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# Nisin Quantification by ELISA Allows the Modeling of Its Apparent Diffusion Coefficient in Model Cheeses

Samar Aly,[†,‡] Juliane Floury,[†,‡] Marie-Hélène Famelart,[†,‡] Marie-Noëlle Madec,[†,‡] Didier Dupont,[†,‡] Yann Le Gouar,[†,‡] Sylvie Lortal,[†,‡] and Sophie Jeanson*[,†,‡]

[†]INRA and [‡]Agrocampus Ouest, UMR1253, Science and Technology of Milk and Eggs, F-35000 Rennes, France

**ABSTRACT:** The diffusion of small solutes in cheese is of key importance for most enzymatic reactions involved in the ripening process. However, only a limited amount of data is available on salt diffusion and practically none on peptide diffusion. Nisin, a bacteriocin peptide, migrated in model cheeses made from ultrafiltered (UF) retentate. A profile concentration device and an enzyme-linked immunosorbent assay (ELISA), specifically developed for nisin quantification in cheese, were used to model the apparent diffusion coefficients for nisin according to Fick's law. This average coefficient was 49.5 $\mu m^2/s$ in UF cheese ($n = 2$). When 10% gelatin was added to the retentate, this value decreased to 34.4 $\mu m^2/s$ ($n = 2$). The two cheeses differed in their macrostructure (rheology) and microstructure (confocal microscopy). This study provides the first apparent diffusion coefficients for a peptide in cheese and supports the hypothesis that composition and structure influence the diffusion of small solutes such as peptides.

**KEYWORDS:** nisin, diffusion, modeling, ELISA, cheese, microstructure

## INTRODUCTION

An essential step in the manufacture of cheese involves the coagulation of caseins to form a gel that entraps the aqueous phase of milk and fat, if present.[1] Bacteria are also entrapped within the curd and develop as bacterial colonies with a three-dimensional spatial distribution.[2] Cheese ripening involves complex microbiological and biochemical reactions within this network, resulting in the development of flavor and texture characteristics specific to each cheese variety.[3] Whatever the cheese, most of the key enzymatic reactions are likely to depend on the diffusion of their substrates. After lysis of bacterial cells, the diffusion of the bacterial enzymes within the matrix could also be a key step in the ripening process. Diffusion limitations may thus create a bottleneck for enzymatic reactions and act as a constraint for bacterial growth and/or metabolic activity.[4] A protein gel structure can act like a sieve, where the gel strands obstruct the diffusing molecules; a denser gel structure can lead to greater obstruction.[5] Furthermore, electrostatic interactions can also modify the migration of charged molecules such as peptides in the casein network.

Limited work has been carried out on the transfer properties of small solutes in cheese. Mainly salt, water, and lactate diffusion coefficients were modeled in cheese matrices, using Fick's diffusion law or similar equations.[6] Only one study on the protein transfer in a solid matrix has been reported so far, that is, the lysozyme diffusion in agarose gels.[7] Evidence exists for the diffusion of two peptidases (phenylalanine amino peptidase and proline iminopeptidase) in Gruyère cheese, from the smear into the edge after 1 and 6 months of ripening. However, the migration properties of these proteins were not examined.[8]

Nisin, a bacteriocin, is a 34 amino acid peptide, which is produced by some strains of *Lactococcus lactis* and shows high antimicrobial activity against a broad spectrum of Gram-positive bacteria.[9] Nisin is able to migrate in gels and model cheese matrices and is used as a food preservative[10] and in food packaging.[11] However, to date, apparent diffusion coefficients for nisin have been determined only in agarose gels.[12,13] In this study, nisin was chosen as a relevant model solute to investigate mass transfer properties of peptides during ripening within the cheese matrix. Peptides are key substrates in the proteolysis process and are also involved in bacterial interactions (quorum-sensing). Ultrafiltered (UF) milk retentate was used as a model cheese, as previously described.[14] This nonfat UF model cheese is a homogeneous matrix unlike real cheese and is therefore a better model system as it is more suited to a homogeneous migration of solutes.

The first objective of our work was to model the apparent diffusion coefficients for nisin in cheese using the profile concentration method. For this purpose, an enzyme-linked immunosorbent assay (ELISA) was developed to specifically quantify nisin within the cheese protein network. ELISA was chosen for its accuracy and its sensitivity of detection. Its specificity to detect a protein within a protein network is well-known in comparison to high-pressure liquid chromatography (HPLC). The second objective was to assess whether a modification of the cheese composition and microstructure could affect this apparent diffusion coefficient, as supported by previous data for water and salt.[6] Gelatin was chosen for incorporation in the curd, as it is already used in the dairy industry to replace fat[15] and to minimize syneresis in yogurt.[16]

A better understanding of the mass transfer properties for key solutes such as peptides, depending on the matrix microstructure, should allow a more generic view of cheese-ripening kinetics for future innovations.

