 4). DLS (D): \equiv 10 millimoles/L bile salts and ||| 10 millimoles/L bile salts with 100 µg/mL nisin (\pm SD, n = 4). Difference in particle size determined by DLS (D) is significant (p = 0.0123, n = 4).



Fig. 4. Schematics of a bile salt, a bile salt primary micelle and a nisin peptide. A bile salt molecule is 2 nm long (Small, 1971) and a nisin peptide is 5 nm long (Sahl, 1994). Drawings of a bile salt and primary micelle are based on those of Small (1968). Bile salt primary micelles can consist of 2 to 10 bile salts (Li et al., 2009). The image of nisin was produced using Protein Database entry 1WCO (Hsu et al., 2004) in conjunction with the NGL Viewer (Rose & Hildebrand, 2015).

In vitro digestion of nisin under physiological gastrointestinal conditions.

Six nisin fragments produced, four of which are bioactive.

Bile forms a complex with nisin.

Bile alters the relative amounts of the nisin fragments produced by digestion.