Diffusion et activité de la nisine en matrice fromage modèle

Samar Aly

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présentée par :

Samar Ibrahim Tawfik ALY

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Président
GAUTIER Michel, Professeur, Agrocampus Ouest, Rennes, France
Rapporteurs
DAUDIN Jean-Dominique, Directeur de Recherches, INRA UR QuaPA Clermont- Ferrand-Theix, France
LACROIX Christophe, Professeur, Institut de l'alimentation, la nutrition et la santé, Zürich, Suisse
Examinateur
VAN IMPE Jan , Professeur, Katholieke Universiteit Leuven, Leuven, Belgique
Directeur de thèse
LORTAL Sylvie, Directrice de Recherches, INRA UMR STLO, Rennes, France

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to obtain the degree of:

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Presented by:

Samar Ibrahim Tawfik Aly

Nisin diffusion and *in situ* activity in model cheese matrix

Thesis prepared at the UMR 1253 Science and Technology of Milk and Egg, INRA-Agrocampus Ouest
65 rue de Saint-Brieuc, 35042 Rennes, France

Public defense on July, 4th 2011 in front of the following commission:

Chairman:
GAUTIER Michel, Professor, Agrocampus Ouest, Rennes, France
Reviewer:
DAUDIN Jean-Dominique, Research Director, INRA UR QuaPA Clermont-Ferrand-Theix, France
LACROIX Christophe, Professor, Institute of Food, Nutrition and Health, Zürich, Switzerland
Examiner
VAN IMPE Jan, Professor, Katholieke Universiteit Leuven, Leuven, Belgium
Thesis Supervisor:
LORTAL Sylvie, Research Director, INRA UMR STLO, Rennes, France

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Abbreviations

a_w	*Water activity*
AU	Arbitrary unit
D	Diffusion Coefficient
Da	Dalton
Dha	Dehydroalanine
Dhb	Dehydrobutyrine
DM	Dry matter
d*B*/dpH	Buffering index
IDF	International Dairy Federation
ELISA	Enzyme-Linked Immune Sorbent Assay
FITC	Fluorescein isothiocyanate
FRAP	Fluorescence Recovery After Photobleaching
HPLC	High performance liquid chromatography
IU	International Unit
LAB	Lactic acid bacteria
Lan	Lanthionine
LC/MS	Liquid chromatography–mass spectrometry
LOD	Limit of detection
MeLan	Methyllanthionine
NMR	Nuclear magnetic resonance
OD	Optical density
PBS-T	Phosphate Buffered Saline with Tween 20
RH	Relative humidity
R^2	Correlation coefficient
RifR	Rifampicin resistant

RFU	Relative fluorescence units
SD	Standard deviation
STLO	Science et Technologie du Lait et de l'Œuf
UF	Ultrafiltrat
UMR	Unité mixte de recherche
UR	Unité de recherche
v/v	volume/volume
w/v	weight/volume
w/w	weight/ weight

Introduction, Objective & Strategy

Introduction générale

L'affinage d'un fromage est un processus naturel au cours duquel des réactions microbiennes et enzymatiques se produisent dans le fromage, depuis la coagulation du lait jusqu'à la consommation du produit. L'affinage résulte en effet de l'activité métabolique de colonies microbiennes immobilisées dans la matrice lipoprotéique au moment de l'étape de coagulation. Cette activité métabolique conduit à la formation des arômes du fromage (Fox *et al.*, 2000). Il s'agit donc d'une étape clé, spécifique à chaque variété de fromage, pour le développement de la saveur, de la texture et de l'aspect final du produit. La structure et la composition de la matrice fromagère varient tout au long du processus d'affinage, conduisant à l'élaboration des propriétés organoleptiques du produit fini. Enfin, chaque variété de fromages possède ses propres conditions d'affinage afin de développer des caractéristiques distinctes.

Les bactéries présentes dans la matrice fromagère, qu'elles soient issues de la population indigène du lait ou bien ajoutées lors de la fabrication, sont les principales actrices de l'affinage du fromage. Quelle que soit la technologie fromagère, les bactéries sont immobilisées dans la matrice laitière et se développent sous forme de colonies au cours de l'étape de coagulation. Les réactions biochimiques et enzymatiques survenant au cours de l'affinage du fromage sont donc catalysées par l'activité métabolique de ces colonies immobilisées et de leurs enzymes. Certains substrats vont devoir diffuser dans la matrice pour atteindre les colonies bactériennes, et les métabolites produits vont ensuite diffuser des colonies bactériennes vers le réseau lipoprotéique. Des limitations de transfert de substrats et/ou de produits sont alors susceptibles d'entrainer des problèmes de limitation de la vitesse des réactions enzymatiques, de la croissance des bactéries immobilisées et/ou de leurs activités métaboliques.

La principale question abordée dans cette thèse est d'arriver à mieux cerner le phénomène de diffusion de solutés métabolites, à savoir les peptides (ici la nisine), en relation avec la composition et la microstructure de la matrice fromagère. La nisine est un peptide antimicrobien naturel produit par certaines souches de *Lactococcus lactis* subsp. *lactis*, qui inhibe un large spectre de bactéries gram-positives. Ce peptide est largement utilisé en fabrication fromagère pour améliorer la conservation du fromage. La deuxième question soulevée dans cette thèse concerne l'influence de la composition du fromage sur l'activité de la nisine *in situ* dans la matrice.

Différentes approches méthodologiques ont été développées au cours de ce travail de thèse pour explorer ces questions avec une vision globale. Pour cela, de nombreuses compétences, en technologie fromagère, microbiologie, biochimie, microscopie confocale, rhéologie, et aussi en transfert de matière, ont dues être mobilisées et intégrées. Cette multidisciplinarité rend bien compte de la complexité des phénomènes physiques, biochimiques et microbiens mis en jeu lors de l'affinage d'un fromage.

Introduction

Cheese ripening is the result of bacterial activity of immobilized colonies within the lipoproteic matrix leading to the flavor development (Fox *et al.*, 2000). Ripening is the natural process of microbial and biochemical reactions occurring in cheese from manufacture to its consumption. It is a key concept concerning the development of specific flavor, texture and appearance for each cheese variety. Almost all cheese varieties have their own ripening conditions in order to develop their distinct attributes. The structure and composition of the cheese change throughout the ripening period leading to the development of distinct organoleptic properties.

