Expression of nisin genes in cheese—A quantitative real-time polymerase chain reaction approach

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ABSTRACT

The role of bacteriocins in different environments has not been thoroughly explained, mainly because of the difficulties related to the detection of their production. Nisin, an antimicrobial peptide produced by *Lactococcus lactis* has a long history of safe use in food products and has been studied from many aspects of genetics, biosynthesis, immunity, regulation, and mode of action. Still, some aspects concerning the dynamics of nisin gene expression remain unknown, especially in complex media like cheese. The main objective of the present study was to quantify in a cheese-like medium the expression of nisin genes in *L. lactis* M78, a well-characterized nisin A producer isolated from raw milk. The expression of all 11 genes involved in nisin biosynthesis was evaluated during cheese production by real-time reverse transcription-PCR. Total RNA was extracted from cheeses using a direct extraction method without prior separation of microbial cells. The M78 strain grew well in experimental cheeses, producing detectable amounts of nisin after 4 h of fermentation. The presence of nisin as an activator modified both the expression of nisin genes and the accumulation of active nisin. Four groups could be distinguished based on gene expression as a function of time: *nisA*, *nisFEG*, *nisRK* and *nisBTCIP*. Based on nisin-producing strain growth, nisin activity, function of nisin genes, and their location, correlations were established that contribute to the explanation of regulation of nisin biosynthesis and immunity. This study is the first in which the evolution of bacteriocin gene transcripts has been quantified rigorously in a cheese-like medium.

Key words: nisin, gene expression, real-time reverse transcription-polymerase chain reaction, cheese

INTRODUCTION

Bacteriocin-producing lactic acid bacteria are gaining trust for use as starter cultures and as means for improving the safety of fermented foods. The role of bacteriocins in different environments and control of the mechanisms of bacteriocin production and activity, however, is still not thoroughly explained, mainly because of the difficulties related to their detection in complex environments. Nisin, a ribosomally synthesized lantibiotic naturally produced by several strains of *Lactococcus lactis*, is one of the best characterized lactic acid bacteria bacteriocins. The potential of nisin-producing starters to control undesirable microorganisms in cheese has been already demonstrated (Maisnier-Patin et al., 1992; Yezzi et al., 1993; Rodríguez et al., 1998; Bouksaim et al., 2000; Ayad, 2009). Several variants of nisin are known, with nisins A and Z being the most common, differing in one nucleotide and in one AA respectively. The nisin gene cluster is located on a large conjugative transposon and is composed of 11 genes, *nisABTCIPRKFEG* (Figure 1), involved in complex biosynthesis, regulation, and immunity. Among these genes, *nisA* encodes a nisin A precursor peptide of 57 AA residues (pre-nisin), which is a structural peptide (pro-nisin) attached to a leader peptide; *nisB* and *nisC* encode modification enzymes (dehydration and cyclization) involved in post-translational modification of pro-nisin; *nisT* encodes a transport protein involved in secretion of modified pre-nisin outside the cell (ABC transporter); *nisP* encodes a membrane-bound serine protease which cleaves pre-nisin, releasing the active pro-nisin; *nisI* encodes a lipoprotein involved in self-protection against nisin; *nisFEG* encode transport proteins also involved in self-protection (ABC transporter); and *nisR* and *nisK* encode a response regulator and a histidine kinase which form a two-component signal transduction system involved in regulation of nisin biosynthesis activated by nisin. Transcription of nisin genes is facilitated from 3 major promoter sites of which the promoter proceeding *nisR* is constitutive, whereas the *nisA* and *nisF* promoters are controlled by the 2-component regulatory system NisRK. An alter-

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Figure 1. Organization of genes involved in nisin production, regulation, and immunity. Black and white triangles indicate nisin-inducible and constitutive promoters, respectively. Hairpins indicate putative transcription terminator loops. Genes are as follows: *nisA*, nisin structural gene; *nisB* and *nisC*, post-translational modification; *nisT*, translocation; *nisP*, activation; *nisR* and *nisK*, regulation; *nisI* and *nisFEG*, immunity.

native induction system exists for the *nisA* promoter that functions independently of NisRK. In fact, the galactose metabolite of the Leloir pathway can induce *nisA* transcription from the same start site. In addition to the *nisR* promoter, another constitutive promoter has been identified before *nisI*. The function of this promoter may be to establish a basal nisin immunity before the cell encounters nisin for the first time (Li and O'Sullivan, 2002, 2006). For a complete review of nisin biosynthesis, immunity, regulation, mode of action, and engineering, see Lubelski et al. (2008).

