Insights in the Antimicrobial Potential of the Natural Nisin Variant Nisin H

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Lantibiotics are a growing class of antimicrobial peptides, which possess antimicrobial activity against mainly Gram-positive bacteria including the highly resistant strains such as methicillin-resistant *Staphylococcus aureus* or vancomycin-resistant enterococci. In the last decades numerous lantibiotics were discovered in natural habitats or designed with bioengineering tools. In this study, we present an insight in the antimicrobial potential of the natural occurring lantibiotic nisin H from *Streptococcus hyointestinalis* as well as the variant nisin H F_1I. We determined the yield of the heterologously expressed peptide and quantified the cleavage efficiency employing the nisin protease NisP. Furthermore, we analyzed the effect on the modification via mass spectrometry analysis. With standardized growth inhibition assays we benchmarked the activity of pure nisin H and the variant nisin H F_1I, and their influence on the activity of the nisin immunity proteins NisI and NisFEG from *Lactococcus lactis* and the nisin resistance proteins *Sa*NSR and *Sa*NsrFP from *Streptococcus agalactiae* COH1. We further checked the antibacterial activity against clinical isolates of *Staphylococcus aureus*, *Enterococcus faecium* and *Enterococcus faecalis* via microdilution method. In summary, nisin H and the nisin H F_1I variant possessed better antimicrobial potency than the natural nisin A.

Keywords: lantibiotics, nisin, nisin H, MS analysis, antimicrobial activity

INTRODUCTION

Lantibiotics (lanthionine containing antibiotics) are a growing class of antimicrobial peptides (AMPs), which posses antimicrobial activity even against highly resistant strains such as methicillin-resistant *Staphylococcus aureus* (MRSA) or vancomycin-resistant *enterococci* (VRE) and some are already in pre-clinical trials (Mota-Meira et al., 2000; Jabes et al., 2011; Dawson and Scott, 2012; Crowther et al., 2013; Dischinger et al., 2014; Ongey et al., 2017; Brunati et al., 2018; Sandiford, 2019). Lantibiotics are peptides, containing 19–38 amino acids and are mainly produced by Gram-positive bacteria (Klaenhammer, 1993; Sahl and Bierbaum, 1998). In the last decades an increasing number of lantibiotic gene clusters were found by data-mining approaches using tools such as BAGEL4 (van Heel et al., 2018).

The best studied lantibiotic is nisin, which was first discovered in 1928 by Rogers and Whittier and belongs to the class I lantibiotics (Rogers, 1928; Rogers and Whittier, 1928; Arnison et al., 2013). It is used in the food industry since 1953 and obtained the status as generally recognized as safe (GRAS) in 1988 from the Food and Drug Administration (FDA) (Delves-Broughton et al., 1996). The class I lantibiotic nisin contains 34 amino acids and five (methyl)-lanthionine rings. These (methyl)-lanthionine rings require multiple posttranslational modifications (PTMs) which are introduced in the precursor peptide. First, the serine and threonine residues in the core peptide are dehydrated by a specific dehydratase NisB (lantibiotic class I LanB dehydratase) (Kaletta and Entian, 1989; Karakas Sen et al., 1999; Koponen et al., 2002; Ortega et al., 2015; Repka et al., 2017). The next step is a Michael-type condensation of dehydrated residues with the thiol group of a cysteine residue, thereby forming (methyl)-lanthionine rings, guided in a regio- and stereospecific manner by the cyclase NisC (class I lantibiotic cyclase) (Okeley et al., 2003; Li et al., 2006; Li and van der Donk, 2007; Repka et al., 2017). These characteristic (methyl)-lanthionine rings give lantibiotics high heat stability, resistance against proteolytic digestion and are responsible for the nanomolar antimicrobial activity (Gross and Morell, 1967; Rollema et al., 1995; Chan et al., 1996; Lu et al., 2010; Oppedijk et al., 2016).

The sequence of nisin A can be subdivided into three parts (see **Figure 1**). The N-terminal part with ring A, B, and C is responsible for binding to the cell wall precursor lipid II (Hsu et al., 2004). The hinge region is very flexible and allows reorientation of the C-terminal part to insert into the membrane (van Heusden et al., 2002; Hasper et al., 2004; Wiedemann et al., 2004; Medeiros-Silva et al., 2018), while changes in this region have a strong impact on the target antimicrobial activity (Zhou et al., 2015; Zaschke-Kriesche et al., 2019b). After penetrating the membrane, the C-terminal part with ring D and E forms a stable pore with a stoichiometry of eight nisin and four lipid II molecules, which subsequently leads to rapid cell death (Hasper et al., 2004; Wiedemann et al., 2004; Alkhatib et al., 2014a; Medeiros-Silva et al., 2018).

Unfortunately, some bacteria established resistance mechanism against lantibiotics. For instance lantibiotic producing strains express the immunity system LanI and LanFEG (Alkhatib et al., 2014a,b), which prevent a suicidal effect after the lantibiotic is secreted. In the case of nisin A from *Lactococcus lactis* these proteins are called NisI and NisFEG. But also non-lantibiotic producing strains showed resistance against lantibiotics like *Streptococcus agalactiae* COH1, which arises from the expression of the membrane-anchored peptidase *Sa*NSR and the ABC transporter *Sa*NsrFP (Khosa et al., 2013, 2016a,b; Reiners et al., 2017).

Several natural nisin variants have been discovered and up to now eight are known. First of all nisin A from *L. lactis* (Rogers and Whittier, 1928), nisin Z from *L. lactis* NIZO 221 86 (Mulders et al., 1991), nisin F from *L. lactis* F10 (de Kwaadsteniet et al., 2008), nisin Q from *L. lactis* 61-14 (Zendo et al., 2003), nisin O_1 to O_4 from *Blautia obeum* A2-162 (Hatziioanou et al., 2017), nisin U and U2 from

Streptococcus uberis 42 and D536 (Wirawan et al., 2006), nisin P from *Streptococcus gallolyticus* subsp. *pasteurianus* (Zhang et al., 2012; Wu et al., 2014), nisin J from *Staphylococcus capitis* APC 2923 (O'Sullivan et al., 2020) and nisin H from *Streptococcus hyointestinalis* DPC 6484 (O'Connor et al., 2015).