Bacteria in the cheese matrix, whether they are indigenous or added, are the major actors of cheese ripening. However, these bacteria are immobilized from the coagulation step, and then they grow as colonies, whatever the type of cheese technology. The ripening of cheese is then catalyzed by the metabolic activity of these immobilized colonies and their enzymes. Substrates have to diffuse in the matrix to reach bacterial colonies, and produced metabolites have then to diffuse from the bacterial colonies into the proteinic network. Diffusion limitations may thus create a bottleneck for enzymatic reactions and act as a constraint for bacterial growth and/or metabolic activity.

The main issue addressed in thesis is to better understand the diffusion phenomenon of metabolite solutes like peptides (i.e. nisin), in relation with the composition and the microstructure of the matrix. Nisin is a natural antimicrobial peptide produced by some strains of *Lactococcus lactis* subsp. *lactis* that essentially inhibits a broad spectrum of gram-positive bacteria. Nisin is widely used for cheese preservation. The second issue is to evaluate the influence of cheese matrix composition on the *in situ* activity of nisin, by combining several exploratory approaches to have a global vision of these topics.

Cheese technology, microbiology, biochemistry, microstructure characterization and mass transfer modeling skills were integrated to take into account the complexity of cheese matrices as biochemical and microbial reactors during cheese ripening.

Strategy and objectives

Enzymatic and chemical reactions in cheese are most likely influenced by diffusion rate of solutes within the matrix. The first objective of this thesis was to summarize the state of the art in that topic. The review [**article 1, published**] presented at the beginning of this introduction highlighted that most of the data published concerned salt and water diffusion. Almost no data exist in dairy products for other solutes and about the potential relationship between these diffusion properties and the composition and microstructure of the cheese. In the literature, all the data are technology and cheese dependent, and no generic or mechanistic conclusions can be drawn so far.

On the other hand, it is well known that bacteriocins of lactic acid bacteria, in particular nisin, are able to diffuse in cheese. Nisin-producing starters are clearly active *in situ* against spoilage or pathogenic microorganisms. This observation led us to the hypothesis that nisin could be an interesting model to further explore solute diffusion in cheese. It can be regarded a “model peptide” of 3.5 kDa, 34 amino acids, positively charged, with a biological activity (bactericidal). In order to quantify properly nisin diffusion in dairy products, two preliminary steps should be addressed: i) a way to quantify the absolute quantity of nisin independently of its biological activity, ii) a model cheese system in which we can easily change the composition and microstructure. Gelatin, a protein that is largely used in the dairy industry, was incorporated in an ultrafiltrated (UF) milk retentate in order to modify the microstructure of the model cheeses. Nisin diffusion was estimated in a UF model cheese with and without gelatin. For that purpose, an ELISA approach was developed to specifically

quantify nisin in cheese. This part of the thesis allowed quantifying for the first time the diffusion coefficient for a peptide (nisin) within a cheese matrix, which was from 2 to 10 times lower than the coefficient for salt. In addition, the incorporation of gelatin was shown to reduce the apparent diffusion coefficient for nisin [**article 2, accepted, in revision**]. However, the most commonly used method to follow diffusion, i.e. the profile diffusion method, is highly time consuming and cannot allow the comparison of diffusion properties in many different cheese compositions or microstructures.

We thus decided to develop an alternative strategy based on a microbial approach. Nisin was shown to be able to diffuse *in situ* in cheese (more details in the next part: review of literature). Moreover, both nisin-producing strains and targeted species are immobilized as colonies within the cheese curd after coagulation. The alternative approach was based on the hypothesis that the rate of death of the targeted species could be related, at least partially, to the diffusion coefficient for nisin in the considered matrix. The advantage of using a couple of strains (nisin-producing strain + nisin-sensitive target strain) was that they could be used in any kind of cheeses. Like previously, the modification of the matrix microstructure was performed by incorporating gelatin in the UF model cheeses. Furthermore, in order to know the distance between the nisin-producing colonies and the nisin-sensitive colonies, the spatial distribution of these colonies in the cheese matrix had to be controlled *via* the inoculation rate of both species. The spatial distribution of bacterial colonies was then experimentally investigated and mathematically modeled within a UF model cheese **[article 3, in annex, published**]. In this latter work, my contribution was mainly to optimize the UF model cheese for further *in situ* observations in the gel cassette system® (Institute of Food Research, Norwich, UK). This work allowed above all to better characterizing nisin activity *in situ* in cheese. It finally showed that the composition of the matrix can drastically influence nisin efficiency, independently of its concentration **[article 4, ongoing submission]**.

Review of Literature

Review of Literature

The Review of literature presented here is divided into two parties. The first part is a state of the art which outlines the current knowledge about solutes diffusion in the cheese. It reviews the data concerning the diffusion coefficients of solutes in different cheese types. It also reviews the experimental methods available to model the mass transfer properties of solutes in a complex matrix such as cheese. This part highlighted the limited data existing on nisin diffusion in the cheese unlike salt and water which their diffusion were surveyed in many cheese types. Following this review, a small part is sited the existed studies deal with the relationship between diffusion of solutes and cheese macro- and microstructure.

The second part of the literature review deals with a given knowledge about nisin as a bacteriocin, its chemical properties, its mode of action, its application, its *in situ* activity and quantification, factors affecting its efficiency as a food preservative and finally nisin diffusion in solid matrix.

In this part, I focused on the most important nisin properties which could cause a reduction in nisin diffusion or its *in situ* activity when implicated in a complex matrix such as cheese. The interest of this part was to understand how the characteristics of a matrix affect the nisin diffusion or activity and what is the relative contribution of other cheese physico-chemical characteristics.

