Controlled release of nisin from Neusilin particles to enhance food safety of sour curd cheese

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Abstract Nisin is frequently added as food additive to soft cheese to increase food safety against foodborne pathogens like *Listeria monocytogenes*. The goal of this study was the extension of the antimicrobial activity of nisin in sour curd cheese (SCC) by self-releasing adsorbed nisin from Neusilin UFL2 over production-based pH shift. First, the antimicrobial activity of nisin adsorbed to Neusilin UFL2 (UFL2-N) and free nisin was investigated in BHI broth at a pH range from 7.5 to 4.5 for each of six *L. monocytogenes* field isolates. UFL2-N showed similar minimal inhibition concentration to *L. monocytogenes* over time as free nisin. Distribution of nebulized, fluorescence-labelled UFL2 was homogenous on SCC surface. Thereafter, SCC surface was inoculated with *L. monocytogenes* and 0.004, 0.013, 0.026, and 0.132 mg mL⁻¹ UFL2-N or free nisin. In SCC, *L. monocytogenes* was below quantification limit at 0.132 mg mL⁻¹ UFL2-N or free nisin after 2 days of ripening. Collectively, UFL2-N enabled a slow release and antilisterial activity in vitro as well as in cheese manufacturing.

Keywords Adsorption · Food safety · *Listeria monocytogenes* · Neusilin UFL2 · Nisin

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Introduction

The foodborne pathogen *Listeria monocytogenes* can remain in food plants due to insufficient cleaning. Many *L. monocytogenes* isolated from food plants are able to survive a broad spectrum of harsh environmental conditions commonly applied by food manufactures such as acidic conditions, high salt concentrations or low temperatures (Gill and Reichel 1989). Therefore, non-thermal treated food products such as sour curd cheese (SCC), which main ingredient is low-fat sour curd, require particular attention to food safety. Soft cheese like SCC is reported in high frequency to be contaminated by *L. monocytogenes* (Martinez-Rios and Dalgaard 2018) and caused many outbreaks like in Austria and Germany in 2009 with eight deaths (Fretz et al. 2010).

Nisin is a 34-residue antibacterial cationic peptide and used to protect food from spoilage of a wide range of Gram-positive bacteria including *L. monocytogenes* (Delves-Broughton et al. 1996; Ferreira and Lund 1996). Novel technologies are continuously developed to immobilize nisin for a targeted, prolonged and cost-effective nisin release from carrier materials to food matrices. Particles such as Neusilin are chemical and physical robust candidates as adsorption material in innovative food protection systems and are based on magnesium aluminometasilicate. Neusilin particles were successfully used as excipient in pharmaceutical formulations (Mallappa et al. 2015), as an adsorbent powder in animal feed administration (Ma et al. 2016), nutritional supplementation (Santaniello and Giannini 2016) and were already approved by US Food and Drug Administration as drug master file. Moreover, Neusilin provide several advantages such as adjustable size, large surface area, pH-dependent release, and reduced interactions to food components of adsorbed

nisin as well as reduced microbial enzymatic degradation (Aasen et al. 2003; Bhatti et al. 2004; Chollet et al. 2008; Sun et al. 2009).

The objective of this study was to evaluate different particle types of Neusilin as suitable carrier material for pH dependent nisin release. Capacity for adsorbing nisin onto the most promising particle Neusilin UFL2, release rates as well as distribution of fluorescence-labeled Neusilin after nebulization was assessed. Susceptibility of *L. monocytogenes* to nisin-adsorbed Neusilin (UFL2-N) or free nisin was determined in vitro at a pH range from 7.5 to 4.5 and on surface of sour curd cheese (SCC) at the end of production.