The structure of the nisin cluster where most of the genes are overlapping (GenBank accession numbers X68307, L16226, X76884, and U17255) indicates translational coupling (Kuipers et al., 1993). However, *nisA* is separated from the rest of the cluster by an inverted repeat which may act either as a transcription terminator or translation processing site (Engelke et al., 1992; Kuipers et al., 1995; Dodd et al., 1996). Two TCT-N₈-TCT repeats are located at the positions -39 to -26 and -107 to -94 upstream of the *nisA* transcription start site. A single TCT-N₈-TCT repeat is also located at the positions -39 to -26 upstream of the *nisF* transcription start site. Although the *nisR* can initiate transcription by binding to one of the TCT-N₈-TCT repeats, it needs 2 repeats for optimal binding, which is the reason for weaker expression of *nisFEG* than *nisA* (de Ruyter et al., 1996; Chandrapati and O'Sullivan, 1999, 2002; Penna et al., 2005). The linear response of the *nisA* and *nisFEG* promoters in response to nisin, together with the constitutive nature of the *nisR* promoter has been demonstrated (Kuipers et al., 1995; de Ruyter et al., 1996).

Kuipers et al. (1995) detected expression controlled by the *nisA* promoter to be 50 times higher for *nisA* than for the genes located after the inverted repeat that separates *nisA* from the other *nis* genes. On the other hand, Runar Ra and Saris (1995) indicated that all nisin genes are transcribed as 2 large polycistronic transcripts, *nisZBTCIPRK* and *nisFEG*, regulated by 2 nisin-dependent promoters (Ra et al., 1996). It is possible that polycistronic transcripts are cleaved during

further processing into separate single-gene transcripts (Buchman et al., 1988; Steen et al., 1991; Engelke et al., 1992). Higher stability of *nisA/Z* transcripts can be another reason for more elevated concentrations of them (Runar Ra and Saris, 1995; Li and O'Sullivan, 2006). Using immunoblotting, Engelke et al. (1994) showed that the peptide encoded by *nisA* is present in the cell before the peptide encoded by *nisB*. According to the speculations of De Vuyst and Vandamme (1992), this may explain why nisin activity coincides more with expression of *nisB* than with expression of *nisA*.

The main objective of the present study was to quantify in a cheese-like medium the expression of bacteriocin genes, using a nisin producer as a model organism. We have chosen a nisin producer because nisin genes have been well characterized, and also because it has already been demonstrated that nisin can be produced in situ in cheese. However, a similar approach may also be used for other bacteriocins. In addition, despite several studies on nisin, the time-dependent expression of all 11 genes involved in nisin biosynthesis and immunity has not yet been reported. Expression of the nisin genes was compared with the growth of producer strain, acid production, and accumulation of active nisin in cheese.

MATERIALS AND METHODS

Bacterial Strains and Culture Conditions

The nisin-producing strain *L. lactis* M78, an isolate from raw milk (Samelis et al., 2009), was kindly provided by J. Samelis (National Agricultural Research Foundation, Dairy Research Institute, Ioannina, Greece). It was confirmed as a nisin A producer by sequence analysis of the entire nisin gene cluster (GenBank accession number HM219853). The nisin-susceptible strain *Lactobacillus sakei* ATCC 15521 was used as the indicator strain in biological assay for nisin quantification. *Lactococcus lactis* S3 (UMR GMPA, INRA, Thiveval-Grignon, France), which does not produce nisin, was used as a negative control. *Lactococcus* and *Lactobacillus* strains were routinely grown under static conditions at 30°C in

M17 lactose broth (Terzaghi and Sandine, 1975) and in De Man, Rogosa, and Sharpe (MRS) broth (De Man et al., 1960), respectively.