In this study we focused on the natural nisin H variant (**Figure 1**). We used a standardized workflow for the characterization of lantibiotics, previous described in Lagedroste et al. (2019) to determine the impact on the expression, modification and antimicrobial properties of this nisin variant. We tested further the antimicrobial activity against some pathogen strains from *Staphylococcus aureus*, *Enterococcus faecium,* and *Enterococcus faecalis* using the microdilution method. As a reference we used the wild-type version of nisin A expressed using the same experimental setup. Furthermore, we exchanged the phenylalanine at position one (F_1) of nisin H to isoleucine, which is the natural amino acid of nisin A at this position (**Figure 1**). This position one was previously analyzed in nisin A and showed a major impact on different levels of the characterization (Lagedroste et al., 2019).

MATERIALS AND METHODS

Microorganisms and Culture Conditions

Cultures of *L. lactis* NZ9000 (Kuipers et al., 1997) containing the plasmids for immunity and resistance proteins were grown in M17 medium (Terzaghi and Sandine, 1975) at 30°C supplemented with 0.5% glucose [GM17 and the appropriate antibiotics described in Alkhatib et al. (2014a,b), Khosa et al. (2016b), Reiners et al. (2017), Lagedroste et al. (2019)]. For pre-nisin secretion, the *L. lactis* strain NZ9000 was grown in minimal medium (Jensen and Hammer, 1993) at 30°C supplemented with 0.5% glucose and the appropriate antibiotics. All bacteria used for minimum inhibitory concentration (MIC) determination of nisin variants [*Bacillus subtilis* 168; *S. aureus*: MSSA strain ATCC 29213, MRSA/VISA strain ATCC 700699; *E. faecium*: ATCC 35667, ATCC 700221 (vancomycin resistant); *E. faecalis*: ATCC 29212, ATCC 51299 (vancomycin resistant)] were cultivated in Mueller-Hinton broth (MHB) at 37°C and shaking at 150 rpm.

Cloning of Nisin H and the F_1I Variant

Nisin H was created as described in Reiners et al. (2017). The substitution of the phenylalanine at position one (F_1I) to an isoleucine was performed by site-directed mutagenesis. Here, we used the following primers (forward: 5′-GTGCATCACCACGCTTTACAAGTATTTCGC-3′; reverse: 5′-GCGAAATACTTGTAAAGCGTGGTGATGCAC-3′). After sequence analysis a competent *L. lactis* NZ9000 strain was transformed with the resulting plasmid via electroporation (Holo and Nes, 1989). The *L. lactis* NZ9000 strain already contain a vector (pil3-BTC) encoding for the modification and secretion proteins (Rink et al., 2005).

FIGURE 1 | Schematic overview of the used lantibiotics nisin A, nisin H, and the nisin H F$_1$I variant. Point mutations in nisin H and nisin H F$_1$I in comparison to nisin A are highlighted in blue. Dehydrated amino acids and cysteine residues involved in (methyl)-lanthionine ring formation (ring A, B, C, D, and E) are labeled in yellow and orange. The mutation of the nisin H F$_1$I variant is highlighted in green.

Expression, Purification and Activation of Pre-nisin Variants

The precursor of nisin H and its variant were expressed and purified as previously described (Abts et al., 2013; Alkhatib et al., 2014b; Lagedroste et al., 2017, 2019). Briefly, for pre-nisin secretion, the *L. lactis* strain NZ9000 was grown in minimal medium (Jensen and Hammer, 1993) supplemented with 0.5% glucose and 5 μg/ml of each erythromycin and chloramphenicol at 30°C. Cells were induced with 10 ng/ml nisin at an OD$_{600}$ of 0.4 and further grown overnight at 30°C without shaking. After harvesting the cells, the 0.45 μm-filtered supernatant was loaded onto a HiTrap SP HP cation exchange chromatography column. After washing with 50 mM lactic acid, the buffer was changed to 50 mM Hepes pH 7 via gradient and the final elution was done with 50 mM Hepes pH 7, 500 mM NaCl buffer. Elution fractions were concentrated in a 3 kDa cutoff filter. With a soluble version of NisP, the activation of all variants was performed overnight at 8°C (Lagedroste et al., 2017). The yield and cleavage efficiency determination was done by RP-HPLC (Agilent Technologies 1260 Infinity II) with a LiChrospher WP 300 RP-18 end-capped column and an acetonitrile/water solvent system (Abts et al., 2013; Lagedroste et al., 2017, 2019).

MALDI-TOF Analysis: Dehydration and (Methyl)-Lanthionine Ring Analysis

With MALDI-TOF analysis we analyzed the modification state of nisin H and its variant. Dehydrations are directly visible in the spectra, due to the loss of mass (−18 Da). For the determination of the presence of (methyl)-lanthionine rings, we used the organic coupling agent CDAP (1-cyano-4 dimethylaminopyridinium tetrafluoroborate) that binds to free cysteine residues (Wu and Watson, 1998). The reaction of the coupling agent to free cysteine

side chains would results in an increased mass in the spectra. The analysis was performed as previously described (Lagedroste et al., 2019). The samples were analyzed with MALDI-TOF (UltrafleXtreme, Bruker Daltonics, Bremen, Software: Compass 1.4) in positive mode.

Tandem Mass Spectrometric Analysis of Nisin H and Nisin H F$_1$I

Nisin H and the nisin H F$_1$I variant were purified using solid phase extraction (Oasis HLB, Waters) and finally resuspended in 0.1% trifluoroacetic acid. The samples were first subjected to liquid chromatography on a rapid separation liquid chromatography system (Ultimate 3000, Thermo Fisher Scientific) using an 1 h gradient and C18 columns as described (PMID 24646099) and further analyzed by a QExactive Plus mass spectrometer (Thermo Fisher Scientific) coupled via a nano-source electrospray interface. First, a precursor spectrum was acquired at a resolution of 140,000 (advanced gain control target 3E6, maximum ion time 50 ms, scan range 200–2000 m/z, profile data type). Subsequently, up to four 4–6-fold charged precursors were selected by the quadrupole (2 m/z isolation window), fragmented by higher-energy collisional dissociation (normalized collision energy 30) and recorded at a resolution of 35,000 (advanced gain control target 1E5, maximum ion time 50 ms, available scan range 200–2000 m/z, centroid data type).