Material and methods

Screening of carrier materials for nisin adsorption

Different porous material particle types of Neusilin (S1, S2, SG2, UFL2, US2; Fuji Chemical Industry Co., Ltd., Japan) were tested. The zeta potential of each Neusilin type was determined as a function of pH. For each type, 100 mg mL⁻¹ were dispersed in 2 mmol L⁻¹ borate buffer (pH 9.0–8.0), 2 mmol L⁻¹ TRIS buffer (pH 8.0–6.0), 2 mmol L⁻¹ sodium acetate buffer (pH 6.0–4.0) or 2 mmol L⁻¹ trifluoroacetate buffer (pH 4.0–2.0) and at steady pH value the zeta potential was measured by Zetasizer 3000 HSA (Malvern Instruments, Germany) with three replicates.

Nisin adsorption and release rate

Neusilin UFL2 (UFL2, lot number 405014; Fuji Chemical Industry) was best in adsorbing and releasing of nisin, which is positively charged until pH 8.52 (Hammami et al. 2010). Diluted UFL2 suspensions were examined by inverse confocal laser scanning microscope (CLSM; Leica TCS SPE, Germany) and images were digitally processed using LAS X (Leica, Germany). The UFL2 particles (100 g L⁻¹) were dispersed in distilled water followed by repeatedly washing with distilled water until a pH of 8.0 was reached. Afterwards, UFL2 suspension was autoclaved (120 °C, 60 min). Two g L⁻¹ nisin (NisinZTM P 95%, lot number 0304 20150412; Handary SA, Belgium) was dissolved in sterile distilled water and was added to UFL2. Thereafter, the pH was slowly increased to 8.0 at room temperature by adding 5 mol L⁻¹ NaOH dropwise. The resulting UFL2 with adsorbed nisin (UFL2-N) was washed twice with sterile distilled water, freeze-dried (VaCo 2; Zirbus GmbH, Germany) and stored at room temperature. UFL2-N stability was confirmed by susceptibility testing to *L. monocytogenes* field isolate BfR L1031 with

0.013 mg mL⁻¹ UFL2-N (see Sect. 2.5.1) after 7, 28 and 35 days of storage at room temperature.

The amount of adsorbed and released nisin was determined in 10 mmol L⁻¹ sodium acetate buffer (pH 5.0) either with or without addition of 0.74 mol L⁻¹ NaCl by the bicinchoninic acid (BCA) protein assay (Thermo Scientific, Germany) over 7 days as described by the manufacturer. The concentration of nisin on the particles was determined as difference of nisin added to the particles and the remaining nisin in the supernatant. A standard curve of albumin from bovine serum (BSA) was prepared as recommended by the manufacturer (Thermo Scientific) and values were corrected as protein amount differed in comparison to BSA standard curve (Fig. S1).

Labeling particles with fluorophores

For the analysis of nisin and particle distribution on the cheese surface by means of confocal laser scanning microscopy (CLSM) the polycation polyallylamine (PAH, M_w 15 kD; Sigma-Aldrich, Germany) was labeled with either tetramethylrhodamin isothiocyanat (PAH-TRITC; Sigma-Aldrich) or cyanine dye Cy5 (PAH-Cy5; Sigma-Aldrich). Subsequently, the Neusilin particles were coated with one PAH-fluorophore by Layer-by-Layer technology as described previously (Peyratout and Dähne 2004). PAH-fluorophores were dried and stored at room temperature as described in Sect. 2.2.

Suspensions of 0.5 mL PAH-TRITC were nebulized with a spray bottle (Carl Roth, Germany). PAH-TRITC was applied as positively charged model compound analogous to nisin. Upon charge reversal of negatively charged Neusilin, PAH-TRITC is released to estimate diffusion kinetics on the SCC surface as well as the depth of penetration into SCC. PAH-Cy5 was used to visualize the spatial distribution of the loaded Neusilin particles after spraying them onto the cheese surface. The amount of fluorophores brought onto the cheese surface was adjusted to the maximal allowed nisin concentration in food (EU 2011). Cheese was stored at 3 °C for 4 days and either the surface was analyzed or the cheese was cut in 0.5 cm slices by a scalpel and the distribution of the particles perpendicular to the surface was analyzed by CLSM.