Cheese Production

Experimental cheeses were made using ultrafiltered milk (5.5% total fat) produced in UMR1253 (STLO, INRA, Rennes, France), as described by Hannon et al. (2006) and stored at -20°C . Cheese cultures were prepared by inoculation of sterile ($100^{\circ}\text{C}/10$ min) reconstituted skim milk (100 g/L; Difco Laboratories, Detroit, MI) with M78 strain and incubation at 30°C for 15 h. Ultrafiltered milk was inoculated at a concentration equivalent to 10^7 cfu/mL and divided into 2 parts. One part was supplemented with 1.2 IU/mL of nisin A (Sigma, St-Quentin-Fallavier, France). After the addition of 60 $\mu\text{L}/\text{L}$ of chymosin (Maxiren 180, DSM Food Specialties, Delft, the Netherlands), each part was divided into 15 aliquots to make 15 small model cheeses of 70 g. Coagulation of ultrafiltered milk resulted in model cheeses being formed without release of the whey. Model cheeses were incubated at 30°C for 24 h, which is near the optimal growth temperature of the nisin-producing strain M78. After 6 h of fermentation, the cheeses were transferred onto grids in sterile crystallizing basins and the incubation continued at 30°C .

Cheese Analysis

Cheeses with and without added nisin were sampled at 4, 6, 9, 12, and 24 h. At each sampling, 3 entire cheeses were taken as 3 separate parallels and each of them was analyzed. Besides RNA extraction, the bacterial counts and nisin production were determined, and pH was measured using a Blue line 27 surface electrode (Schott, VWR, Fontenay-sous-Bois, France).

Bacterial Growth. One gram of cheese was homogenized with 9 mL of 2% trisodium citrate dihydrate (pH 7.5) using a mechanical blender (Ultra-Turrax T25, Ika Labortechnik, Staufen, Germany). The resulting suspension was 10-fold serially diluted in tryptone-salt broth (9.5 g/L) and plated in triplicate on M17 agar plates. Colonies were enumerated after 48 h of incubation at 30°C .

Nisin Production. The procedure introduced for nisin quantification in cheese was based on previously published methods (Tramer and Fowler, 1964; Wolf and Gibbons, 1996; Pongtharangkul and Demirci, 2004). Cheese extracts were prepared by homogenizing 1 g of cheese in 1 mL of 0.02M HCl using a mechanical blender. The cheese homogenates were heated in a water bath at 90°C for 5 min. After cooling to room

temperature, they were centrifuged (10 min at $5,000 \times g$) and the resulting supernatants were filter-sterilized (0.22 μm). Cheese extracts were obtained also from cheeses produced with strain S3, which does not produce nisin. For the standard curve, standard samples were prepared from cheese homogenates supplemented with a known amount of nisin. Agar plates for nisin activity assay were prepared from 2 layers of MRS agar; the base layer contained 1.5% of agar-agar, and the cover layer 0.75% of agar-agar. In addition, the cover layer was supplemented with 1% (wt/wt) of Tween 40. After sterilization ($120^{\circ}\text{C}/20$ min) and cooling to 55°C , the base layer (20 mL) was poured into the plates (90 mm in diameter) and left to solidify and dry (12 h) at room temperature. The cover soft agar was incubated at 55°C (12 h) and before being poured (20 mL) over the base layer, it was inoculated with 2% of a fresh culture (18 h) of the indicator strain *L. sakei* ATCC 15521. After solidification of the soft agar at room temperature (2 h), 5-mm wells were punched into the cover layer using the back end of a Pasteur pipette. The wells were immediately filled with 70 μL of cheese extract and plates placed at 4°C for a 24-h pre-diffusion step. After pre-diffusion, the plates were incubated at 30°C for 24 h and the diameter of the inhibition halos measured using a caliper horizontally and vertically to the nearest 0.1 mm.