Recorded spectra were analyzed by the MASCOT search engine (version 2.4.1, Matrix Science) and searches triggered by Proteome Discoverer (version 2.4.0.305, Thermo Fisher Scientific). A database was generated for the searches including 1000 randomly generated sequence entries each 34 amino acid long and the sequences of nisin H and nisin H F$_1$I. Methionine oxidation and dehydration of serine and threonine residues were

considered as variable modifications and the precursor mass tolerance set to 10 ppm and the fragment mass tolerance set to 0.02 Da. For peptide validation, the Fixed Value PSM validator was used (1% false discovery rate) and the IMP-ptmRS node for site validation (PMID 22073976). No random sequences were found by the search.

Determination of the Antimicrobial Activity by Growth Inhibition Assay

The determination of the antimicrobial activity of the different nisin variants was tested using a growth inhibition assay. The used strains were described in Alkhatib et al. (2014a,b), Reiners et al. (2017), and Lagedroste et al. (2019).

Briefly, the *L. lactis* NZ9000 strains were grown in M17 medium (Terzaghi and Sandine, 1975) at 30°C supplemented with 0.5% glucose (GM17 and the appropriate antibiotics) overnight with 1 ng/ml nisin. In a 96-well plate, a serial dilution of the nisin variant was applied and incubated with the test strains at a final OD_{600} of 0.1 for 5 h at 30°C. Later on, the optical density was measured at 584 nm via 96-well plate reader BMG. The normalized optical density was plotted against the logarithm of the nisin concentration and the resulting inhibitory concentration (IC_{50}), represents the value where 50% of the cells died in the presence of the different nisin variants. By dividing the IC_{50} values of strains expressing the immunity or resistance proteins from the IC_{50} value of the sensitive strain we calculated the fold of immunity/resistance, which is an indicator for the recognition of nisin H or its variant by the immunity or resistance proteins.

Minimum Inhibitory Concentration Determination of Nisin Variants

Nisin variants were tested for antibacterial capabilities against *B. subtilis* and different strains from *S. aureus, E. faecium,* and *E. faecalis* using the microdilution method, according to the recommendations of Clinical and Laboratory Standards Institute (2012). Briefly, fresh cultures prepared from overnight cultures were incubated until exponential phase (OD ∼ 0.6) and seeded at 5×10^4 CFU/well in 96-well round-bottom microplates, in a total volume of 100 μL containing twofold serially diluted test peptides. Moxifloxacin was used as a positive control. Plates were incubated statically and aerobically for 24 h at 37°C. MICs were determined macroscopically by identifying the least concentration of peptides that resulted in complete inhibition of bacterial visual growth.

SYTOX Green Nucleic Acids Binding Assay

The cells of NZ9000Cm were grown overnight in GM17 supplemented with 5 mg/ml chloramphenicol. The overnight culture was diluted to an OD_{600} of 0.1 in fresh media and the cultures were grown until the OD_{600} reaches 0.3. The SYTOX green dye was added at a final concentration of 2.5 mM according to the manual of the manufacturer (Invitrogen). After reaching a stable baseline (∼200 s) we added 100 nM of the nisin variants. The fluorescence signal was measured at an excitation wavelength

of 504 nm and emission wavelength of 523 nm, respectively (using a fluorolog Horiba III). After a stable baseline was reached, the nisin variant was added and the fluorescence was monitored over an additional time period. The measurement was performed at 30°C.

RESULTS

O'Connor et al. (2015) described a new natural nisin variant from *S. hyointestinalis* DPC 6484 and named it nisin H. In following, we compared nisin A and its natural variant nisin H, both heterologously produced in *L. lactis*, following the protocol of lantibiotic characterization (Lagedroste et al., 2019). We also included the nisin H F_1I variant.

The characterization starts with the expression, secretion and purification of the lantibiotic and its comparison to nisin A. The heterologously expressed and secreted nisin A and the variants nisin H and nisin H F_1I can be purified with high purity as observed on the Tricine-SDS-PAGE gel (**Figure 2A**). Nisin A was purified with a yield of 6.0 ± 0.3 mg/L of cell culture (Lagedroste et al., 2019), nisin H was expressed and purified with a yield of 5.3 ± 0.6 mg/L of cell culture, which is identical within experimental error. The nisin H F_1I variant displayed a slightly reduced yield of 4.9 ± 0.2 mg/L of cell culture (**Figure 2B** and **Table 1**).

An important step prior to the activity assay is the cleavage of the leader sequence form the pre-nisin variants, resulting in biologically active compounds. For the cleavage reaction, we used the peptidase NisP and monitored the cleavage efficiency via RP-HPLC (**Figures 2C–E**).

Intriguingly, the natural variant nisin H showed only a low cleavage efficiency of 15.6 ± 1.4%, compared to nisin A with 94.6 ± 2.0% (**Figure 2C** and **Table 1**). In comparison to nisin A, nisin H contains a phenylalanine at the first position (O'Connor et al., 2015), which apparently leads to a significant reduction in cleavage efficiency. The first residue of nisin A is an isoleucine, and as demonstrated before (Lagedroste et al., 2019), the introduction of aromatic residues at position one results in a clearly reduced cleavage efficiency. To counteract the lower cleavage efficiency of nisin H by NisP, we created a mutant of nisin H, in which the phenylalanine was substituted by isoleucine and termed it nisin H F_1I. For this variant, nisin H F_1I, the cleavage efficiency of the pre-lantibiotic was restored with an efficiency of 91.8 ± 0.8% (**Figure 2C** and **Table 1**), which corresponds to levels previously observed for nisin A. We monitored the cleavage via RP-HPLC, the pre-nisin elutes normally between 18 and 22 min (shown as blue dashed lines, **Figures 2D,E**). After cleavage by NisP, the leader peptide can be detected at 14.5 and 15.5 min in the chromatogram (shown as black lines, **Figures 2D,E**). For nisin H there was a high amount of uncleaved nisin H visible (eluting from 18 to 21 min) and only a small amount of cleaved product at 23–24 min (black lines, **Figure 2D**). For the nisin H F_1I variant, high amounts of leader peptide and cleaved product could be detected in the chromatogram, indicating high cleavage efficiency (black lines, **Figure 2E**). This efficiency was similar as observed for nisin A and in line with previous results that the position