Listeria monocytogenes field isolates

Six *L. strains* isolated from food products were selected according to serotypes most frequently associated with human listeriosis, i.e. IIa, IIb and IVb (Allerberger 2003). Isolates are deposited at the strain collection of the National Reference Laboratory for *L. monocytogenes* (German Federal Institute of Risk Assessment, Germany). *Listeria monocytogenes* field isolates were maintained in

BHI broth (Carl Roth) added with 15% glycerol (Carl Roth) at -80°C until further use. Field isolates were plated on BHI agar plates (Carl Roth) and were routinely grown for 1 day at 30°C (Noll et al. 2018).

Antilisterial activity

In vitro susceptibility testing of UFL2-N and free nisin

A stock solution of 0.132 mg mL^{-1} UFL2-N or free nisin (Handary) was prepared in pH adjusted (0.1 mol L^{-1} HCl) sterile BHI broth (pH 7.5, 7.0, 6.5, 6.0, 5.5, 5.0, and 4.5). The nisin concentration in UFL2-N was calculated based on mass of nisin per mg UFL2 particle. Before susceptibility testing, UFL2-N was dispersed in ultrasonic bath (USR 30 H; Merck KGaA, Germany).

Each *L. monocytogenes* working suspension was added into fresh BHI broth. Thereafter, 0.1 mL of each *L. monocytogenes* field isolate was added to each well of a sterile 96-well microtiter plate (approximately 5 log CFU mL^{-1} per well), which was centrifuged at $2200\times g$ and 4°C for 10 min. The supernatants were discarded and cells were resuspended in pH-adjusted BHI broth containing 0.004, 0.013, 0.026, and 0.132 mg mL^{-1} UFL2-N or free nisin. Microtiter plate was sealed with Breathe-Easy® membrane (Carl Roth) followed by incubation for 7 days. The optical density (OD) of each well was measured daily at a wavelength of 595 nm after 5 s of shaking by FLUOstar OPTIMA microplate reader (BMG Labtech, Germany). Controls were provided by incubating bacterial suspensions without supplementation of UFL2-N, free nisin, or UFL2. Cutoff value for bacterial growth was set to $\Delta\text{OD}_{595} > 0.13$ as described earlier (Noll et al. 2018). The lowest nisin concentration with no growth was defined as minimal inhibition concentration (MIC) in six replicate measurements.

Monitoring pH during SCC ripening, susceptibility testing of UFL2-N and free nisin on *L. monocytogenes* contaminated SCC

Sour curd, 2-weeks ripened SCC ('culture cheese'), sodium chloride and sodium hydrogen carbonate were manually mixed in sterile beakers at room temperature according to a traditional Hessian recipe of SCC. The surface pH was measured over time. Loaves of SCC were formed from portions of 25 g and were ripened at 98% relative humidity. After 2, 6, 20, 24, 43, 48 and 72 h of ripening the upper surface of a loaf was cut off in thin slices and 0.5 g SCC as well as 0.5 g sour curd (0 h) was homogenized in 10 mL distilled water. After continuous stirring, the pH value was determined. Different commercial Hessian SCCs were analyzed in the same manner.

SCC loaves produced under laboratory conditions were further subjected to *L. monocytogenes* contamination. Therefore, UFL2-N or free nisin were applied onto the upper surface of each loaf (see Sect. 2.5.1) in four independent SCC incubations. *Listeria monocytogenes* field isolate BfR L1031 (5 log CFU mL^{-1}) was added onto the SCC topside surface. After contamination, SCC was ripened for 2 days and macroscopic appearance as well as texture were inspected.

Sampled surface area was approximately 0.45 cm^2 and isolation of genomic DNA (gDNA) from homogenized SCC was conducted according to manufacturer's procedures using KingFisher™ cell and tissue DNA kit (Thermo Scientific). To characterize the bacterial community of SCC, amplicon sequencing and sequence data analysis were carried out as explained earlier (Buettner and Noll 2018).