Expression of Nisin Genes. The priority of cheese sampling was to get cheese samples through the first step of extracting RNA. Extraction of RNA from cheeses was done as described previously (Monnet et al., 2008), except that the amount of cheese processed in each tube was increased to 500 mg, using 7-mL bead-beating tubes that contained 3.2 g of zirconium beads (diameter, 0.1 mm; Biospec Products, Bartlesville, OK). After addition of 5 mL of TRIzol reagent (Invitrogen, Cergy Pontoise, France), the tubes were vigorously shaken in a bead beater (Precellys24 Dual, Saint-Quentin-en-Yvelines, France) by using two 20-s mixing sequences at a speed of 6,500 rpm. The tubes were cooled on ice for 5 min after each mixing sequence. After the bead-beating procedure, the tubes were placed at -80°C until all of the samples for RNA extraction had been collected. To ensure a sufficient amount of extracted RNA, the content of 2 tubes was subsequently pooled and processed using 15-mL Phase Lock Gel Heavy tubes (Eppendorf, Hamburg, Germany), and RNA was purified using a single RNeasy spin column (Qiagen, Courtaboeuf, France). The RNA was eluted from spin columns with 30 μL of RNase-free water.

Purified RNA was quantified at 260 nm using NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE). The RNA integrity number

Table 1. Sequences and target genes of primers (F = forward, R = reverse) used in this study

Gene	Gene product	Primer sequence (5'→3')	Reference
16S rRNA	16S ribosomal RNA	F: GCTCACCAAGGCGATGATACATA R: ACCAACGTTCTTCTCTACCAACA	Monnet et al., 2008
23S rRNA	23S ribosomal RNA	F: ACAGGATAGGTAGGAGCCATT R: CGATTATGCCAGCGGGTTAG	Monnet et al., 2008
<i>nisA</i>	Nisin structural gene	F: TCAGGTGCATCACCACGCATTA R: TTACGTGAATACTACAATGACAAGTTGCT	This study
<i>nisB</i>	Nisin biosynthesis protein	F: GGGAGAGTTGCCGATGTTGT R: TAAAGCCACTCGTTAAAGGGCAAT	This study
<i>nisT</i>	Nisin transport ATP-binding protein	F: CAACTTTTCAGGAGGTCAGTGG R: AGCTACAGGATCGAGTGCAG	This study
<i>nisC</i>	Nisin biosynthesis protein	F: CAAATCGGAGAATCAGATGTCTCAAT R: CTAAAGATACAGCCCACTCCAG	This study
<i>nisI</i>	Nisin immunity protein	F: CTGAAGTACGTGTGTTTGATTTCAGTTAG R: TCGCCATAATCCCATTCCGTC	This study
<i>nisP</i>	Nisin leader peptide-processing serine protease	F: TGCACCGAGTGTATTTGAGGATGT R: GCAGGAGCATAAATTGCATCCG	This study
<i>nisR</i>	Nisin biosynthesis regulatory protein	F: GAGAGGAACGCAATAAACATGCAG R: GTTTGACCATTGACACAGACTCG	This study
<i>nisK</i>	Nisin biosynthesis sensor	F: CGAACGTGATATGAAACACTACCC R: ACTAGGTACATCCGACTTGACATAA	This study
<i>nisF</i>	Nisin transport/immunity protein	F: TGGATCCTGATGGTATTGCGG R: AGCTACTTTACTTATTTCTGCAACTG	This study
<i>nisE</i>	Nisin transport/immunity protein	F: TTGATTTGGCTAAGAGGTATACTAGCG R: ATAAAGGGTAAATTCCAAGAAGCTGC	This study
<i>nisG</i>	Nisin transport/immunity protein	F: GGCCAATCGTTTCCAAGAAATAAATGT R: CCCTACTGCCTGTGAAACTCC	This study