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## MATERIALS AND METHODS

**Model Cheese Manufacture.** *Milk Microfiltration and Ultrafiltration.* Skimmed milk was microfiltered to remove the indigenous microflora and then ultrafiltered as already described,[17] except that neither NaCl nor cream was added. The UF retentate was then stored at −20 °C. The total milk proteins were concentrated 4.2 times, resulting in the following retentate composition: 208.5 g/kg dry matter, 146.4 g/kg total nitrogen, of which 118.6 g/kg were caseins and 26.1 g/kg whey proteins. The pH was 6.64 (±0.01).

*UF Model Cheeses.* UF retentate was heated to 93 °C for 15 min while stirring. This heat treatment resulted in the denaturation of whey proteins, an increase in the water restraint in the protein network, and consequently a reduction in the syneresis of the model cheese matrix during the diffusion experiment. It was important to calculate the denaturation percentage to prove the reproducibility of the heat treatment and also to evaluate the rate of denatured soluble proteins as they are responsible for the decrease of syneresis when denatured. The percentage of denatured soluble proteins was calculated using the following equation:[18]

$$\text{denaturation}\ (\%) = \frac{(\text{NCN}_{RR} - \text{NPN}_{RR}) - (\text{NCN}_{HTR} - \text{NPN}_{HTR})}{(\text{NCN}_{RR} - \text{NPN}_{RR})^{RR}} \times 100 \quad (1)$$

where $X_{RR}$ are the variables for the raw retentate, $X_{HTR}$ are the variables for the heat-treated retentate, NCN is non-casein nitrogen (g/kg), and NPN is non-protein nitrogen (g/kg). The rate of denaturation was calculated following five heat treatments and was 83.8 ± 1.3%. Total proteins and protein fragments were analyzed using the Kjeldahl method.[19]

This heat-treated UF retentate was then coagulated with 0.03% v/v final concentration of a chymosin agent Maxiren (DSM Food Specialties, France). After molding in plastic cylinders, the UF model cheeses were all incubated at 30 °C for 1 h for the coagulation process.

To modify the micro- and macrostructure, 46.25% (v/v) of the UF retentate was replaced by a solution of gelatin 210 g/L (gelatin type B from bovine skin, Bloom 225, Sigma-Aldrich, Germany). The final concentration of gelatin in the mixture represented 10% of the dry matter, maintaining the same initial dry matter. Two different model cheeses were made: R cheese, made only from UF retentate; and R-G, cheese made from UF retentate in which gelatin partially replaced milk proteins.

**Nisin Diffusion: Concentration Profile Device.** *Nisin Solution Preparation.* Commercial nisin (Shanghai Richem International Co., China) was used. This powder contains nisin Z as the unique peptide as shown by electrospray ions/liquid chromatography−mass spectroscopy (ESI/LC-MS). The purity of this commercial nisin was 40% (data not shown) on the basis of the analysis of amino acids.[20] Nisin powder was dissolved in the permeate of the milk ultrafiltration (pH 6.6) to a final target concentration of 500 mg/L of nisin.

*Experimental Device for Nisin Diffusion.* The experimental device was chosen to allow unidirectional solute mass transfer from a nisin solution into cylindrical blocks of cheese when put in contact with the nisin solution (Figure 1). This device was performed with one repetition leading to two sets of data for each model cheese, including values for the two durations of incubation (3 and 6 days). Therefore, four cylindrical blocks of each type of model cheese (R and R-G) were molded and coagulated in impermeable plastic cylinders of 3 cm in diameter and 7 cm in length (Krehalon, France). After 1 h of incubation at 30 °C for complete coagulation, one side of the cheese round was carefully cut to produce a plane surface. Each cheese cylinder was then hung to allow its plane surface to come into contact with the nisin solution (500 mg/L) as shown in Figure 1. Nisin diffusion in the model cheeses began as soon as the surface of the cheese cylinder came into contact with the nisin solution. After both 3 and 6 days of incubation at 19 °C with the nisin solution, two cylinders of each cheese (R and R-G) were totally used for the quantification of nisin. As a result, the experiment includes independent duplicates for each model cheese (R and R-G) and for each incubation time (3 and 6 days).