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Review

Determination of the diffusion coefficients of small solutes in cheese: A review

Juliane Floury[1,2,3*], Sophie Jeanson[1,2], Samar Aly[1,2], Sylvie Lortal[1,2]

[1] INRA, UMR1253, F-35000 Rennes, France
[2] AGROCAMPUS OUEST, UMR1253, F-35000 Rennes, France
[3] Université Européenne de Bretagne, France

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Abstract – In cheese technology, the mass transfer of small solutes, such as salt, moisture and metabolites during brining and ripening, is very important for the final quality of the cheese. This paper has the following objectives: (i) to review the data concerning the diffusion coefficients of solutes in different cheese types; (ii) to review the experimental methods available to model the mass transfer properties of small solutes in complex matrices such as cheese; and (iii) to consider some potential alternative approaches. Numerous studies have reported the transfer of salt in cheese during brining and ripening. Regardless of the type of cheese and its composition, the effective diffusion coefficients of salt have been reported to be between 1 and $5.3 \times 10^{-10}\ m^2{\cdot}s^{-1}$ at 10–15 °C. However, few papers have dealt with the mass transfer properties of other small solutes in cheese. Most of the reported effective diffusion coefficient values have been obtained by macroscopic and destructive concentration profile methods. More recently, some other promising techniques, such as nuclear magnetic resonance, magnetic resonance imaging or fluorescence recovery after photobleaching, are currently being developed to measure the mass transfer properties of solutes in heterogeneous media at microscopic scales. However, these methods are still difficult to apply to complex matrices such as cheese. Further research needs to focus on: (i) the development of non-destructive techniques to determine the mass transfer properties of small solutes at a microscopic level in complex matrices such as cheese; and (ii) the determination of the mass transfer properties of metabolites that are involved in enzymatic reactions during cheese ripening.

cheese / mass transfer / diffusion / modelling / solute

摘要 – 干酪中少量溶质扩散系数的测定–综述。在干酪技术中，通过盐渍和成熟过程的控制来调整少量溶质 (盐、水分和代谢产物) 的传质，将对最终干酪的质量具有非常重要的作用。本文综述了溶质在不同类型干酪中的扩散系数，以及综述了少量溶质在干酪这一复杂基质中质量传递的数学模型。关于盐渍和成熟过程盐的迁移已有大量的文献报道，无论是何种类型的干酪，盐的有效扩散系数在 $1 \sim 5.3 \times 10^{-10}\ m^2{\cdot}s^{-1}$ (10 ~ 15 °C) 范围内。但是关于干酪中其他少量溶质传质特性的报道非常有限。大多数的有效扩散系数是通过显微镜或者破坏性浓度分布曲线的方法获得。一些新的测定技术，如核磁共振、磁共振成像或者光脱色荧光恢复技术等已经在显微技术的水平下用于测定不同介质中溶质的质量传递特性。然而，这些技术还很难应用于象干酪这样复杂的介质中。将来的研究将主要在：(i) 基于干酪这一复杂介质，在显微水平下采用非破坏性分析技术测定少量溶质的质量传递性质；(ii) 测定干酪成熟过程中代谢产物的质量传递特性。

干酪 / 质量传递 / 扩散 / 模型 / 溶质

*Corresponding author (通讯作者): Juliane.Floury@agrocampus-ouest.fr

Résumé – Détermination des coefficients de diffusion de petits solutés dans le fromage : une synthèse. En technologie fromagère, le transfert de petits solutés, tels que le sel, l'eau et les métabolites au cours du saumurage et de l'affinage, joue un rôle majeur sur la qualité finale du fromage. Cette revue bibliographique a pour objectifs principaux : (i) de faire le bilan des valeurs publiées des coefficients de diffusion de différents solutés dans les fromages ; (ii) de passer en revue les méthodes expérimentales disponibles pour déterminer les propriétés de transfert des petits solutés dans des milieux complexes comme le fromage ; (iii) de considérer les méthodes alternatives potentiellement applicables aux fromages. Dans la littérature, de nombreuses études ont été publiées au sujet du transfert de sel dans les fromages au cours du saumurage et de l'affinage. En fonction du type de fromage et de sa composition, les coefficients de diffusion effectifs du sel sont compris entre 1 et 5,3 × 10^{-10} $m^2 \cdot s^{-1}$ à des températures comprises entre 10 et 15 °C. Très peu d'études concernant les propriétés de transfert d'autres petits solutés dans les fromages ont été publiées. La plupart des coefficients de diffusion effectifs ont été obtenus à l'aide de la méthode classique dite « des profils de concentration », méthode macroscopique présentant l'inconvénient d'être destructive. D'autres techniques, telles que la résonance magnétique nucléaire, l'imagerie par résonance magnétique ou la redistribution de fluorescence après photo-blanchiment sont actuellement développées pour mesurer des propriétés de transfert de matière de solutés à une échelle microscopique. Cependant, elles sont encore difficilement applicables aux matrices complexes comme le fromage. Les perspectives en matière de recherche dans ce domaine sont donc les suivantes : (i) le développement de nouvelles techniques expérimentales pour modéliser à l'échelle microscopique les propriétés de transfert de solutés dans des milieux complexes comme le fromage ; (ii) la détermination des propriétés de transfert des métabolites impliqués dans les réactions enzymatiques pendant l'affinage du fromage.

fromage / transfert de matière / diffusion / modélisation / soluté

1. INTRODUCTION

In cheese, transport of water and aqueous solutes has a crucial role during cheese making and cheese ripening (NaCl, transfer of substrates or reaction products like lactic acid). Cheese ripening is the result of bacterial activity of immobilized colonies in the lipoproteic matrix. Substrates have to diffuse in the matrix to reach bacterial colonies, and produced metabolites have then to diffuse from the bacterial colonies into the proteinic network. In case of diffusional limitations, microgradients of concentration, pH or water activity can be created around and in between the immobilized colonies, modifying bacterial and enzymatic activities.

Diffusion properties of cheese solutes can depend on (i) their physicochemical characteristics and (ii) the composition and microstructure of the matrix. In food matrices and notably in cheese, transfers of small molecules can occur between two heterogeneous phases of the matrix, heterogeneous in terms of composition or physical state (liquid, solid or gaseous). To measure these transfers, diffusion coefficients (D) must be modelled [80].

Analysis of the literature reveals a strong lack of data concerning the migration rates of key molecules in cheese, such as sugars, organic acids and peptides, which can be decisive in the ripening process. Most of the data related to mass transport of small solutes in cheese deal with the salting process. Indeed, salt concentration distribution is an important parameter affecting cheese quality and acceptability. Salt affects the water activity of cheese, the growth and survival of bacteria and the activity of cheese enzymes [7].

Many different mechanisms can be involved during cheese processing, like multicomponent diffusion of solutes and water during salting. Due to technical difficulties to follow solute migration and modelling

difficulties inherent to the physical model chosen, working out diffusion properties of solutes is a complicated task, especially in complex heterogeneous matrices like cheese.