was determined with the 2100 Bioanalyzer (Agilent, Palo Alto, CA, USA) using RNA 6000 NANO chips according to the manufacturer's instructions. The concentration of extracted RNA was adjusted to 25 ng/ μ L, and DNase treatment was performed using the TURBO DNA-free kit (Ambion, Austin, TX) according to the manufacturer's instructions. Absence of DNA contamination in RNA samples was confirmed with non-reverse transcribed samples (controls). Reverse transcription was performed using the SuperScript III First-Strand Synthesis System (Invitrogen, Cergy Pontoise, France) with random hexamer priming and RNaseH treatment. The reaction mixture contained 200 ng of DNase-treated RNA in the final volume of 20 μ L. The reverse transcription procedure was performed according to the manufacturer's recommendations.

Oligonucleotide primers were designed using the LightCycler probe design software (v1.0; Roche Applied Science, Mannheim, Germany) and synthesized by Eurogentec (Seraing, Belgium). The thermocycling program consisted of initial denaturation at 95°C for 8 min, followed by 45 cycles of denaturation (95°C/10 s), annealing (60°C/7 s), and extension (72°C/6 s). Fluorescence acquisition (530 nm) was done at the end of each extension. After real-time PCR, a melting curve analysis was performed by measuring fluorescence during heating from 65 to 95°C at a transition rate of 0.1°C/s. Quantification cycle (C_q) values were determined with LightCycler software (version 3.3), using the second

derivative method. Standard curves were generated by plotting C_q values as a function of the log cDNA concentrations. From the slopes of the standard curves, the PCR efficiency (E) was calculated for each primer pair using the following formula: $E = 10^{-1/\text{slope}}$ (Pfaffl, 2004). The genes investigated in the present study and the corresponding primer pairs are presented in Table 1. Real-time PCR measurements of each sample were performed using 1/5 and 1/10 dilutions of the reverse-transcribed RNA. The resulting C_q values were used to calculate the expression of selected genes by relative quantification using a reference gene and a calibrator (Eq. 3.5; Pfaffl, 2004). We tested 16S rRNA and 23S rRNA respectively to be used as reference genes. The last sampling (24 h), corresponding to the cheese without added nisin, was chosen as the calibrator. Absence of inhibitors in both reverse transcription and real-time PCR was confirmed by comparison of standard curves obtained with serially diluted samples before and after reverse transcription.

Statistical Analyses

At each sampling time, 3 separate cheeses were sampled. For the determination of nisin activity, each cheese was used to produce one cheese extract and one end result. For the determination of nisin gene expression, each cheese was used to obtain one RNA extract and real-time PCR analyses were performed using 2

Figure 2. Dynamics of growth of *Lactococcus lactis* M78 and of pH in the model cheeses. Results are expressed as mean values of 3 repetitions. Closed and open symbols correspond to cheeses with and without addition of 1.2 IU/mg of nisin, respectively. Circles correspond to cell counts and squares to pH.

dilutions of the cDNA. Results from the 3 replicate cheeses were used to calculate mean values and standard deviations, which represented both the methodological and the experimental errors.

RESULTS

Bacterial Growth and Nisin Production in Milk and Cheese

Expression of nisin genes was measured during 24 h of manufacturing cheese with and without addition of nisin. Growth of *L. lactis* M78 was similar in cheeses with and without added nisin (Figure 2). Exponential phase was observed until 6 h of fermentation with growth rate constants 0.94 and 0.91/h, respectively for cheeses with and without added nisin. After about 12 h, *L. lactis* M78 reached the stationary phase in both types of experimental cheeses. Final cell count in cheeses with and without added nisin was 9.12 ± 0.06 and 9.19 ± 0.04 log cfu/g, respectively. The pH value at 24 h was 5.83 ± 0.1 . Nisin production was very different between the 2 types of experimental cheeses (Figure 3). As expected, addition of a small amount of nisin (1.2 IU/g) increased production of nisin.