Quantitative PCR amplification (qPCR) of the *hlyA* gene, which encodes the *Listeria* specific virulence factor listeriolysin O, and bacterial 16S rRNA gene from the gDNA was performed with two technical replicates in thermal cycler CFX96 (BioRad Laboratories GmbH, Germany). Primer sequences (Eub 341f, Eub 534r; *hlyA*f, *hlyA*r) and preparation of standard curves were published elsewhere (Muyzer et al. 1993; Nogva et al. 2000). Reaction mixtures of $25\text{ }\mu\text{L}$ contained $2\text{ }\mu\text{L}$ of gDNA, $0.3\text{ }\mu\text{mol L}^{-1}$ of each primer, iTaq Universal SYBR Green Supermix (BioRad) and nuclease-free water (Carl Roth). Thermal profile for *hlyA* gene amplification was 5 min initial denaturation at 95°C , followed by 40 cycles of denaturation at 95°C for 20 s, annealing at 58.7°C for 20 s, and elongation at 72°C for 20 s; while for 16S rRNA gene amplification 8 min initial denaturation at 95°C was followed by 40 cycles of denaturation at 95°C for 30 s, annealing at 54.3°C for 30 s, and elongation at 72°C for 30 s.

Statistics

The bacterial growth expressed in CFU mL^{-1} was log-transformed to test statistical significance by a two-sided Wilcoxon–Mann–Whitney-Test at 95% confident interval (R; v. 3.3.3, R Core Team, Austria).

Results

Adsorption of nisin on UFL2

All of the tested Neusilin particle types comprised a negative surface potential in the range of pH 9.0–5.3. Neusilin particles have a typical clay structure (alumosilicates, Supplementary Information Fig. S2), for which isoelectric

points between 4 and 7 are common. Within this range, an efficient adsorption of free nisin at pH 8.0 was observed for several Neusilin types but only minimal release of nisin could be achieved from Neusilin S1, S2, SG2, and US2. Given the isoelectrical point at pH 5.3 (Fig. 1) and the mean particle size of 8–20 μm (Supplementary Information Fig. S3), UFL2 was well suited for a release by reversing its zeta potential from positive to negative at sour curd pH. Different adsorption procedures were tested and nisin adsorption to UFL2 at a constant pH of 8.0 had a low adsorbing of below 30%. The highest yield of nisin adsorption was achieved by slowly increasing pH to 8.0, resulting of up to 45% (wt/wt) nisin uptake per mg UFL2 particle.

Nisin release and evaluation of particle nebulization

The accumulative release of nisin from UFL2-N at pH 5.0 was determined both without and with NaCl addition, which is typical in SCC (Fig. 2). Nisin release rate gradually increased over time and was higher in presence of NaCl compared to absence within initial 48 h of ripening (Fig. 2). MICs of lyophilized UFL2-N after 5 weeks of storage at room temperature was similar to freshly prepared UFL2-N indicating temporal stability of nisin activity.

To implement UFL2-N in SCC production, particle nebulization was tested with fluorescently labeled PAH-Cy5 and PAH-TRITC (Fig. 3a–d). The particles were homogeneously distributed on the surface of SCC after nebulization (Fig. 3a, c). Some pores in the cheese surface allowed the penetration of particles up to 300 μm inside the cheese (Fig. 3b). In order to determine the density of the particles on the cheese surface, the diffusion of released nisin into the SCC matrix was studied by fluorescently labeled polycation PAH-TRITC (Fig. 3e). Diffusion was

Fig. 1 Zeta potential of Neusilin types S1 (filled square), S2 (filled circle), UFL2 (filled triangle), US2 (filled inverted triangle), and SG2 (filled diamond) as a function of pH; (n = 3)

Fig. 2 Time dependent release of nisin from Neusilin UFL2-N in 10 mmol L⁻¹ sodium acetate buffer at pH 5.0 (filled square) and in same buffer with 0.74 mol L⁻¹ NaCl added as common in sour curd cheese (open circle); (n = 3)

up to 100 μm inside the cheese. Hence, the density of the sprayed particles is more as sufficient to ensure the diffusion of released nisin into the particle free areas within less than 1 h.