After a theoretical reminder concerning mass transfer phenomena, this paper reviews different methods available in the literature to determine diffusion coefficients of small solutes in cheese products. Values of the diffusion coefficients are then discussed for solutes in different cheese types, with details concerning the modelling methods. Finally, alternative techniques potentially applicable to cheese are presented.

2. THEORY OF MASS TRANSFER

2.1. Definitions

Mass transfer by diffusion is the transport of molecules caused by a random molecular motion in a region where composition gradient exists [82].

2.1.1. Steady-state diffusion

In a macroscopic, motionless (without internal movement and deformation), homogeneous (made up of one phase) and isotropic medium (uniform structure in all directions), solutes diffuse in the direction of their decreasing chemical potentials, until thermodynamic equilibrium is reached. Fick's first law links the diffusive flux to the concentration field, by postulating that the flux goes from high-concentrated regions to low-concentrated regions, with a magnitude that is proportional to the concentration gradient (spatial derivative). In one spatial dimension, this leads to

$$J_i = -D_{im} \cdot \frac{\partial C_i}{\partial x}, \qquad (1)$$

where J_i is the molar diffusion flux of component i (kg or mol·s^{-1}·m^{-2}), C_i is the concentration of component i (kg or mol·m^{-3}), x is the position (m) and D_{im} is the diffusion coefficient of component i in the medium (m^2·s^{-1}). J_i measures the amount of substance that will flow through a small area during a short time interval.

The driving force for the one-dimensional diffusion is the quantity $-\frac{\partial C_i}{\partial x}$. To solve transfer equations, a simplification is generally made, considering chemical potential as a concentration or partial pressure (in the gas phase).

In two or more dimensions, the gradient operator ∇ can be used. This leads to

$$J_i = -D_{im} \cdot \nabla C_i. \qquad (2)$$

Molecular diffusion coefficient D_{im} at a constant temperature may be adequately predicted in very diluted solutions using the well-known Stokes-Einstein equation, provided the molecular radius of the solute, solvent viscosity and absolute temperature are known [19]:

$$D_{im} = \frac{k_B T}{6\pi\mu R_0}, \qquad (3)$$

where k_B is the Boltzmann constant (1.38×10^{-23} J·mol^{-1}·K^{-1}), T is the absolute temperature (K), μ is the viscosity of the phase (Pa·s) and R_0 is the radius of the diffusing molecule (m).

The Stokes-Einstein equation (equation (3)) does not take the intermolecular interactions between solutes and between solvent and solute molecules into account (that may be significant for small solutes). Diffusion through a heterogeneous matrix is more complicated. Solutes will have to diffuse in the liquid or gas phase contained within that porous matrix. Subsequently, the Stokes-Einstein equation has little use in the prediction of diffusion properties in food [77].

Some phenomena that cannot be distinguished from molecular diffusion must also be considered in heterogeneous matrices in terms of composition and structure, such as

capillary or Knudsen diffusion, diffusion modification due to matrix changes (obstruction, retraction, etc.) or interactions of the solute with other components. The term "apparent" or "effective diffusion" is then generally preferred to "diffusion" alone. Effective diffusivities are the most convenient way to describe mass transfer process through porous matrices, which have an intricate network of pores where diffusing species take a tortuous path [77].

If we consider liquid diffusion through porous matrices in which the pores are large, Fick's diffusion model is able to correctly describe the mass transfer within the liquid contained in the pores. The flux can be described in terms of an effective diffusion coefficient D_{eff} ($m^2 \cdot s^{-1}$), defined as

$$D_{\text{eff}} = \frac{\varepsilon}{\tau} D_{im}, \qquad (4)$$

where D_{im} is the diffusion coefficient of i in the medium m ($m^2 \cdot s^{-1}$), τ is the tortuosity and ε is the porosity [82].

In a porous matrix, the effective diffusion coefficient D_{eff} is then significantly smaller than the molecular diffusion coefficient D_{im} because of (i) tortuosity effects (the more tortuous the region the more devious the route between two points) and (ii) interactions between the solute and the matrix if they are both charged (ionic strength, hydrophobic and electrostatic interactions) [69, 77]. Note that equation (4) does not take chemical or electrostatic interactions into account, but only structural incidence of the matrix on solute diffusion properties.

Various alternative equations have been subsequently developed incorporating factors for molecular interactions and physical interferences [67]. To consider charged molecules, a general flux model can be used [38]:

$$J_i = D_{\text{eff}} \frac{C}{RT} \left(\frac{\partial \mu_i(x,t)}{\partial x} \right), \qquad (5)$$

where D_{eff} is the effective diffusion coefficient ($m^2 \cdot s^{-1}$), which does not depend on the electrostatic forces. μ_i is the chemical potential of the solute ($J \cdot mol^{-1}$), which is a function of solute concentration, ionic strength and pH. The charge dependence is thus moved from the diffusion coefficient to the chemical potential. Neglecting pressure and temperature contributions, the chemical potential is defined by [83]

$$\mu_i = \mu_i^0 + RT \ln a + \mu_{el}, \qquad (6)$$

where a is the activity and μ_{el} is the contribution of the electrostatic charges to the chemical potential. In dilute solutions, the activity can be replaced by the concentration and if no electrical charges are present, $\frac{\partial \mu_{el}}{\partial x} = 0$, leading to Fick's law according to equation (1).

However, due to the difficulty in quantifying such factors in real food matrices, equation (5) has poor prediction accuracy [77].

2.1.2. Unsteady-state diffusion

In order to be able to predict the concentration profiles of solutes in the matrix, Fick's first law is associated to a local mass balance to obtain Fick's second law

$$\frac{\partial C_i}{\partial t} = \nabla (D_{im} \cdot \nabla (C_i)). \qquad (7)$$

Considering both unidirectional mass transfer along the x axis and a constant diffusion coefficient value, the previous equation becomes

$$\frac{\partial C_i(x,t)}{\partial t} = D_{im} \cdot \frac{\partial^2 (C_i(x,t))}{\partial x^2}. \qquad (8)$$

Analogous equations can be written in spherical or cylindrical shapes, and two or three dimensions, in order to find the solute concentration as a function of time and position [17].