Extraction of RNA from Cheese and Real-Time Reverse Transcription-PCR Analysis

A prerequisite for successful expression quantification is the extraction of good quality RNA from cheese. The yield of RNA after extraction was $4.9 (\pm 0.7) \times 10^{-6}$ ng/cfu and $2.4 (\pm 0.2) \times 10^{-6}$ ng/cfu for samples obtained

at 4 and 24 h, respectively. The RNA integrity number values of the RNA samples were always above 8.5, which indicates that RNA was not degraded and was, thus, appropriate for real-time reverse transcription-PCR analysis. The absence of contaminating DNA in RNA samples was confirmed by the absence of amplification in samples that were not subjected to reverse transcription. The absence of inhibitors of reverse transcription and of amplification reactions in the samples was confirmed by comparison of the standard curves obtained using serial dilutions of samples both before and after reverse transcription, which had identical slopes and intercepts. The efficiency of the real-time PCR amplifications was always at least 94% and not more than 100%. The C_q values of the non-template controls were always above 38 cycles, and more than 10 cycles above the C_q values of the samples with the lowest target concentration. No significant variation of 16S and 23S rRNA abundance in the 2 experimental cheeses (the amount of RNA that was reverse-transcribed was standardized) existed and the 16S rRNA gene was used as the reference gene in expression quantification. Although we did not calculate the detection limit we always worked within the range of standard curves developed for this study.

Expression of Nisin Genes During Cheese Manufacture

Relative expression ratios of *nis* genes in cheeses with and without added nisin are presented in Figure 4. From these data, 4 expression groups of nisin genes can

Figure 3. Nisin production by *Lactococcus lactis* M78 in the model cheeses. Results are expressed as mean values of 3 repetitions. Closed and open symbols correspond to cheeses with and without addition of 1.2 IU/mg of nisin, respectively.

Figure 4. Evolution of the relative expression ratio of nisin genes in model cheeses without (A) or with (B) addition of 1.2 IU/g of nisin, and ratio between the 2 types of cheeses (C). Symbol designations are as follows: 1: *nisA* ($\times$), *nisF* ($\circ$), *nisE* ($\blacklozenge$), and *nisG* ($\square$); 2: *nisR* ($\blacktriangledown$) and *nisK* ($\triangle$); 3: *nisB* ($\bullet$), *nisT* (∇), *nisC* ($\blacksquare$), *nisI* ($\diamond$), and *nisP* ($\blacktriangle$). Expressions are relative to the sample at 24 h without added nisin (calibrator), and error bars represent the standard deviations of 3 biological repetitions. When error bars are not visible, they fall within the symbol.

be distinguished: *nisA*, *nisFEG*, *nisRK*, and *nisBTCIP*. Expression of the *nisA* gene is higher in the presence of external nisin. The highest difference was at 4 h of fermentation, which corresponds to a 10-fold difference in expression. The ratio decreased gradually to a 2-fold difference at 24 h of fermentation (Figure 4, C1). The 3 immunity genes *nisFEG* showed a similar expression evolution as a function of time, whereas different profiles were observed between fermentations with or without added nisin. In cheeses without addition of nisin, an increase from 4 to 12 h and a decrease at 24 h was observed (Figure 4, A1). In the presence of nisin, expression determined at 4 h was higher by factors from 18 to 26 (Figure 4, C1). For each of the 2 types of fermentation, the genes *nisRK* showed a similar evolution of expression as a function of time (Figure 4, A2

and B2). A slight difference in *nisRK* expression was observed at 4 h, where it was approximately 2 times higher in cheeses with added nisin (Figure 4, C2). For the last group of nisin genes (*nisBTCIP*), expression reached its maximum at 6 h of fermentation (Figure 4, A3 and B3). At 4 h, expression in cheeses with added nisin was about 5 times higher than in cheeses without added nisin (Figure 4, C3).