Antilisterial activity of UFL2-N and free nisin in vitro

Nisin susceptibility of UFL2-N and free nisin were similar to each other and isolate specific (Table 1). MICs of UFL2-N as well as free nisin were affected by pH and were lower at decreasing pH. MIC of *L. monocytogenes* field isolate BfR L261 increased over time for both UFL2-N and free nisin at pH 6.5–5.5, while *L. monocytogenes* field isolate BfR L32 was completely inhibited. Lag phase of *L. monocytogenes* field isolates was extended at pH ≤ 5.5 (Supplementary Information Fig. S4) likewise after addition of UFL2 (Table 1). Addition of already 0.004 mg mL⁻¹ free nisin decreased cell conglomeration of *L. monocytogenes* compared to incubations without nisin irrespective of the presence of 0.74 mol L⁻¹ NaCl (Supplementary Information Fig. S5).

Nisin addition on surface of SCC

Surface pH of SCC rapidly increased from 4.6 in sour curd to 6.3 after addition of curing salts and thereafter steadily increased to pH 7.0 (Fig. 4a). Surface pH of commercial Hessian SCC was similar (Fig. 4b). Addition of UFL2-N or free nisin up to 0.132 mg mL⁻¹ to SCC did not alter the texture and appearance (size, color in curd core and rind) when compared to commercial SCC. In addition, we have sequenced the bacterial community of SCC, and obtained 103 OTUs including added *L. monocytogenes* as only

Fig. 3 CLSM images of PAH-Cy5 filled UFL2 particles sprayed on sour curd cheese (SCC) surface. **a** Particle (bright) distribution on SCC surface. **b** Particle distribution 100 μm below the cheese surface within a SCC pore. **c** Cut through the cheese on a none-porous part showing high particle concentration on the surface. **d** Control image of pure cheese surface without fluorescent particles. **e** Depth resolved PAH-TRITC polymer penetration after 1 day diffusion from the surface into SCC. **f** Control image of cheese without PAH-TRITC. CLSM images **c**, **e** and **f** were obtained from a vertical cut through SCC

pathogenic OTU (0.003%). The relative abundance of the OTUs in the sequence data set was highest for the genera *Lactobacillus*, *Streptococcus* and *Weissella* while other OTUs were below $\geq 0.1\%$ (Supplementary Information Table S1). The presence of the microflora was not significantly reduced although *L. monocytogenes* and UFL2-N or free nisin were present ($P > 0.05$, Table 2).

MICs of UFL2-N and free nisin (Table 1) were added on top of SCC. With increasing concentration of UFL2-N or free nisin, respective log-reduction of *L. monocytogenes* field isolate BfR L1031 also increased (Table 2). By addition of 0.132 mg mL^{-1} of UFL2-N, *L. monocytogenes* was below limit of quantification.

Discussion

Recipes of SSC has been first described in a cookbook from the 16th century, and therefore its quite simple production has a long tradition especially in Germany and neighboring states. Sour curd is fermented without any additional heat treatments by lactic acid bacteria as starter cultures ripens the sour curd from the rind to the inner fluffy body. During ripening of few days to several weeks the initial white color shifts to orange-yellow color and the pH increase from approximately 4.4 to 7.2 (Belitz et al. 2001). In addition, ripening changes the attributes from a creamy texture and a salty flavor towards a less-flexible texture and intensified cheese flavor and taste. Some SCC

varieties have additions of mold cheese, herbs and/or salt at the initial stage of ripening, which also shifts appearance, flavor and texture compared to the traditional SCC.