FIGURE 2 | Determination of purity, yield and cleavage efficiency of the pre-nisin variants. **(A)** Purity of the purified nisin A and the variants nisin H and nisin H F_1I (Marker: Precision Plus Protein Dual Xtra standards Bio-Rad). **(B)** Yields after purification of nisin A and their corresponding variants via cation-exchange chromatography. **(C)** Quantification of the cleavage efficiency of NisP. **(D)** Chromatogram of nisin H. **(E)** Chromatogram of the nisin H F_1I variant. The pre-nisin variants before NisP cleavage were shown by blue dashed lines and after NisP cleavage by black lines. Error bars represent the standard deviation of at least three biological replicates.

one is important for the cleavage reaction (Lagedroste et al., 2019). Thus, we assume, that the four other mutations naturally occurring in nisin H (compared to nisin A) do not interfere with cleavage, however the isoleucine at position one does.

The next step was to determine the modification state of the heterologous produced nisin H and its F_1I variant. As the natural variant nisin H contains ten possible residues in the core peptide that can be dehydrated, we were curious to determine if the modification machinery of nisin A was able to modify the peptide

as efficiently (O'Connor et al., 2015). In the MALDI-TOF spectra, a dominant species of eightfold dehydrated residues was observed for nisin H, followed by a minor species containing nine- and sevenfold dehydrations. The possible 10-fold dehydrated species however was not observed (**Figure 3A** and **Table 1**). Furthermore no CDAP-coupling products were observed, which indicates that all cysteine residues are linked in (methyl)-lanthionine rings. We proved the functionality of the assay with unmodified pre-nisin A as demonstrated in Lagedroste et al. (2019). Thus, the modification enzymes were able to modify nisin H proving the promiscuity of the nisin modification machinery. Interestingly, the nisin H F_1I variant showed a dominant ninefold dehydrated species in comparison the nisin H wild-type (**Figure 3B** and **Table 1**) and also showed no CDAP-coupling products, indicating that all cysteine residues are closed to (methyl)-lanthionine rings. The difference in the dehydration pattern for the nisin H F_1I variant indicates a different accessibility of the serine and/or threonine residues in the core peptide for at least the dehydratase NisB. To validate which serine or threonine residues is dehydrated, we performed a tandem mass spectrometric

TABLE 1 | Determination of the yield, cleavage efficiency, dehydrations, and (methyl)-lanthionine ring formation for nisin A, nisin H, and nisin H F_1I.

Variant	Yield (mg/L culture)	Cleavage (%)	Dehydrations	Lanthionine rings
Nisin A	6.0 ± 0.3	94.6 ± 2.0	**8**, 7	5
Nisin H	5.3 ± 0.6	15.6 ± 1.4	9, **8**, 7	5
Nisin H F_1I	4.9 ± 0.2	91.8 ± 0.8	**9**, 8, 7, 6	5

Main species found in MALDI-TOF analysis are marked in bold.
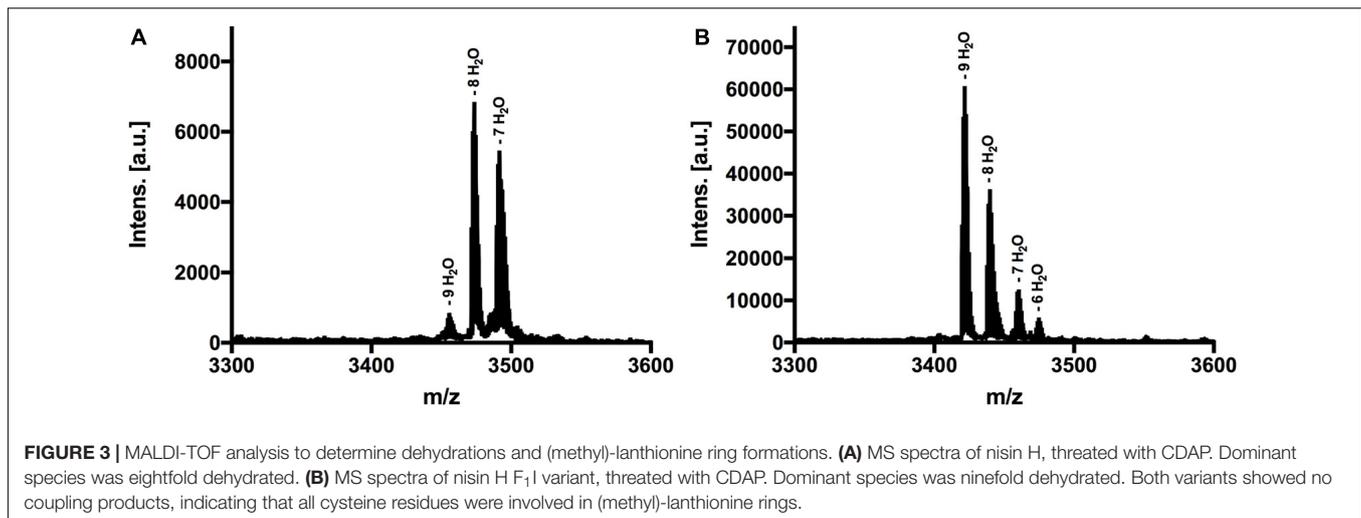
FIGURE 3 | MALDI-TOF analysis to determine dehydrations and (methyl)-lanthionine ring formations. **(A)** MS spectra of nisin H, threated with CDAP. Dominant species was eightfold dehydrated. **(B)** MS spectra of nisin H F_1I variant, threated with CDAP. Dominant species was ninefold dehydrated. Both variants showed no coupling products, indicating that all cysteine residues were involved in (methyl)-lanthionine rings.

analysis of nisin H and the F_1I variant. Here we found that the Thr_2 partially escape the dehydration in nisin H. Only in the small amount of the ninefold dehydrated species the Thr_2 is dehydrated, in all other species we found a mix in the dehydration pattern, where Thr_2 partially escape the dehydration. For example in the eightfold species we have a mix of dehydrated Thr_2 or Ser_{33}. In the nisin H F_1I variant the Thr_2 was in all species dehydrated, which suggests that, the phenylalanine at position one in nisin H is critical for the dehydratase NisB. Ser_{29} was never dehydrated in the found species.