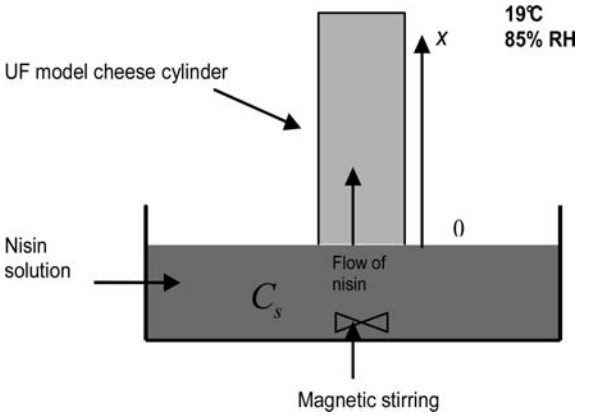


**Figure 1.** Schematic experimental device for nisin diffusion in two model cheeses in an incubator with controlled temperature and relative humidity.

The volume of the nisin solution was large enough to maintain constant nisin concentration during the diffusion process. The nisin solution was covered and sufficiently agitated to avoid a fluctuation of the nisin concentration on the surface of the cheese. The experiment was performed in a thermostatically and hydrometrically controlled incubator for 3 or 6 days at 19 °C and 85% relative humidity (RH).

**Physicochemical Analysis.** *Dry Matter Content and pH.* Four cylindrical blocks of each type of cheese (R and R-G) were prepared as described under UF model cheeses. The moisture content was measured in duplicate in 1.5 ± 0.2 g slices of the R and R-G cheeses after 3 and 6 days of incubation with the nisin solution.[19] Both model cheeses had the same initial dry matter (21.0 ± 0.5%) and the same pH, which was measured using a pH-meter (inoLab pH Level 1, WTW, Germany) with an accuracy of ±0.01.

Nisin concentration was recalculated for the aqueous phase of the cheese matrix, taking the dry matter content of each slice into account. The dry matter content in all slices was uniform throughout the cheese cylinder prior to the diffusion process. However, it increased in the slices close to the exposed surface after a few days of incubation due to a water intake from the nisin solution into the product (data not shown).

*Water Activity Measurement ($a_w$).* The water activity measurement for R and R-G cheeses was performed at 19 °C, after 1 h of incubation at 30 °C, using an activity meter instrument (Fast lab, GBX, France) based on the dew-point method, with an absolute error of ±0.003. Measurements were carried out in triplicates. Means of $a_w$ of the R and R-G cheeses were statistically compared using a Student's $t$ test in Microsoft Excel.

**Quantitative Inhibition ELISA.** *Nisin Extraction from Model Cheese Slices.* Beginning with the exposed surface in contact with the nisin solution, the cheese cylinders were cut into thin slices. Each slice was weighed (1.5 ± 0.2 g) and its thickness precisely measured using a caliper rule (about 2 mm thick). The slices were then grated and diluted 1:10 (w/w) in acidified citrated water (pH 5) before homogenization with an Ultra-Turrax T25 (IKA Labortechnik, Staufen, Germany) for 2 min at 8500 rpm. The extract was centrifuged at 6000*g* for 10 min. For each sample, 1 mL aliquots of the supernatant were stored for several weeks at −80 °C until analysis.

*Test Development.* An inhibition ELISA was developed for the quantification of nisin. The main steps of this inhibition assay are as follows: (i) a known amount of antigen solution (commercial nisin) was

used to coat microtiter plate wells; (ii) an excess of unlabeled antibodies (antinisin antibodies) was incubated with their antigen in the sample (cheese extract); (iii) these bound antibody/antigen complexes were then added to the antigen-coated wells; (iv) the plate was washed so that unbound antibodies were removed; therefore the more antigen in the sample, the less antibody was able to bind to the antigen in the well; (v) a secondary antibody (enzyme-labeled anti-IgG) specific to the primary antinisin antibody was added; (vi) a substrate was added, and the remaining enzymes produced a chromogenic or fluorescent signal. For inhibition ELISA, the higher the sample antigen concentration, the weaker the eventual signal.