Once organized sampling regime revealed that cheese has been contaminated by *L. monocytogenes* every production plant usually has a defined action plan, which is part of the plant-specific HACCP concept and was summarized previously (McElhatton and Marshall 2007). Such action plans include information of the staff, painstaking sampling and thereafter disinfection campaigns in the production plant, no food delivery and call back of potential spoiled food charges. To circumvent such time-consuming, expensive and may defamatory actions, and to enhance food safety, addition of food additives such as nisin have been used since more than 50 years (Delves-Broughton et al. 1996). However, free nisin showed a low bio-availability as it interacts with food components (Aasen et al. 2003; Bhatti et al. 2004; Chollet et al. 2008). Moreover, rapid proteolytic degradation processes limited the use of nisin in food industry (Sun et al. 2009). Thus, the highly porous Neusilin was employed as carrier material for controlled slow release of adsorbed nisin to minimize its food interaction and exposition to degradation. Different Neusilin types were previously applied as excipient and improved solubility of less water soluble drugs (Mallappa et al. 2015). Hence, the poor solubility of nisin at neutral pH was circumvented by UFL2. The adsorption and subsequent desorption of free nisin on UFL2 should be based on electrostatic interactions. Beside the negative zeta

Table 1 Minimal inhibition concentration (MIC) of UFL2-N, free nisin or UFL2 for six *L. monocytogenes* field isolates at pH of 7.5–4.5 over time (n = 6)

pH-value	Field isolate	MIC (mg mL ⁻¹)						UFL2		
		UFL2-N			Free nisin					
		1 day	4 days	7 days	1 day	4 days	7 days	1 day	4 days	7 days
7.5	BfR L32	0.026	0.132	0.132	0.026	0.132	0.132	+	+	+
	BfR L261	0.026	0.132	0.132	0.013	0.132	0.132	+	+	+
	BfR L308	0.026	0.132	0.132	0.026	0.132	0.132	+	+	+
	BfR L451	0.026	0.132	0.132	0.026	0.026	0.026	+	+	+
	BfR L493	0.026	0.026	0.026	0.013	0.026	0.026	+	+	+
	BfR L1031	0.026	0.026	0.026	0.013	0.013	0.013	+	+	+
7.0	BfR L32	0.026	0.132	0.132	0.026	0.026	0.026	+	+	+
	BfR L261	0.026	0.132	0.132	0.013	0.026	0.026	+	+	+
	BfR L308	0.026	0.026	0.026	0.026	0.026	0.026	+	+	+
	BfR L451	0.026	0.132	0.132	0.013	0.026	0.026	+	+	+
	BfR L493	0.026	0.026	0.026	0.013	0.026	0.026	+	+	+
	BfR L1031	0.026	0.026	0.026	0.013	0.013	0.013	+	+	+
6.5	BfR L32	0.026	0.026	0.026	0.026	0.026	0.026	+	+	+
	BfR L261	0.013	0.026	0.132	0.013	0.026	0.026	+	+	+
	BfR L308	0.026	0.026	0.026	0.013	0.026	0.026	+	+	+
	BfR L451	0.026	0.026	0.026	0.013	0.026	0.026	+	+	+
	BfR L493	0.013	0.026	0.026	0.013	0.026	0.026	+	+	+
	BfR L1031	0.013	0.013	0.013	0.013	0.013	0.013	+	+	+
6.0	BfR L32	0.026	0.026	0.026	0.026	0.026	0.026	+	+	+
	BfR L261	0.013	0.026	0.132	0.013	0.026	0.026	+	+	+
	BfR L308	0.026	0.026	0.026	0.013	0.026	0.026	+	+	+
	BfR L451	0.026	0.026	0.026	0.013	0.026	0.026	+	+	+
	BfR L493	0.013	0.026	0.026	0.013	0.026	0.026	+	+	+
	BfR L1031	0.013	0.026	0.026	0.013	0.013	0.013	+	+	+
5.5	BfR L32	0.013	0.013	0.013	0.013	0.013	0.013	+	+	+
	BfR L261	0.013	0.013	0.026	0.004	0.013	0.013	+	+	+
	BfR L308	0.013	0.013	0.013	0.013	0.013	0.013	+	+	+
	BfR L451	0.013	0.013	0.013	0.013	0.013	0.013	+	+	+
	BfR L493	0.013	0.013	0.013	0.004	0.013	0.013	+	+	+
	BfR L1031	0.004	0.013	0.013	0.004	0.013	0.013	–	–	+
5.0	BfR L32	0.004	0.004	0.004	0	0.013	0.013	–	–	–
	BfR L261	0	0.013	0.013	0	0.004	0.004	–	+	+
	BfR L308	0	0.013	0.013	0.004	0.004	0.004	–	–	–
	BfR L451	0.004	0.004	0.004	0.004	0.004	0.004	–	–	–
	BfR L493	0.004	0.013	0.013	0.004	0.013	0.013	+	+	+
	BfR L1031	0	0.013	0.013	0	0.004	0.004	–	–	+
4.5	BfR L32	0	0.004	0.004	0	0.004	0.004	–	–	–
	BfR L261	0	0.004	0.004	0	0.004	0.004	–	–	+
	BfR L308	0	0.004	0.004	0	0.004	0.004	–	+	–
	BfR L451	0	0.004	0.004	0	0.004	0.004	–	–	–
	BfR L493	0	0.004	0.004	0	0	0	–	–	–
	BfR L1031	0	0.004	0.004	0	0.004	0.004	–	–	–