Lantibiotics are very potent and possess an antimicrobial activity in the nanomolar range (Gross and Morell, 1967; Rollema et al., 1995; Chan et al., 1996; Lu et al., 2010; Oppedijk et al., 2016). To verify this potential for nisin H and the nisin H F_1I variant we used a standardized growth inhibition assay and first screened against the nisin sensitive *L. lactis* strain NZ9000Cm. Here, Cm stands for chloramphenicol resistance, which arises from the empty plasmid, which was transformed. In comparison to nisin A (IC_{50} value: 4.8 ± 0.7 nM), the heterologous expressed variant nisin H possessed a comparable IC_{50} value (5.3 ± 1.0 nM) (**Figure 4** and **Table 2**). Both values are in line with previously determined IC_{50} values for the strain NZ9000Cm (Reiners et al., 2017). The effect of the nisin H F_1I variant was more pronounced. For the NZ9000Cm sensitive strain we calculated an IC_{50} value of 14.2 ± 0.2 nM, approximately threefold lower than the wild-type nisin H (**Figure 4** and **Table 2**).

To test the effect of the nisin variants on the immunity proteins NisI (Alkhatib et al., 2014a) and NisFEG (Alkhatib et al., 2014b), as well as the resistance proteins *Sa*NSR (Khosa et al., 2016a) and *Sa*NsrFP (Reiners et al., 2017), we expressed each of them in a plasmid-based system in a *L. lactis* NZ9000 strain. We termed these strains NZ9000NisI, NZ9000NisFEG, NZ9000*Sa*NSR and NZ9000*Sa*NsrFP, respectively.

Nisin A displayed an IC_{50} value of 48.6 ± 6.3 nM against strain NZ9000NisI and 53.0 ± 4.5 nM against strain NZ9000NisFEG. For the resistance strains NZ9000*Sa*NSR and NZ9000*Sa*NsrFP nisin A displayed IC_{50} values of 73.0 ± 3.6 and 82.1 ± 3.1 nM, respectively (**Figure 4** and **Table 2**). By comparing theses values,

we calculated the fold of immunity/resistance to 10.1 ± 2.0 for NZ9000NisI, 11.1 ± 1.9 for NZ9000NisFEG, 15.2 ± 2.5 for NZ9000*Sa*NSR and 17.1 ± 2.7 for NZ9000*Sa*NsrFP (**Table 2**). After the first screen against the sensitive strain NZ9000Cm, nisin H and its variant were used to screen against the strains expressing the immunity or resistance proteins.

Nisin H revealed an IC_{50} value of 43.2 ± 8.7 nM against the NZ9000NisI strain, similar to nisin A, and a fold of immunity of 8.1 ± 2.2. Against the NZ9000NisFEG strain we determined an IC_{50} value of 23.4 ± 3.3 nM for nisin H which displayed a fold of resistance of 4.4 ± 1.0. Against the NZ9000*Sa*NSR strain we obtained an IC_{50} value of 52.4 ± 9.9 nM, resulting in a fold of resistance of 9.8 ± 2.6. Nisin H showed an IC_{50} value of 86.4 ± 4.0 nM for the NZ9000*Sa*NsrFP strain, resulting in a fold of resistance of 16.2 ± 3.1 (**Figure 4** and **Table 2**). NZ9000*Sa*NsrFP showed the highest fold of resistance for nisin H [in-line with a previous report (Reiners et al., 2017)]. Intriguingly, strain NZ9000NisFEG showed a reduced immunity and consequently we propose that nisin H is not recognized as efficiently as nisin A. Even NZ9000*Sa*NSR had a reduced resistance. That could be due to the exchange of His_{31} against lysine in the C-terminal part of nisin H (**Figure 1**).

Surprisingly, all strains displayed a reducing resistance/immunity effect for the nisin H F_1I variant in comparison to nisin A and also, with exception of NZ9000FEG for nisin H. Against the NZ9000NisI strain we determined an IC_{50} value of 34.1 ± 0.3 nM for the nisin H F_1I variant, with a fold of resistance 2.4 ± 0.1, which is nearly threefold lower than for nisin A. We obtained an IC_{50} value of 32.1 ± 0.8 nM against the ABC transporter NZ9000NisFEG, with a fold of resistance 2.3 ± 0.1 (**Figure 4** and **Table 2**). Nisin H F_1I showed for the resistance strain NZ9000*Sa*NSR and NZ9000*Sa*NsrFP an IC_{50} value of 44.2 ± 1.3 and 50.2 ± 1.6 nM, respectively. The calculated folds of resistance were 3.1 ± 0.1 and 3.5 ± 0.1, respectively (**Figure 4** and **Table 2**) and both are fivefold less than the observed fold of resistances for nisin A.