A polystyrene microtiter plate (Maxisorp, Nunc, Denmark) was coated with 100 $\mu$L/well of 0.5 $\mu$g/mL nisin in 0.1 M sodium carbonate−bicarbonate buffer (pH 9.6). For each step, the microplates were incubated at 37 °C for 1 h and washed three times by filling all wells simultaneously with 300 $\mu$L of washing solution (0.05% Tween20 in 0.05 M phosphate-buffered saline, PBS-T) using an automatic microplate washer (ELx50 Filter Microplate Washer, BioTek, Winooski, VT). To reduce nonspecific binding, the microplates were blocked with 250 $\mu$L of 1% (w/v) porcine gelatin (gelatin from porcine skin, Sigma-Aldrich, France). At the same time, serial 10-fold dilutions of samples (from 1:20 to 1:5000) were made in PBS-T and incubated at 37 °C for 1 h in test tubes with the same volume of rabbit polyclonal antiserum and anti-nisin[21] diluted at 1:1250 in PBS-T.[22]

After incubation, 100 $\mu$L of each mixture was added to the microtiter plate wells. Wells were then incubated with anti-rabbit IgG−alkaline phosphatase diluted 1:5000 in PBS-T (Sigma-Aldrich, Germany). Finally, the reaction was initiated with 100 $\mu$L of substrate (1 mg/mL tablet of *p*-nitrophenyl phosphate, KPL USA, diluted in 1 M diethanolamine). Following incubation, the absorbance was measured at 405 nm against a blank and using a microplate reader (ELx800 BioTek). The curve fits were performed by Gen5 data analysis software (BioTek). Both negative and positive controls were added to several microplates to test cross-reactions with the UF model cheese samples. A UF cheese model manufactured with a known amount of nisin was the positive control, whereas a UF model cheese manufactured without nisin was the negative control. Extracts from these two controls were prepared as described previously for model cheese slices. Each cheese sample was quantified at least four times on different microtiter plates.

*ELISA Validation.* The limit of detection (LOD) is defined as the lowest nisin concentration that can be distinguished from a nisin-free sample (negative control). The LOD was calculated on the basis of the mean of 20 measurements, carried out on 5 different days, of the negative control extracts plus 3 times the standard deviation (SD) of the mean (mean + 3SD).[23] Samples used for the standard curve were prepared by mixing known nisin concentrations to the UF retentate before manufacture of the UF model cheeses and by preparing the cheese extracts as described previously for model cheese slices. Final nisin concentrations in these model cheeses ranged from 0.8 to 80 mg/kg.

The accuracy of the assay was assessed by determining the recoveries of nisin from the spiked cheese samples. These samples were prepared by mixing known nisin concentrations with UF retentate before the UF model cheeses were manufactured in duplicates. Nisin concentrations in these spiked cheeses were 10, 50, 100, 150, 250, and 500 mg/kg.

Nisin was extracted from spiked cheeses (either for the standard curve or to test the accuracy) as already described for the model cheese slices. The supernatants were used to perform an inhibition ELISA in duplicate.

**Determination of the Apparent Diffusion Coefficient for Nisin.** Assuming no convective fluxes, Fick's diffusion equation describing un-steady-state mass transfer can be written as[24]

$$\frac{\partial C}{\partial t} = \nabla(D_{app}\nabla(C)) \tag{2}$$

where $t$ is the time (s) of diffusion, $C$ is the concentration (mg/kg) of nisin in the aqueous phase of the model cheese matrix, and $D_{app}$ is the apparent diffusion coefficient ($m^2/s$) for nisin in the model cheese matrix. This equation has already been used for studying nisin diffusion in agarose gels.[12,13]

Using the present experimental device for studying nisin diffusion (Figure 1), it can be assumed that the mass transfer is unidirectional along the $x$-axis. It can also be assumed that the apparent diffusion coefficient is constant with time. Equation 2 then becomes

$$\frac{\partial C(x,t)}{\partial t} = D_{app}\nabla^2(C(x,t)) \tag{3}$$

where $x$ (m) is the position along the $x$-axis of the UF model cheese.

The initial and boundary conditions are as follows:

$$\text{at } t = 0 \rightarrow C(x,t) = 0$$

The duration of the experiment was assumed to be such that the solute (nisin) did not reach the extremity of the matrix. The matrix was thus considered as a semi-infinite medium.

$$\text{at } t > 0 \rightarrow C(0,t) = C_s \text{ and } C(\infty,t) = 0$$

$C_s$ is the concentration (mg/kg) of nisin at the interface of the UF model cheese with the nisin solution.

The solution of eq 3 is then

$$\frac{C(x,t)}{C_s} = \text{erfc}\left(\frac{x}{\sqrt[2]{D_{app}t}}\right) \tag{4}$$

where erfc is the complementary error function.

Experimental nisin concentrations measured using inhibition ELISA were plotted versus the distance of diffusion $x$ (m) of the slice from the nisin solution.