2.2. Using Fick's law solutions to estimate diffusion coefficients

Most research publications on mass transfer in cheese are using Fick's model with some specific geometries [10]. Diffusion coefficients in food matrices can be evaluated by different methods involving defined geometries and well-defined experimental conditions (steady or transient state and boundary conditions). To determine the diffusion coefficient of a solute in a given matrix, an experimental device generating a flux of the diffusing substance is set up. An average flux (mass variation) or a profile of concentration of the diffusing substance is measured, using either a destructive (slicing and analyzing samples) or a non-destructive method (nuclear magnetic resonance, NMR; fluorescence recovery after photobleaching, FRAP; radioactive tracer; etc.). A mathematical method, adapted to the experiment and generally based on Fick's laws, gives an average diffusion coefficient or diffusion coefficient versus concentration. Table I presents a summary of the principles, advantages and drawbacks of some existing methods for the determination of diffusion properties in cheese-like matrices.

The majority of macroscopic model studies can be divided into measurements in a diffusion cell (steady-state diffusion type of studies) and in cheese cylinders (transient diffusion type of studies).

2.2.1. Steady state

Zorrilla and Rubiolo [88] used the diffusion cell model developed by Djelveh et al. [20]. The diffusion cell consists of two compartments where perfectly mixed solutions A and B of equal volume V but different solute concentrations are separated by a matrix slab with thickness L and cross-section S. The solute migrates through the slab from the higher concentration solution A to the lower concentration solution B.

Assuming a one-dimensional diffusion process through the slab and perfectly mixed compartments, the effective diffusion coefficient of the migrating solute can be modelled thanks to Fick's model. Equation (1) is transformed into equation (9) by applying a mass balance, assuming that there is no change in volume and that the effective diffusion coefficient is constant

$$V_A \frac{\partial C_A}{\partial t} = -D_{eff} \cdot S \cdot \frac{C_A - C_B}{L}, \qquad (9)$$

where D_{eff} is the effective diffusion coefficient of the solute ($m^2 \cdot s^{-1}$), V_A is the liquid volume in the compartment from which the solute diffuses (m^3), S is the matrix area through which the diffusion takes place (m^2) and C_A and C_B are the solute concentrations, respectively, in the upper and lower compartments A and B (mol or $kg \cdot m^{-3}$).

By measuring the solute concentration in the upper compartment A and, via a mass balance, calculating the concentration in the lower compartment B at different times, an effective diffusion coefficient can be calculated by fitting equation (9) to the experimental data.

2.2.2. Unsteady or transient state

Gros and Rüegg [29] reviewed the various experimental techniques and appropriate mathematical treatments proposed to obtain effective diffusion coefficients in food matrices. Measuring unidirectional diffusion from a semi-infinite food cylinder geometry with different boundary conditions is the most frequently applied method to determine the effective diffusion coefficient of a solute in cheese. If the semi-infinite cylinder, containing an initial concentration C_0 of the solute, is in contact

Table I. Principles, advantages and drawbacks of existing methods for the determination of effective diffusion properties in cheese-like matrices.

Technique	Principle	Model	Advantages	Drawbacks	Refs.
Infinite cylinder in contact with a perfectly mixed solution Touching semi-infinite cylinders	A semi-infinite cylinder of the matrix, initially free from the diffusing solute, is in contact: – either with a well-stirred solution containing a constant concentration C_s of the solute at the interface – or with another semi-infinite cylinder of matrix containing a concentration C_s of the solute	– One-dimensional diffusion – macroscopic scale – measurement of the concentration profiles of the migrating solutes along the x axis as a function of time – effective diffusion coefficient with Fick's second law of diffusion – Maxwell-Stefan diffusivities with the Maxwell-Stefan multicomponent approach	– Can be adapted for various small molecules – easy to implement	– Destructive and low resolution: thin slicing of the sample gives spatial resolution of 1 mm – slow: several days of diffusion – a lot of analyses are required to obtain concentration profiles as a function of the distance and the time – a large number of assumptions are required when using the Maxwell-Stefan multicomponent approach – lack of physical interpretation of the Maxwell-Stefan diffusivities	[29] [61] [85] [23]
Diffusion cell	A slab of matrix is placed in between two compartments of perfectly mixed solutions A and B of different solute concentrations	– One-dimensional diffusion – macroscopic scale – evaluation of the solute quantity having migrated through the product slab in a given time – effective diffusion coefficient with Fick's second law of diffusion	– Quite inexpensive – can be adapted to a large range of products – can be adapted to a multicomponent system (simultaneous diffusion of several components)	– Slow: several days of diffusion – accurate determination of solute concentrations is required in both compartments	[20] [89] [90] [88]

continued on next page

Table I. Continued.

Technique	Principle	Model	Advantages	Drawbacks	Refs.
SL-NVRK	– Based on the on-line monitoring of release kinetics of NaCl from a matrix containing a salt concentration C_s into water – a conductivity probe, immersed in the well-stirred aqueous solution, continuously measured the electrolytes released until thermodynamic equilibrium	– One-dimensional diffusion – macroscopic scale – effective diffusion coefficient with Fick's second law of diffusion	– Non-destructive – non-invasive – easy and fast (no analytical technique to quantify concentrations)	– Lack of specificity of the measure with the conductivity probe – modelling difficulties because of the two unknown parameters: the effective diffusion coefficients of salt and of the other electrolytes of the product – can be applied to ionic solutes only	[46] [47]
PFG-NMR	– Based on the attenuation of individual proton resonances under the influence of linear field gradients – the amplitude of the signal is directly related to the self-diffusion coefficient of the molecule	– Microscopic scale – measurement of the **self-diffusion coefficient of small molecules** (random **translational** motion of molecules driven by internal kinetic energy)	– No initial gradient of concentration – non-destructive – non-invasive – promising approach for characterizing the structural modifications during the coagulation process	– High cost – difficulty to sample the product in the thin NMR tubes – high complexity of the spectral data obtained with real food products – difficulty to establish the physical link between the self-diffusion coefficient and the effective diffusivity estimated with classical methods	[13] [55] [56] [16] [22]
NMR imaging	– Imaging technique used primarily in medical settings to produce high-quality images of the inside of the human body – MRI is based on the principles of NMR – MRI primarily images the NMR signal from the hydrogen nuclei ^{23}Na-MRI is based on the paramagnetic properties of the naturally occurring ^{23}Na isotope	– Microscopic scale – measurement of the **self-diffusion coefficient of water or Na** – or visualization of water or Na distribution	– No initial gradient of concentration – non-destructive – non-invasive	– High cost – complex calibration and data handling work – insensitive technique to molecules with low mobility – difficulty to establish the physical link between self-diffusion coefficient and effective diffusivity	[79] [78] [45]

continued on next page

Table I. Continued.