Since a similar activity for nisin H and nisin A was observed it became obvious that both exhibit the same mode of action. In
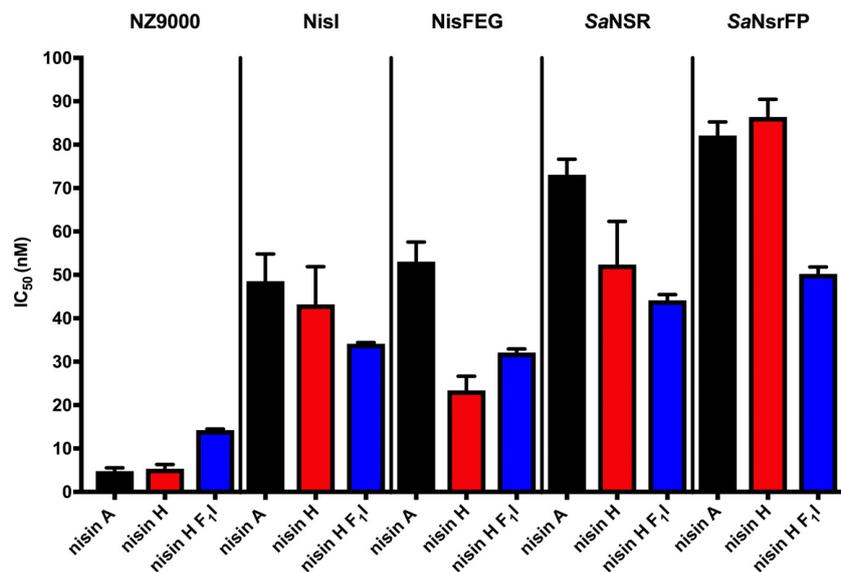
FIGURE 4 | Growth inhibition assays in the presence of nisin A and the nisin H varants. The lantibiotic nisin A (black bars), the heterologous expressed nisin H (red bars) and the position 1 variant of nisin H F_1I (blue bars) were used for growth inhibition (IC_{50}) with the strains NZ9000Cm, NZ9000NisI, NZ9000NisFEG, NZ9000SaNSR, and NZ9000SaNsrFP. Values represent the average of at least five biological independent measurements and the errors report the standard deviation of the mean (SDM).

TABLE 2 | IC_{50} values (nM) for nisin A, nisin H, and nisin H F_1I with the corresponding fold of resistance (FR) against the strains NZ9000Cm, NZ9000NisI, NZ9000NisFEG, NZ9000SaNSR, and NZ9000SaNsrFP.

Variant	NZ9000Cm	NZ9000NisI		NZ9000NisFEG		NZ9000SaNSR		NZ9000SaNsrFP	
		IC_{50}	FR	IC_{50}	FR	IC_{50}	FR	IC_{50}	FR
Nisin A	4.8 ± 0.7	48.6 ± 6.3	10.1 ± 2.0	53.0 ± 4.5	11.1 ± 1.9	73.0 ± 3.6	15.2 ± 2.5	82.1 ± 3.1	17.1 ± 2.7
Nisin H	5.3 ± 1.0	43.2 ± 8.7	8.1 ± 2.2	23.4 ± 3.3	4.4 ± 1.0	52.4 ± 9.9	9.8 ± 2.6	86.4 ± 4.1	16.2 ± 3.1
Nisin H F_1I	14.2 ± 0.2	34.1 ± 0.3	2.4 ± 0.1	32.1 ± 0.8	2.3 ± 0.1	44.2 ± 1.3	3.1 ± 0.1	50.2 ± 1.6	3.5 ± 0.1

the case of nisin A this combines growth inhibition with pore formation in the membrane with subsequent cell death. To test this we performed a SYTOX assay previously used for nisin A (Reiners et al., 2017). Here the SYTOX dye was added to *L. lactis* cells and displayed an increased fluorescence signal upon binding of DNA which is released from the cell upon pore formation (Roth et al., 1997). We use 100 nM of nisin A, nisin H and nisin H F_1I variant respectively and observed an almost instant fluorescence increase similar to the signal increase observed for nisin A (**Figure 5**). This shows that nisin H as well as its F_1I variant form pores in the membrane of *L. lactis* cell.

The nisin variants were further tested for antibacterial capabilities against *B. subtilis* and different pathogenic strains from *S. aureus, E. faecium,* and *E. faecalis* using the microdilution method, according to the recommendations of CLSI (2012). Here we found that nisin H and the F_1I variant performed almost identically or in most cases even better than the natural nisin A. Especially against the MSSA and MRSA strains, nisin H had significant lower MIC values of 0.19 and 0.78 µM, in comparison to 0.78 and 6.25 µM for nisin A, respectively (**Table 3**). Also, against *E. faecium* ATCC 35667, *B. subtilis* 168 as well as *E. faecium* ATCC 700221 (VRE), nisin H showed more potency

with about two to eightfold lower MIC values than nisin A [0.39, 0.1, and 0.39 µM compared to 1.56, 0.78, and 0.78 µM for nisin A, respectively (**Table 3**)]. While the nisin H F_1I variant and nisin A had similar MIC values against both MSSA and MRSA strains, the nisin H F_1I variant only performed better than nisin A or nisin H against *E. faecalis* ATCC 51299 (VRE) with a MIC value of 0.78 µM compared to 1.56 µM for nisin A and nisin H. Against *E. faecium* ATCC 35667 and *B. subtilis* 168 nisin H F_1I was less efficient than nisin H, but still better than nisin A (**Table 3**).

DISCUSSION

We focused in this study on the natural variant nisin H and the nisin H F_1I mutant. Nisin H was discovered from the gut-derived strain *S. hyointestinalis* DPC6484 in 2015 by O'Connor et al. (2015). Here, we showed the heterologous expression of nisin H and the F_1I variant with the NICE-system in *L. lactis* (Eichenbaum et al., 1998; Mierau and Kleerebezem, 2005; Rink et al., 2005; Zhou et al., 2006; Lagedroste et al., 2019) and extended the characterization in terms of cleavage efficiency by the protease NisP and the antimicrobial activity against
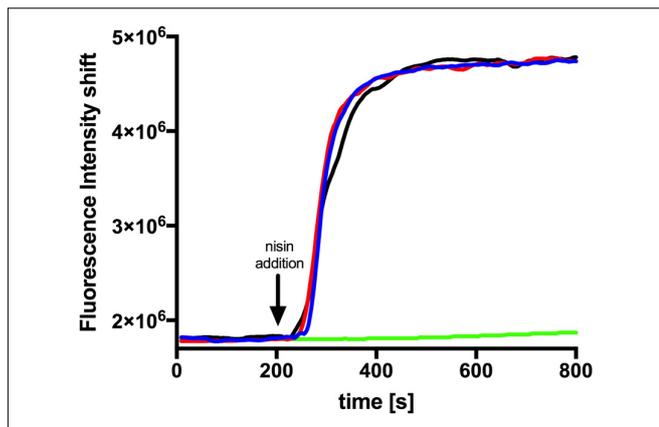
FIGURE 5 | Nisin mediated pore formation, visualized with the SYTOX green assay. The NZ9000Cm strain incubated with the SYTOX dye. After a stable baseline (~200 s), one of the nisin variants (100 nM) was added (indicated with an arrow). The fluorescence signal was measured using a fluorolog (Horiba III). The rapid increase in fluorescence indicates pore formation. The black line represents the addition of nisin A, the red line nisin H, and the blue line nisin H F_1I. As a control we added buffer shown as green line.