DISCUSSION

The aim of the present study was to evaluate the expression of the genes involved in the production of nisin, a well-characterized bacteriocin, in cheese. We used a nisin-producing strain, *L. lactis* M78, which was isolated from raw milk. This strain was found to

demonstrate better growth and acidifying activity in milk and cheese than the other 2 nisin-producers tested (results not shown), which are generally regarded as weak acid producers and unable to coagulate milk (Froseth and McKay, 1991; De Vuyst and Vandamme, 1992; Takala and Saris, 2007). The fact that this strain is proteinase positive (A. Trmčić, unpublished data) and able to ferment lactose (Samelis et al., 2009) may explain its ability to grow well in milk and cheese. The growth was also expected to be influenced by nisin that was added in a commercial preparation form to one series of experimental cheeses. According to Rauch et al. (1994), nisin is supposed to prolong the lag phase of growth without affecting the growth rate or cell yield of nisin-producing strains. This phenomenon was not observed in our study because our test strain entered the exponential growth phase immediately after inoculation in the milk, with or without nisin supplementation.

The second and main effect expected from addition of nisin to the cheese-milk was stimulation of its own production by nisin producer present in cheese. Nisin is a known self-inducer and this characteristic was also confirmed in our study. Nisin activity at the first sampling (4 h) was about 10-times higher when nisin was present in cheese-milk at inoculation. The concentration of added nisin (1.2 IU/g, which is equivalent to 0.08 log IU/g) was below detection limit and was, thus, negligible in comparison to that of the nisin produced by strain M78, at least after 4 h of fermentation. We also observed a strong correlation between production of nisin and expression of its genes. In cheese with added nisin, an overexpression of the nisin genes *nis-ABTCIPFEG* was detected. This overexpression was the highest at 4 h of fermentation and from that point on, it varied considerably as a function of time. Four different groups were distinguished according to expression as a function of time: *nisA*, *nisBTCIP*, *nisRK*, and *nisFEG*.

Genes *nisFEG* are under the control of a nisin-dependent promoter and this is in agreement with our results. The presence of nisin in cheese stimulated the initial expression of *nisFEG* by 18 to 26 times, and expression was at least 2-times higher by the end of the experiment. Additionally, in cheese with no nisin added, an increase of *nisFEG* expression was observed from 4 to 12 h. This was probably due to bacterial production of nisin and its accumulation in cheese. The genes *nisFEG* encode for nisin immunity proteins; therefore, the likely explanation is that higher concentrations of nisin also demand a higher protection of the nisin-producing strain and, consequently, a higher production of immunity proteins.

The expression of the second nisin-dependent promoter, located upstream of *nisA*, was strongly induced

by the presence of nisin in cheese. In contrast to the expression stimulation of *nisFEG*, where expression increased during the first 12 h of fermentation, *nisA* showed an increase in expression during the entire 24 h of fermentation. Although in both types of cheeses the increase of *nisA* expression was observed, in those without added nisin the increase was more pronounced (10-fold) compared with the 2-fold increase in cheeses with added nisin. Some other studies reported that expression of *nisA* and production of nisin were most extensive during the exponential growth phase and ceased completely in the stationary phase (Engelke et al., 1994; Takala and Saris, 2007). However, the results of this study showed different dynamics. Both *nisA* expression and nisin production by *L. lactis* M78 were still observed during the stationary phase and even increased in cheeses without added nisin. Expression of *nisA* and production of nisin at 4 h of fermentation were about 10-fold higher in cheeses with added nisin. A linear correlation between the expression of *nisA* and nisin activity in cheeses could be observed throughout the entire experiment. Linear correlation is presented by the equation $y = 0.735x$, with y representing the relative expression ratio and x representing nisin activity (log). Such a correlation was expected because the *nisA* gene encodes for the structural protein that is converted to active nisin. Kuipers et al. (1995) and de Ruyter et al. (1996) also observed a linear correlation between expression and activity of nisin in the study on *nisA* promoter activity in the presence of nisin.