Technique	Principle	Model	Advantages	Drawbacks	Refs.
FRAP technique	– A certain region within a fluorescently labelled sample is irreversibly photobleached with a short intense light pulse – measurement of the fluorescence recovery inside the bleached area as a result of diffusional exchange of bleached fluorophores by unbleached molecules	– Microscopic scale – analysis of the fluorescence recovery inside the bleached area with Fick's law of diffusion – effective diffusion coefficient and fraction of mobile species	– No initial gradient of concentration – simple – non-destructive and slightly invasive	– High cost: a CLSM is necessary – the migrating molecule has to be fluorescent or it must be marked by a fluorescent probe – not adapted to complex and opaque media like cheese	[57] [14] [43]

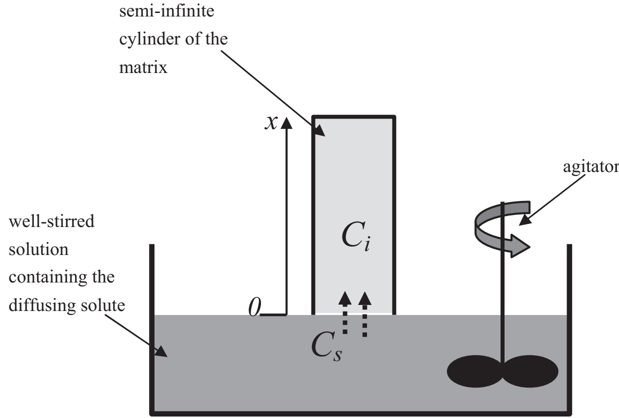

Figure 1. Diagram of the semi-infinite cylinder experimental device.

with a well-stirred solution containing a constant concentration C_s of the solute at the interface ($C_s > C_0$) (Fig. 1), the external mass transfer resistance can be neglected [71] and the boundary conditions are as follows:

$$t = 0 \quad C_i = C_0 \tag{10}$$

$$x = 0 \quad C_i = C_s \;\text{ for }\; t > 0, \tag{11}$$

$$x \to \infty \quad C_i = C_0 \;\text{ for }\; t > 0, \tag{12}$$

where t is the time (s), x is the position (m), C_i is the concentration of solute i in the matrix (kg or mol·m^{-3}), C_0 is the initial concentration of the solute i in the matrix (kg or mol·m^{-3}) and C_s is the concentration of the same solute at the interface (kg or mol·m^{-3}).

The duration of experiments is assumed to be such as the solute does not reach the extremity of the matrix. The matrix is thus considered as a semi-infinite medium. This boundary condition is only valid for Fourier number $\left(F_0 = \frac{D_{\text{eff}} \cdot t}{L^2}\right)$ under 0.05, where L is the length of the semi-infinite cylinder along the x axis (m).

The solution of equation (8) is then

$$\frac{C(x,t) - C_0}{C_s - C_0} = erfc\left(\frac{x}{2\sqrt{D_{\text{eff}} t}}\right), \tag{13}$$

where *erfc* is the complementary error function.

The value of D_{eff} is then determined from concentration profiles by minimizing the sum of squares of the deviations between the experimental (C_{exp}) and model values (C_{model})

$$Crit = \sum_{i=1}^{N} \left(C_{\text{exp}} - C_{\text{model}}\right)^2. \tag{14}$$

If $F_0 > 0.05$, then the assumption of a semi-infinite medium no longer applies and the last boundary condition must be changed. The solution of equation (8) and its boundary conditions can be found in Crank [17] or in Gros and Rüegg [29].

An alternative method, called the "touching semi-infinite cylinders technique", is based on a similar approach [29, 85].

This method consists in bringing into contact two cylinders of the same matrix, each of them having a different initial concentration of the migrating solutes. The concentration profiles are measured from their distance to the interface, as a function of time, along a one-dimensional axis. Crank [17], Gros and Rüegg [29] or Wilde et al. [85] gave the solution of equation (8) and boundary conditions for this unidirectional diffusion from a semi-infinite matrix cylinder, containing an initially uniform concentration of the diffusing substance into a contiguous semi-infinite cylinder initially free of solute or containing lower concentration.

The main drawback of these types of experiments is that they are generally destructive. Thin slicing of the sample gives spatial resolution of about 1 mm. Some studies are less precise with a slice thickness up to 1 cm [74]. Moreover, the measurement in each slice of the solute concentration at different given times of the diffusion process is very time-consuming. This explains why such operations are not extensively repeated. In addition, the thinner the slices, the longer the operation and the higher the number of measurements have to be further performed. Reducing the slice thickness also increases uncertainty on the slice position along the direction of transfer and possibly on concentration measurement (due to less matter) [50]. However, these Fickian approaches based on the concentration profiles of the diffusing solute can be adapted for various small molecules, ionized or not, easy to detect and quantify (water, solutes, colourants and aroma compounds) [15].

Lauverjat et al. [47] recently developed a method, also based on the Fickian approach, for easier and faster determination of diffusion properties of salt in complex matrices. This method, called the solid liquid non-volatile release kinetic method (SL-NVRK), is based on the on-line monitoring of release kinetics of NaCl from a product containing a salt concentration C_s into water. A conductivity probe, immersed in the well-stirred aqueous solution, continuously measured the electrolytes released until thermodynamic equilibrium. The adjustment of a mechanistic model, ensuing from the analysis of mass transfer to the experimental kinetics, led to the determination of the effective diffusion coefficient of NaCl. However, the main limit is the lack of measurement specificity. Indeed, besides NaCl, the cheese-like model matrices contained other solutes such as KCl, calcium, phosphates, citrates and lactates. Because all these species contribute to the conductivity signal and it was not possible to dissociate the respective contribution of each one, two independent diffusion equations for NaCl and for other electrolytes were necessary. The main difficulty was that the model had to be adjusted to experimental conductivity data using two unknown parameters, the effective diffusion coefficients of NaCl and of the other electrolytes. The other drawback is that this method is specific to measuring diffusion properties of ionic species only.