the immunity proteins NisFEG (Alkhatib et al., 2014b) and NisI (Alkhatib et al., 2014a), as well as the resistance proteins *Sa*NSR (Khosa et al., 2013; Khosa et al., 2016a,b) and *Sa*NsrFP (Khosa et al., 2016a; Reiners et al., 2017). We further tested for antibacterial capabilities against *B. subtilis* and different pathogenic strains from *S. aureus*, *E. faecium,* and *E. faecalis.*

Both lantibiotics, nisin H and the F_1I variant were purified in high amounts and purity with 5.3 ± 0.6 mg/L of cell culture for nisin H and 4.9 ± 0.2 mg/L of cell culture for nisin H F_1I variant, respectively (**Figure 2B** and **Table 1**). In comparison, the homologous expression of nisin H in *S. hyointestinalis* and nisin A in *L. lactis* NZ9700 results in a very low amount of 0.15 mg/L of cell culture and 0.50 mg/L of cell culture (O'Connor et al., 2015), respectively, which demonstrates the enormous potential of the NICE-system, for lantibiotic and even non-lantibiotic expression (Eichenbaum et al., 1998; Mierau and Kleerebezem, 2005; Rink et al., 2005; Zhou et al., 2006; Lagedroste et al., 2019).

An important step in the maturation of a lantibiotic is the cleavage of the leader peptide to become biological active. The cleavage efficiency of the natural substrate nisin A was determined with 94.6 ± 2.0%. For nisin H the cleaving efficiency was drastically reduced to 15.6 ± 1.4%. The first residue of nisin A is an isoleucine, while the corresponding residue in nisin H is phenylalanine and as demonstrated before (Lagedroste et al., 2019), aromatic residues prevent efficient cleavage likely by interfering with the S1′ binding pocket of NisP. With the nisin H F_1I variant, the cleavage efficiency was restored to 91.8 ± 0.8% (**Figures 1**, **2C** and **Table 1**). This indicated that the other point mutations naturally occurring in nisin H did not affect cleavage by NisP. With respect to other natural nisin variants, NisP cleavage could be a critical step. For example nisin O1 to O4 from *B. obeum* A2-162 (Hatziioanou et al., 2017) has a tyrosine or a threonine, respectively, at position one, which should also result in a low NisP cleavage efficiency. Natural variants such as nisin U (Wirawan et al., 2006), nisin J (O'Sullivan et al., 2019, 2020), nisin Q (Zendo et al., 2003), nisin Z (Mulders et al., 1991) and nisin F (de Kwaadsteniet et al., 2008) have an isoleucine and nisin U2 (Wirawan et al., 2006) and nisin P (Zhang et al., 2012; Wu et al., 2014) a valine at position one, which should result in high NisP cleavage efficiency.

Furthermore, we made a sequence alignment with Clustal Omega (Madeira et al., 2019) for the NshP (the natural protease for the nisin H cleavage) from *S. hyointestinalis* and NisP from *L. lactis* to see if there is any difference in the active site, which could be the reason for the reduced cleavage efficiency (**Figure 6A** and **Supplementary Figure 1**). Here the three important residues His_{306}, Asp_{259}, and Ser_{512} which build up the catalytic triad in NisP are conserved in NshP. We also calculated a homology model of NshP based on the known NisP structure (PDB code 4MZD) using Phyre2 (Kelley et al., 2015; **Figure 6B** and **Supplementary Figure 2**). Here, no significant differences are found within the overall fold as well as the active site which would explain the difference in the cleavage site. This is intriguing since the recognition site within the leader peptide differs between nisin A (sequence is ASPR) and nisin H (sequence is ASTR) (see **Supplementary Figure 3**). This suggests that the proteases NisP and NshP recognize their substrate by small difference in their active site.

Nevertheless, for an efficient cleavage, (methyl)-lanthionine rings have to be present. This even holds true in light of the presence of all (methyl)-lanthionine rings, which is generally assumed as the prerequisite for fast and efficient conversion of the pre-nisin to modified nisin (Plat et al., 2011;

TABLE 3 | MIC values for nisin A, nisin H, and nisin H F_1I against different pathogenic strains.

Organisms	Minimum inhibitory concentration (μM)		
	Nisin A	Nisin H	Nisin H F_1I
Staphylococcus aureus ATCC 29213 (MSSA)	0.78	0.19	0.78
Staphylococcus aureus ATCC 700699 (MRSA)	6.25	0.78	6.25
Enterococcus faecalis ATCC 29212	1.56	1.56	1.56
Enterococcus faecium ATCC 35667	1.56	0.39	0.78
Bacillus subtilis 168	0.78	0.1	0.39
Enterococcus faecalis ATCC 51299 (VRE)	1.56	1.56	0.78
Enterococcus faecium ATCC 700221 (VRE)	0.78	0.39	0.78

FIGURE 6 | Sequence alignment of NisP from *Lactococcus lactis* and NshP from *Streptococcus hyointestinalis*. **(A)** The important residues from the active site (Asp$_{259}$, His$_{306}$, and Ser$_{512}$ for NisP and Asp$_{178}$, His$_{225}$, and Ser$_{431}$ for NshP) are marked in red, shown together with the close neighborhood residues. The sequence alignment was done with Clustal Omega (Madeira et al., 2019). **(B)** Zoom-in into the active sites of NshP (magenta) and NisP (cyan). The important residues for the catalytic activity (Asp$_{259}$, His$_{306}$, and Ser$_{512}$ for NisP and Asp$_{178}$, His$_{225}$, and Ser$_{431}$ for NshP) are shown in stick representation. Figure was generated using Pymol (2015). For a full alignment and a corresponding homology model, please see **Supplementary Material**.