Equation 4 was used in Microsoft Excel. The unknown parameter $D_{app}$ was fitted using the Microsoft Excel Solver Tool by minimizing the sum of squares of the deviations between the experimental ($C_{exptl}$) and theoretical model values ($C_{model}$) of nisin concentrations (mg/kg).

$$\text{crit} = \sum_{i=1}^{N}(C_{exptl} - C_{model})^2 \tag{5}$$

To evaluate the adequacy between experimental and theoretical data, theoretical concentrations were plotted versus experimentally determined concentrations; the coefficient ($R^2$) was then calculated. If $R^2$ tends toward 1, the dispersion between experimental and theoretical data is weak; therefore, the mathematical model fits the experimental data.

Two sets of data for each model cheese were modeled, leading to two values of apparent diffusion coefficient for each model cheese (R and R-G).

**Macro- and Microstructure Analysis.** *Rheological Measurements.* The macrostructure of the R and R-G cheeses was assessed by measuring their rheological behavior during large strain compression. Cylinders of model cheeses were made, as described under UF model cheeses, specifically for the rheology experiment. They were cut into 2 cm long slices after coagulation at 30 °C for 1 h and then incubation at 19 °C for 2 h. The macrostructure of the cheese slices was then examined. Compression tests were performed using an Instron instrument 4501 (Instron France S.A.S., Elancourt, France) equipped with a 100 N sensor and a 6 cm diameter plate geometry. Operating at 19 °C, 10 mm/min, and 90% compression, measurements were performed on six replicates. The compression stress was calculated using the corrected area of the slices assuming the volume of each slice was constant during compression.

*Microstructural Examinations.* The microstructure of both R and R-G cheeses was investigated by confocal laser scanning microscopy

(CLSM) using an Eclipse TE2000-C1si inverted microscope (Nikon, Champigny-sur-Marne, France). All samples were observed using a lens ×40 magnification (oil immersion) directly on a 2 mm thick slice of model cheese. Nile blue (1% aqueous solution, Sigma-Aldrich, Germany) was used to stain the cheese protein network, but it did not stain the gelatin. Nile blue was excited with a helium/neon laser (excitation at 633 nm wavelength, fluorescence emission detected over 650 nm). At the same time, for the purpose of the confocal examination, gelatin was labeled using fluorescein isothiocyanate (FITC) 0.6% (w/v) aqueous solution (Sigma-Aldrich, Germany) before being added to the R-G model cheese. The gelatin solution was first diluted 2-fold to reach a final pH of 8.45 and then incubated with FITC for 4 h at 30 °C. Free FITC was eliminated by dialysis (cutoff 12−14 kDa) in Tris buffer (10 mM/NaCl, 0.6 mM, pH 7) at 30 °C. Labeled gelatin was then concentrated by evaporation to reach an initial dry matter content of 21% and kept in liquid form to be incorporated in the R-G cheese. FITC−gelatin was excited using an argon laser (excitation at wavelength 488 nm, fluorescence emission detected between 500 and 530 nm).

## RESULTS AND DISCUSSION

**Quantification of Nisin in Model Cheeses Using Inhibition ELISA.** The calibration curve of the assay was created from UF model cheeses made with known concentrations of nisin. The curve was linear between 0.8 and 80 mg/kg with a correlation coefficient $R^2$ of 0.99 (data not shown). All cheese samples were diluted to measure the nisin concentration within the linear range of the ELISA calibration curve. The LOD of this assay was 0.626 mg/kg.

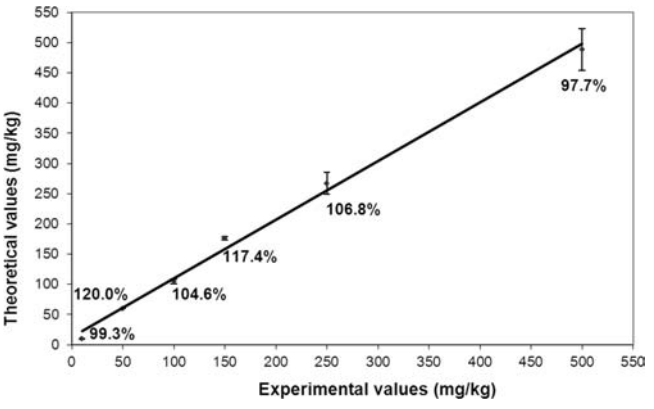


**Figure 2.** Theoretical versus experimentally measured values for nisin concentrations, determined by measuring the percentage of nisin recovered in spiked cheeses by inhibition ELISA ($n$ = 4).