+ bacterial growth; – no bacterial growth

potential between pH 9.0 and 5.3, the size distribution of UFL2 was well suited.

In fact, time lapsed increase of pH beyond the isoelectric point of UFL2 resulted in maximal adsorption amount of

Fig. 4 Measurement of pH value on sour curd cheese (SCC) surface during **a** SCC production under laboratory conditions and **b** on surface after commercial production ($n = 6$). Ripening was carried out in foil over 33 days at 3 °C. Dashed lines refer to 95% confidence interval

cationically charged nisin to negatively charged UFL2 after contact for several hours. Such effect was not reported for the efficient loading of the small molecule domperidon into Neusilin UFL2 particles (Kudamala and Murthy 2017). Obviously, due to the small average pore size of 15 nm of Neusilin UFL2 the immediate strong interaction between the highly negative charged pore surface and the large cationic nisin led obviously to a blocking of pores. In contrast, a weak interaction at lower pH values enables a smooth diffusion of the nisin into the pores resulting in the 1.5 higher loading amount. Maybe, similar blocking phenomena influences the release behavior as well.

After applying the particles to a sour curd likely environment, the pH decreases below the isoelectric point of UFL2. This leads to a reversal of the surface potential of UFL2 and subsequently to a release of the positively charged nisin by electrostatic repulsion superposed by the diffusion rates from the nanometer pores. The presence of higher concentration of sodium chloride reduced the release of nisin after longer incubation time. In line with this result, the electrostatic repulsion is reduced by the shielding effect of the chloride ions leading to less release (Fig. 2). In addition, higher ion strength will influence the structure of nisin itself, which might lead to a more coiled

structure. This could explain the slightly accelerated release in the first day (Fig. 2).

Similarly, Prombutara et al. (2012) reported a burst release from nisin-loaded solid lipid nanoparticles in presence of 0.5 mol L⁻¹ sodium chloride, which might be reduced by the necessary diffusion out of the pores in our case. Furthermore, non-electrostatic interactions like hydrophobic interactions and/or hydrogen bonding might additionally affect release kinetics and were previously associated to adsorbed nisin on clays (Ibarguren et al. 2014; Meira et al. 2015). Probably explaining the poor release of nisin from Neusilin S1, S2, SG2, and US2. Moreover, increased salt contents did not alter the nisin efficacy (Table 2) as shown earlier (Chollet et al. 2008; Pawar et al. 2000). However, De Martinis et al. (1997) and Yen et al. (1991) described a protective effect to *L. monocytogenes* after addition of similar salt concentrations, which is supported by our preliminary results (Supplementary Information Fig. S5).