Lagedroste et al., 2017). To check the amount of dehydrations, necessary for (methyl)-lanthionine ring formation we applied MALDI-TOF analysis. The loss of water molecules within the peptide is directly visible in the reduced molecular weight, but not the (methyl)-lanthionine ring formation. Here we used 1-cyano-4 dimethylaminopyridinium tetrafluoroborate (CDAP) (Wu and Watson, 1998), which binds to free cysteine residues, indicating that these cysteines are not involved in a (methyl)-lanthionine ring. For both lantibiotics, nisin H and the nisin H F_1I variant no CDAP coupling products were found, indicating that no (methyl)-lanthionine ring is lacking (**Figure 3** and **Table 1**). Nisin H has 10 possible dehydration sites and is predicated to be ninefold dehydrated when expressed homologous (O'Connor et al., 2015). A minor species of nine dehydrations was found, but the dominant species was eightfold dehydrated. The dehydration pattern of the F_1I variant is changed in comparison to nisin H. Here we determined a dominant ninefold species (**Figure 3** and **Table 1**). This provides a hint, that position two of wild-type nisin H might not have been previously dehydrated, due to steric hindrance of the phenylalanine. To validate which serine or threonine residues is dehydrated, we perform a tandem mass spectrometric analysis of nisin H and the F_1I variant. Here we found that the Thr$_2$ partially escape the dehydration in nisin H. In the nisin H F_1I variant the Thr$_2$ was in all species dehydrated, which gives a hint that, the phenylalanine at position one in nisin H is critical for the dehydratase NisB. This is in line with previous data from the I_1F variant of nisin A, where the dominant species was sevenfold dehydrated and not eightfold as wild-type nisin A (Lagedroste et al., 2019). It has also been reported for the natural nisin Z (Mulders et al., 1991), that the I_1W mutation showed a partial inhibition of dehydration of the Thr$_2$ (Breukink et al., 1998), which could also be the case for nisin H with the aromatic phenylalanine at position one, resulting in eight dehydrations. A dehydration of position Ser$_{29}$ normally goes in line with the lack of ring E (Lubelski et al., 2009), which drastically reduces the antimicrobial activity of nisin A against the sensitive NZ9000Cm

strain (Alkhatib et al., 2014b; Khosa et al., 2016a; Reiners et al., 2017). Since the activity was high for nisin H and the F_1I variant, we expected that Ser$_{29}$ was not dehydrated and tandem mass spectrometric analysis supported this.

Nisin H showed nearly the same activity as nisin A against the sensitive NZ9000Cm strain but the nisin H F_1I variant is roughly threefold less active. For the immunity protein NisI, it was revealed that nisin H has an identical activity like nisin A within experimental error. However, the nisin H F_1I variant exhibited a lower IC$_{50}$ value of 34.1 ± 0.3 nM and due to the weaker wild-type activity more than a threefold lower fold of resistance (2.4 ± 0.1 compared to 8.1 ± 2.2) (**Figure 4** and **Table 2**). NisI recognizes the N-terminus of nisin (Wiedemann et al., 2001) and the lower IC$_{50}$ could be due to the fact that Thr$_2$ is dehydrated in the nisin H F_1I variant in contrast to nisin H. An additional change is the leucine at position 6 against methionine in nisin H and the nisin H F_1I variant, which could be responsible for the better recognition by NisI.

The immunity protein NisFEG, in comparison to nisin A, showed a strong reduction in immunity in the presence of nisin H and the nisin H F_1I variant. NisFEG recognizes the C-terminus of nisin (Alkhatib et al., 2014b), which indicates that the point mutations of nisin H affect NisFEG. So, we suppose that nisin H and the nisin H F_1I variant are not recognized and subsequently transported out of the membrane like nisin A.

The resistance protein *Sa*NSR also recognizes the C-terminus of nisin (Khosa et al., 2016a), and cleaves nisin between the positions 28 and 29. Other studies showed that mutations in this area of the nisin molecule, e.g., S$_{29}$P or C$_{28}$P strongly reduce the efficiency of *Sa*NSR (Field et al., 2019; Zaschke-Kriesche et al., 2019a). We assume that the exchange of His$_{31}$ against lysine in nisin H and the nisin H F_1I variant (**Figure 1**) has the same effect on *Sa*NSR thereby lowering the resistance efficiency to an IC$_{50}$ value of 52.4 ± 2.6 nM for nisin H and 44.2 ± 1.3 nM for the nisin H F_1I variant, respectively (**Figure 4** and **Table 2**).

For the resistance protein SaNsrFP, we observed an activity for nisin H identical to nisin A. SaNsrFP recognizes the N-terminus of nisin (Reiners et al., 2017), which is affected due to the different dehydration pattern in wild-type nisin H in comparison to the nisin H F_1I variant. The nisin H F_1I variant showed a lower IC_{50} value of 50.2 ± 1.6 nM, compared to 86.4 ± 4.1 nM for nisin H. This effect is even more pronounced when comparing the fold of resistances of 16.2 ± 3.1 for nisin H to 3.5 ± 0.1 for the nisin H F_1I variant, respectively (**Figure 4** and **Table 2**).

This study demonstrated again that only a complete characterization of a lantibiotic reveals the full antimicrobial potential. Based on the IC_{50} value of the sensitive NZ9000Cm strain the F_1I variant might be classified as weakly antimicrobial active, but with respect to the immunity and resistance proteins, it becomes more interesting, due to its high activity even against the immunity proteins NisI and NisFEG from $L.$ $lactis$ and the nisin resistance proteins SaNSR and SaNsrFP from $S.$ $agalactiae$ COH1. Against the tested pathogenic bacteria, we found that nisin H and the nisin H F_1I variant performed almost identically or in the most cases even better than the natural nisin A. Nisin H displayed high antimicrobial potential against both methicillin-resistant and –susceptible the $S.$ $aureus$ strains, both vancomycin-resistant and -susceptible, $E.$ $faecium$ strains, as well as $B.$ $subtilis.$

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

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AUTHOR CONTRIBUTIONS

FUNDING

ACKNOWLEDGMENTS

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2020.573614/full#supplementary-material

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