The accuracy of the ELISA was estimated on the basis of nisin recovery varying from 98 to 120% with a correlation coefficient $R^2$ of 0.99 (Figure 2). These results are consistent with results from previous studies in which the recovery rate was 105 ± 15% for nisin from milk samples[25] and from 96.7 to 104.2% for other proteins in milk or cheese samples.[23]

**Apparent Diffusion Coefficients for Nisin in UF Model Cheeses.** Figure 3A shows the experimental and theoretical profiles of nisin concentrations after migration for 3 and 6 days in the R cheese. Results clearly showed that nisin effectively migrated in this cheese matrix. The results also confirmed that Fick's second law can be accurately applied for modeling the apparent diffusion of nisin under these experimental conditions. The correlation coefficient ($R^2$ = 0.85) was close to 1, indicating that the experimental points were not too dispersed when taking into account all of the data (two set of data and two sampling days) compared to the theoretical model. Nisin could reach migration distances of about 11 mm with a nisin concentration of 7.46 ± 0.11 mg/kg in the farthest slice after 6 days. The obtained apparent diffusion coefficients for nisin were 49 $\mu m^2/s$ (with $R^2$ = 0.79) and 50 $\mu m^2/s$ (with $R^2$ = 0.97) with an average diffusion coefficient of 49.5 $\mu m^2/s$ at 19 °C and 85% RH in the R cheese. This is the first time an apparent diffusion coefficient for nisin has been reported in a cheese matrix. This value is of the same order as the apparent diffusion coefficients calculated for nisin in agarose gels, ranging from 13 to 81 $\mu m^2/s$ depending on incubation temperature and agarose content.[12,13] In cheese, data are only available for diffusion of salt (often measured as sodium diffusion) and water.[6] Apparent diffusion coefficients for salt ranged from 100 to 530 $\mu m^2/s$ at around 10−15 °C, depending on the composition of the cheese.[6] In comparison, the apparent diffusion coefficient for nisin is about 2−10 times less in similar conditions. This difference could easily be explained by the size and charge of the diffusing molecules. Nisin is a positively charged peptide with a molecular weight of 3500 g/mol, whereas sodium is also positively charged but with a molecular weight of 23 g/mol, that is, 152 times smaller.

When gelatin was incorporated in the R-G cheese (Figure 3B), the calculated apparent diffusion coefficients for nisin drastically

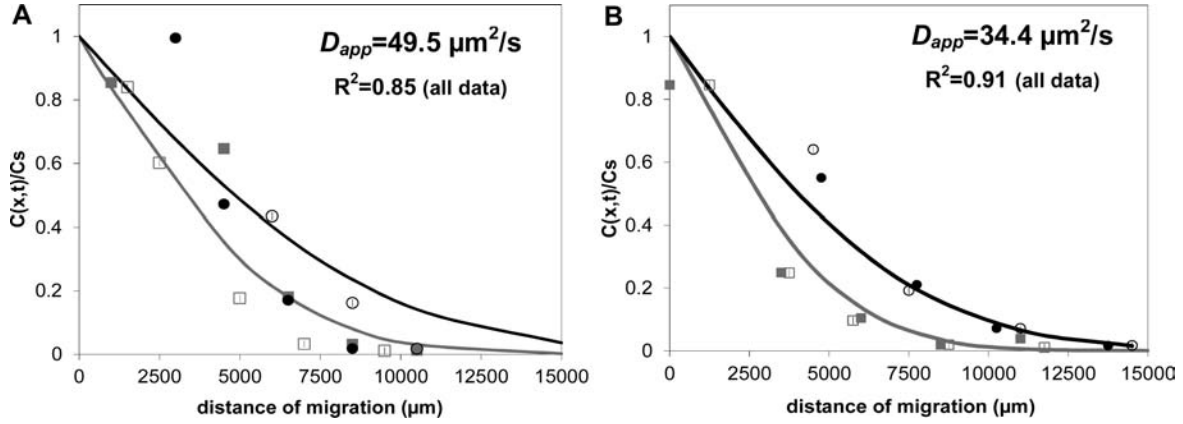


**Figure 3.** Concentration profiles of nisin in R cheese without gelatin (A) and in R-G cheese with 10% gelatin (B) after 3 days (gray and white squares, gray lines) and 6 days (black and white circles, black lines) in contact with a 500 mg/L nisin solution: experimental data for the two repetitions (solid and empty symbols) and theoretical data (continuous line).