All of the genes involved in the formation of active nisin, together with the immunity gene *nisI*, form a cluster of overlapping genes, which are located downstream of *nisA* (Kuipers et al., 1993; Li and O'Sullivan, 2006). The gene overlapping and, consequently, the transcription coupling, also were confirmed in this study by the equal expression dynamics of these genes. Additionally, the expression dynamics of *nisBTCIP* genes differed from the expression of *nisA*, which points out the presence of individual *nisA* transcripts besides large polycistronic transcripts, as already reported by Runar Ra and Saris (1995) and Ra et al. (1996). The transcription of *nisA* into 2 different types of transcripts may be due to the inverted repeat that is present between *nisA* and *nisB*, and may cause a limited read-through during transcription from the *nisA* promoter (Kuipers et al., 1995; de Ruyter et al., 1996). Steen et al. (1991) also mentioned the possibility of *nisA* being released from a larger polycistronic transcript after processing, where the inverted repeat may also have a role. Dependence of *nisBTCIP* genes on the induction by nisin was confirmed by the results of measured expression at 4 h of fermentation, when the levels of expression were 4- to 6-times higher in cheeses with added nisin,

compared with those without added nisin. Maximum expression was reached after 6 h of fermentation. The point of maximum expression does not seem to affect the correlation between *nisA* expression and accumulation of active nisin as would be expected (De Vuyst and Vandamme, 1992; Engelke et al., 1994). No obvious correlation between nisin production and expression of *nisB* was found, although the expression dynamics of *nisB* were completely comparable with those reported by Engelke et al. (1994). Although the experimental design did not allow us to make absolute comparisons among expressions of different nisin genes, we could estimate the absolute expression from the C_q values. The C_q values for the *nisA* gene were always the lowest of all examined nisin genes, which means that this transcript was the most abundant one. Several mechanisms may explain this observation. One reasonable conclusion would be that *nisA* transcripts are more stable than the other nisin gene transcripts (De Vuyst and Vandamme, 1992; Li and O'Sullivan, 2006). The promoter upstream of *nisA* can also be activated by galactose or lactose, which can account for higher expression of this gene in milk and cheese (Chandrapati and O'Sullivan, 1999; Penna et al., 2005; Li and O'Sullivan, 2006). Lower expression of *nisFEG* genes compared with *nisA* expression is consistent with the results of de Ruyter et al. (1996) and the fact that the promoter upstream of *nisF* contains only one of 2 binding sites present in the nisin-dependent promoter located upstream of *nisA* (Chandrapati and O'Sullivan, 2002). A second constitutive promoter located upstream of *nisI* has been reported, enabling nisin-producing strains to establish immunity before the presence of nisin in the environment (Li and O'Sullivan, 2002, 2006). Because our sampling started during the exponential growth phase, the nisin present at this time already acted as an inducer and might, thus, mask any transcription initiation from this constitutive promoter. In fact, no difference of expression dynamics could be observed between *nisIP* and *nisBTC*.

Genes *nisRK* encode for regulatory proteins that transfer the signal from extracellular nisin to the promoters upstream of *nisA* and *nisF*. These regulatory proteins genes have to be expressed constantly, to enable the nisin-producing strain to respond to the presence of nisin in the surrounding environment. Because of this, *nisRK* genes are under the control of a nisin-independent promoter. But this constitutive expression was only partly confirmed in the present study because the number of transcripts at the beginning and the end of the experiment was about 2-times higher in the cheeses with added nisin. In addition, a significant variation of *nisRK* transcript quantity as a function of time was found. If this is not a result of a change in RNA stability, it could raise some doubt about the

exclusive constitutive expression of these 2 genes. The study of Ra et al. (1996) showed that these genes may be part of a large *nisZBTCIPRK* transcript. In that case, *nisRK* could be transcribed as 2 different transcripts, which might explain the observed variation of transcript quantity as a function of time. Furthermore, it was noticed that, except at 4 h, the transcription profile of *nisRK* was similar to that of *nisBTCIP*.

CONCLUSIONS

In the present study, we succeeded in following the expression of 11 nisin genes from *L. lactis* M78 in model cheeses by real-time reverse transcription-PCR. This study is the first one in which the evolution of transcripts has been quantified rigorously in a cheese-like medium. Beside the contribution to the explanation of regulation of nisin biosynthesis and immunity, this study presents an example of what could be done in the future to better understand the role of bacteriocins in different environments and the physiology of microorganisms growing in cheese.

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