In accordance with previous studies decreasing antimicrobial activity of nisin was observed when pH was increased (De Martinis et al. 1997; Ferreira and Lund 1996). The pH typically in sour curd enabled initial high release rates of nisin from UFL2-N as pH 4.4 was below the isoelectric point and neutral surface charge of UFL2. In BHI broth at a pH of 5.0 and 4.5, UFL2-N showed enhanced antilisterial activity (Table 1). The carrier material did not show any antilisterial activity in tested pH range where *L. monocytogenes* field isolates readily grew. At slightly acidic conditions (pH < 5.0) a combination of pH and positive zeta potential of UFL2 resulted in attraction of negatively charged bacterial cell walls (Supplementary Information Fig. S3) thereby impairing bacterial growth, which was previously reported (Were et al. 2004). Antilisterial activity of UFL2-N and free nisin decreased isolate specific over time. Similarly the capability of *L. monocytogenes* to become spontaneously less susceptible to free nisin has been shown before (Ming and Daeschel

Table 2 Reduction based on median of *L. monocytogenes* field isolate Bfr L1031 (*hlyA* gene qPCR) and microbiota (16S rRNA gene qPCR) after addition of UFL2-N or free nisin on top of sour curd cheese subsequent to 2 days of ripening

MIC (mg mL ⁻¹)	log-reduction in CFU mL ⁻¹			
	<i>hlyA</i> gene		16S rRNA gene	
	UFL2-N	Free nisin	UFL2-N	Free nisin
0.004	0.28 ^a	0.71 ^a	0.04	No reduction
0.013	1.25	1.33 ^a	0.08	No reduction
0.026	1.39 ^a	1.07 ^a	No reduction	0.001
0.132	n.d.	n.d.	0.17	0.040

Logarithmic reduction is referred to samples without addition of UFL2-N or free nisin ($n = 4$). Detection limit was 2.64 log CFU mL⁻¹

n.d. No amplification detected by qPCR or signal lay below limit of quantification

^aSignificantly difference at $P < 0.05$ within treatment

1993). Growth of *L. monocytogenes* at pH 5.0 and 4.5 was also isolate specific, which is in line with previous findings of extended lag phase at pH 5.6–3.8 in vitro and in vivo (Cheroutre-Vialette et al. 1998; Rogga et al. 2005).

Experimental data from autoclaved SCC were very promising (data not shown) and application of fluorescence-labelled UFL2 by spraying indicated a proper and simple technique for a homogenous distribution on SCC surface. Results obtained from SCC showed in practice log-reduction ($P < 0.05$) to virtually complete inhibition of *L. monocytogenes* field isolate BfR L1031 at 0.132 mg mL^{-1} and MICs did not exceed the limit of 12.5 mg kg^{-1} set by the EU (EU 2011). The outcome of no complete *L. monocytogenes* field isolate BfR L1031 inhibition below 0.132 mg mL^{-1} might have been a combination of increased pH and nisin release rate from UFL2-N as well as presence of proteolytic or nisin-degrading activity from members of the cheese microbial community (Ramsaran et al. 1998; Sulzer and Busse 1991).

The production of UFL2-N is a simple and cost-effective process, which can be easily upscaled. The excipient UFL2 as well as nisin have a low price, and only few and inexpensive equipment is needed. Moreover, both materials can be implemented in existing good manufacture practice environments. UFL2-N can also be sprayed on surfaces of low pH processed and ripened cheese or can be directly incorporated to dairy produce to support food safety. However, the worldwide legislative requirements for application of UFL2 itself should be also taken into account before launching into market.

Conclusion

Neusilin UFL 2 has a size below the detection limit of the human tongue and was found to be an ideal commercial carrier for adsorption of free nisin. Slow release of nisin from UFL2-N was accomplished by pH-shift. Subsequently, UFL2-N and free nisin showed antilisterial activity in vitro as well as in SCC. Particles sprayed on contact surfaces did not penetrate deeply into SCC. UFL2-N can be stored at room temperature for more than 1 month as no loss of antimicrobial activity in application was recorded. Moreover, the appearance and texture of the SCC remained similar after addition of UFL2-N, which meet customers' expectations. Thus, condition-tailored UFL2-N would provide improvements to present hurdle technology and nebulization would ease implementation in food production.

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