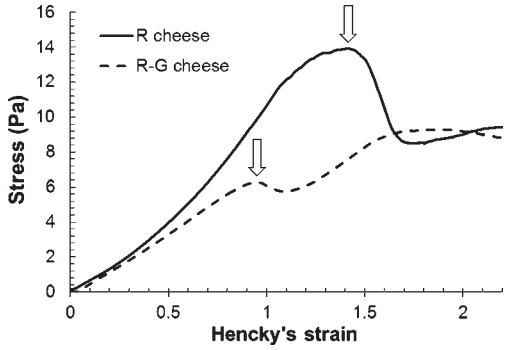


**Figure 4.** Rheological behavior of the two model cheeses obtained under large strain compression; stress versus Hencky strain for R cheese (continuous line) and R-G cheese (dashed line). The arrows show the fracture point.

dropped to 33 $\mu m^2/s$ (with $R^2 = 0.93$) and 36 $\mu m^2/s$ (with $R^2 = 0.93$) with an average diffusion coefficient of 34.4 $\mu m^2/s$. The correlation coefficients $R^2$ were even closer to 1 for this matrix, which also indicated that experimental points were not dispersed when compared to the theoretical model. Nisin could almost reach the same distance (15 mm) with a nisin concentration of 6.09 ± 0.05 mg/kg in the farthest slice after 6 days. Changes in the macro- and microstructure may be responsible for this difference. It was shown, for example, that the agarose content affected the apparent diffusion coefficients for nisin; the latter decreased when the agarose content increased.[12] Apparent diffusion coefficients for nisin were calculated at 42 and 25 $\mu m^2/s$ after 6 days at 10 °C in a 300 mg/L nisin solution, for agarose contents of 3.2 and 6.7% w/w, respectively. These values are of the same order of magnitude as those obtained in our model cheeses.

**Macro- and Microstructure Characterization.** *Water Activity ($a_w$).* Water activity is an important physicochemical parameter that influences microbiological and biochemical changes during cheese ripening.[1] The presence of gelatin resulted in a significant decrease ($p < 0.01$) in average values of $a_w$, from 0.989 ± 0.003 in the R cheese to 0.967 ± 0.004 in the R-G cheese. This occurred despite the fact that their initial dry matter content (21%) and pH (6.64 ± 0.01) were identical. Water sorption is a known property of gelatin due to its polar groups.[26] This reduction in $a_w$ could thus explain the reduced apparent diffusion of nisin in the presence of gelatin. However, $a_w$ was found to be unrelated to salt diffusion in Camembert-type cheese, in which $a_w$ values ranged from 0.969 to 0.977.[27]

*Rheological Assay.* The curves of the stress versus Hencky strain (Figure 4) show the general rheological behavior of the two model cheese matrices under compression. The stress increased until fracture of the matrix occurred, as observed at the maximum of the first peak of the curve (arrows in Figure 4). Only the section of the curves before the fracture point was examined. The presence of gelatin reduced both the firmness (stress at fracture) and the suppleness (strain at fracture) of the R-G model cheese. One hypothesis could be that gelatin impaired the formation of the casein network by preventing its reorganization and, therefore, resulting in lower resistance of the final product to compression. This hypothesis is supported by other authors[28] who studied the effect of added gelatin (type B) on the rheology of sodium caseinate gels acidified using gluconо-δ-lactone. The addition of gelatin (from 0.1 to 1.5%) led to a reduction in gel strength and a moderate increase in gelation time. According to these authors, gelatin addition resulted in the inhibition of casein network rearrangement due to the presence of a new gelatin−casein interaction.

*Microstructural Properties.* Confocal microscope examinations of the two model cheeses are presented in Figure 5. Staining by Nile blue (white parts in Figure 5A) indicated that the cheese protein network of the R cheese appeared perfectly homogeneous. To investigate the distribution of gelatin within the R-G cheese protein network, gelatin was specifically FITC-labeled before being incorporated into the R-G cheese. Panels B and C of Figure 5 represent the same confocal microscopy examination of the R-G cheese with both FITC-labeled gelatin and Nile blue stained cheese proteins. Figure 5B shows the micrograph of Nile blue fluorescence only, whereas Figure 5C shows both Nile blue and FITC fluorescence, showing both the cheese protein network and the FITC-labeled gelatin in the same image. Figure 5B demonstrates that the cheese protein network was heterogeneous, displaying dark regions evenly spread in the R-G cheese (white regions represent cheese proteins). Figure 5C confirmed that the dark regions in Figure 5B were actually the FITC-labeled gelatin (green regions in Figure 5C) evenly distributed throughout the cheese protein network (blue regions in Figure 5C), resulting in a heterogeneous microstructure. On a microscopic scale, the R-G cheese matrix was composed of two different networks: the gelatin network and the milk protein network. A previous study[29] showed that when skim milk−gelatin mixtures (gelatin varied from 0 to 1%) stained by FITC (0.5%) and Nile red (0.025%) were examined by confocal microscopy, an inhomogeneous microstructure was observed, consisting of large dispersed cheese protein-rich regions distributed in a gelatin-rich continuous phase. The present results as well as the former ones suggest that gelatin does not interact with the cheese proteins, resulting in a heterogeneous mixture of two networks on a microscopic scale. This heterogeneity, due to the integration rather than the interaction of two different protein networks, may also be responsible for the decrease in firmness and suppleness of the R-G cheese, compared to the R cheese on a macroscopic level. The presence of gelatin in the R-G cheese resulted in several changes both on a microscopic scale with a heterogeneous microstructure and on a macroscopic scale with a reduction in both $a_w$ and firmness/suppleness. All of these variations, taken separately or combined, may partly explain the decrease in the apparent diffusion coefficient for nisin.