Vestergaard et al. [78] were the first to develop a ^{22}Na-radioisotope non-destructive method for studying NaCl diffusion in meat. Reliable sodium diffusion profiles in meat were obtained by scanning a cylindrical geometry of meat where diffusion of sodium took place from one end to the other end of the cylinder. The use of radioisotopes in the biological and medical sciences is well established. By administering a suitable compound marked with a radioactive tracer it is, for example, possible to locate abnormalities in specific organs. Since the technique was first applied in cancer diagnostics, it has been extensively developed and it is presently known as Single Photon Emission Computerized Tomography.

Despite the disadvantage of the tracer being radioactive and requiring precautions in its handling, Vestergaard et al. [78] concluded that ^{22}Na measurements are a promising methodology for studying salt diffusion

in meat. This method may be transposed to cheese in order to study the diffusion of salt or other solutes where an atom can be radioactively marked.

2.2.3. *Drawbacks of the Fickian approach*

The classical Fickian approach of transport phenomena is difficult to apply to food matrices because of their specific characteristics, structure, properties, etc. In fact, even considering cheese as a food matrix with saline solution occluded in the pores, parameters such as porosity, tortuosity and phase ratios are not sufficient to describe the mass transfer process accurately. Some typical pitfalls with the Fickian approach in foods were reported by Doulia et al. [21]:

- The dependence of D_{eff} on the concentration of the component being transferred. In this case, the driving force for mass transfer is the difference in chemical potential and not the difference in concentration.
- The dependence of D_{eff} on temperature. The application of an Arrhenius-type relation is questionable, in case of sudden changes in the matrix microstructure.
- The dependence of D_{eff} on volume changes occurring during dehydration (shrinkage) or rehydration (swelling). In most cases, the influence of volume changes is ignored and implicitly included in D_{eff} value.
- The evaluation of D_{eff} entails that mass transfer is mainly a molecular diffusion mechanism, whereas several other mechanisms are also often involved, such as capillary or Knudsen diffusion.
- In initial and boundary conditions, the distribution coefficient between the two phases should be taken into account. The latter coefficient is the quotient of the concentrations resulting from the equilibrium experiments and reflects the allegation that the driving force is not the concentration difference. In equilibrium conditions, the distribution coefficient in terms of chemical potential should be equal to 1.

The perverse effect of calculating a D_{eff} (which may be correctly defined as a mass transfer coefficient) from experimental data is then that no effort is made to understand the actual mechanism for mass transfer [1]. In fact, some researchers have correctly noted that it is worthless to calculate diffusion coefficients unless the structure is resolved [26]. It is very probable that the quantification of food microstructure using image analysis will assist in finding the mechanisms and their relative contributions to the transport phenomena, and better modelling [1].

In order to improve modelling of mass transfer phenomena in cheese, several other methods were proposed in the literature, which are reviewed thereafter.

3. MULTICOMPONENT DIFFUSION

3.1. Generalized Fick's model

Zorrilla and Rubiolo [88–90] were the first to develop a model for a multicomponent system (where many components diffuse simultaneously), using the diffusion cell, for determining apparent diffusion coefficients of both NaCl and KCl in cheese during salting and ripening processes.

From a theoretical point of view, mass transport phenomena for a multicomponent system can be physically modelled using three different approaches: (i) the generalization of Fick's law, (ii) the use of irreversible thermodynamics and (iii) the use of Stefan-Maxwell equation. These three approaches are based on kinetic, thermodynamic and hydrodynamic considerations, respectively [12].

The generalized Fick's law is, as indicated by its name, a generalization of Fick's

law initially formulated for binary diffusion [73]. For example, in the case of a ternary mixture, the mass diffusion fluxes J_i^* ($kg{\cdot}m^{-2}{\cdot}s^{-1}$) can be calculated from mass fractions of each species ω_i and mass content of the mixture ρ ($kg{\cdot}m^{-3}$) using

$$\begin{bmatrix} \vec{J}_1^* \\ \vec{J}_2^* \end{bmatrix} = \rho \begin{bmatrix} D_{11} & D_{12} \\ D_{21} & D_{22} \end{bmatrix} \begin{bmatrix} \vec{\nabla}\omega_1 \\ \vec{\nabla}\omega_2 \end{bmatrix}. \quad (15)$$

Note that for the third component (arbitrarily chosen as a reference species)

$$\vec{J}_3^* = \vec{J}_1^* - \vec{J}_2^*. \quad (16)$$

The values of the multicomponent diffusion coefficients D_{ii} (main diffusion coefficients, $m^2{\cdot}s^{-1}$) and D_{ij} (cross diffusion coefficients, $m^2{\cdot}s^{-1}$) depend on (i) the reference velocity chosen to express the diffusion velocity of each species with respect to the bulk flow of the mixture (molar, mass or volume average velocity), (ii) the state variable chosen to describe the composition of the system (molar, mass or volume fraction) and (iii) the arbitrary choice made when designing a reference species. This point considerably restricts the use of multicomponent diffusion coefficients found in the literature since these precisions are often lacking. Note that relationships between these coefficients and the binary values are not known a priori [12].

In Zorrilla and Rubiolo [88–90], the generalized Fick's law form was used as a constitutive equation for the diffusive molar flux of NaCl and KCl during brining and ripening in the cheese. From a physical point of view, using Fick's model is not ideal in that case, but it was used because of its simplicity in the experimental and mathematical works [19]. Generally, for highly dissociable solutes such as NaCl and KCl, the cross diffusion coefficients are smaller than the main ones [25]. Consequently, the main effective diffusion coefficients of NaCl and KCl were much larger ($\sim 4 \times 10^{-10}$ $m^2{\cdot}s^{-1}$) than the cross diffusion coefficients between NaCl and KCl ($\sim 0.1 \times 10^{-10}$ $m^2{\cdot}s^{-1}$) in the semi-hard cheese type. Zorrilla and Rubiolo [88–90] observed that main diffusion coefficients of both NaCl and KCl were very similar because of their chemical similarities.