There are two possible hypotheses for the effect of gelatin on nisin diffusion: the path length for nisin diffusion increased in the presence of an additional network, or the presence of gelatin decreased the free water, which is important for solute diffusion, and therefore decreased nisin diffusion.

In this study, nisin was used as a model solute, which can mimic peptide diffusion, such as those produced by bacterial proteolytic enzymes. Quantifying the apparent diffusion coefficient for nisin in cheeses with different structures is an important step in understanding cheese-ripening mechanisms. Using these results, it is possible to quantitatively compare apparent diffusion coefficients for molecules of various sizes and charges (water, salt, lactates, peptides, etc.) within a cheese matrix. However, the profile concentration device can also provide information on the macroscopic resolution. Because tests are rather time-consuming, it is only feasible to compare a limited number of different cheese compositions. Future work

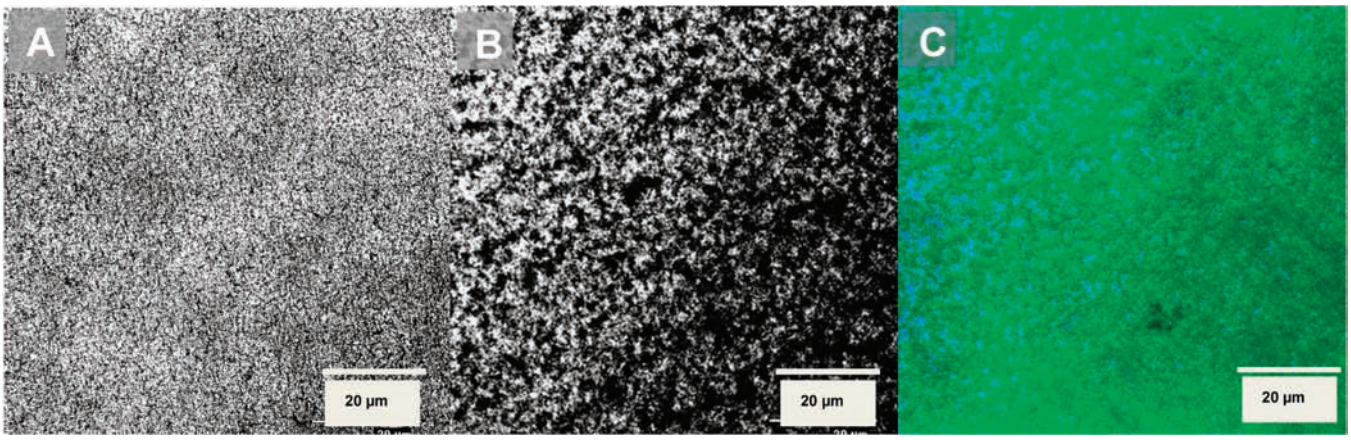


**Figure 5.** Confocal microscopy images of R cheese (A) and R-G cheese (B and C, prepared with FITC-labeled gelatin): (A) black and white micrograph of R cheese stained with Nile blue; (B, C) one micrograph (same microscopic field) of R-G cheese made with FITC-labeled gelatin and cheese protein stained with Nile blue; (B) black and white micrograph only showing Nile blue fluorescence; (C) same micrograph as (B) but in color showing both FITC and Nile blue fluorescence.

should focus on developing nondestructive and in situ techniques on a more microscopic scale.

## AUTHOR INFORMATION

### Corresponding Author

*Phone: +33-(0)2-23485337. Fax: +33-(0)2-23485350. E-mail: sophie.jeanson@rennes.inra.fr.

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