Gerla and Rubiolo [25] also studied multicomponent mass transport of lactic acid and NaCl in a solid-liquid system through the brining process of Pategras cheese. This was done to predict changes in acid concentration during the salting process. The NaCl diffusion rate was independent from the lactic acid concentration gradient, while the lactic acid diffusion rate increased 12 times due to NaCl concentration changes in the cheese. Therefore, in processes involving the simultaneous diffusion of several solutes, the largest solute gradient can cause the modifications of the diffusion properties of minor solutes. If these solutes are important for ripening, the modifications of their diffusion properties can have consequences on the sensorial properties of the cheese. These results established the importance of using multicomponent mass transport models. However, interactions between protons, Na^+ and Cl^- ions within cheese matrices can be explained by other arguments than the magnitude of their gradients since they can all interact with the proteinic network. In that case, Na^+ and Cl^- probably modify electrical charges of proteins and thus their buffering capacity, which in turn affect lactic acid diffusion properties.

Simal et al. [70] and Bona et al. [9, 10] described a mathematical procedure to obtain the diffusion coefficients of different species (salt and water) that simultaneously diffuse in cheese in such a situation that each mass flux is affected by the existence of the others. The correspondent local mass balances combined with Fick's law were simultaneously solved in one dimension [70] or in three dimensions using a numerical finite

difference method [9, 10]. Indeed, with the development of high-performance computers, it is possible to simulate a process close to reality using three-dimensional geometries and numerical techniques such as the finite element method (FEM) [9, 10]. Water losses and salt gain during brining could be adequately simulated using the proposed model. Although the experimental data of water and salt contents were in good agreement with calculated values, the main drawback of the proposed model was the high number of unknown parameters that had to be numerically identified.

The multicomponent analysis of mass transfer phenomena is an alternative to the classical modelling method presented in the Section 4.2.3. However, it was previously reported that from a physical point of view, the use of Fick's model may give misleading results when the Fickian analysis is applied in a complex system like food products. Indeed, the simplifications imposed on the model may affect its accuracy. Alternative methods described by irreversible thermodynamics and the Stefan-Maxwell theory have then come into force. In these approaches, the driving force is the chemical potential.

3.2. Stefan-Maxwell approach

Payne and Morison [61] developed a Stefan-Maxwell multicomponent approach to model salt and water diffusion in cheese. Stefan-Maxwell's model expresses the chemical gradient of potential like a linear function of the matter flux. A full description of this equation is given by [73]:

$$\frac{x_i}{RT}\left(\frac{\partial \mu_i}{\partial x}\right) = \sum_{j=1}^{n} \frac{x_i x_j}{D_{ij}^{\mathrm{SM}}}\left(v_j - v_i\right), \quad (17)$$

where D_{ij}^{SM} are the Stefan-Maxwell diffusion coefficients between components i and j ($\mathrm{m^2{\cdot}s^{-1}}$), R is the ideal gas constant, 8.31414 $\mathrm{J{\cdot}mol^{-1}{\cdot}K^{-1}}$, T is the temperature (K), x_i, μ_i and v_i are respectively the molar fraction, the molar chemical potential ($\mathrm{J{\cdot}mol^{-1}}$) and velocity relative to stationary coordinates ($\mathrm{m{\cdot}s^{-1}}$), of the component i.

Payne and Morison [61] considered cheese as a three-component system consisting of NaCl (component 1), water (component 2) and a matrix of protein and fat (component 3).

In regard to the Fickian approach, the main advantage of Stefan-Maxwell equation is that no reference species is needed. Secondly, as corrections for thermodynamic non-ideality are included in this analysis, the concentration dependence of Stefan-Maxwell diffusion coefficients is not as strong as that of Fickian diffusion coefficients. In the case of dilute gases, the Stefan-Maxwell diffusion coefficients correspond to the binary values (Fickian diffusion coefficients). However, when applied to concentrated aqueous solutions or food matrices like cheese, the Stefan-Maxwell diffusion coefficients are no longer equal to the binary values.

For Payne and Morison [61], the main difficulties encountered with this model were the determination of water activity and the activity coefficient of salt in cheese. The value of cheese matrix activity was not required because it could be assumed that the diffusional flux of the matrix was insignificant. To solve the model, values for the Stefan-Maxwell diffusion coefficients between salt, water and the cheese matrix were required. However, there are very little data available in the literature for the Stefan-Maxwell diffusion coefficients, and none were found for cheese, salt and water. This does present a number of problems, the most significant being that the accuracy of the model is limited by the accuracy of these values [61]. Stefan-Maxwell diffusion coefficients are mainly determined empirically by doing a large number of assumptions. Payne and Morison [61] fitted experimental data from Geurts et al. [27] and Wesselingh et al. [84] to model

Table II. Literature review of effective diffusion coefficients found for small solutes in different cheese types.

Cheese	Composition dry matter (DM) ($g \cdot kg^{-1}$), fat/DM ($g \cdot 100\ g^{-1}$) and pH	Brining and/or ripening conditions			Geometry	Model	Effective diffusion coefficient (D_{eff}) ($\times 10^{-10}\ m^2 \cdot s^{-1}$)	Refs.
		Process considered	Temperature (°C)	Brine composition				
Solute: NaCl								
Camembert (soft-type cheese)	DM 410 fat/DM 45	Brining and ripening	14	300 $g \cdot kg^{-1}$ NaCl pH 4.6	Slab	Fick (1D)	~ 2.54	[41]
Cuartirolo Argentino (soft-type cheese)	DM 480 fat/DM 51.7	Brining and ripening	7.5	205 $g \cdot kg^{-1}$ NaCl agitated or brine at rest	Finite rigid slab	Fick (1D)	3.6	[51, 52]
Feta	DM 440 fat/DM 43	Dry-salted	13	–	Semi-finite geometry	Fick (1D)	2.3	[87]
White cheese (semi-hard, Turkey)	DM 450 fat/DM 42 pH 5.3	Brining	4, 12.5 and 20	150–200 $g \cdot kg^{-1}$ NaCl	Finite slab	Fick (1D)	2.1, 3 and 4 (no effect of brine concentration)	[74]
White cheese (semi-hard, Turkey)	DM 450 fat/DM 42 pH 5.3	Brining	4–20	150–200 $g \cdot kg^{-1}$ NaCl	Finite slab	Fick (1D)	2.2–4.2	[75]
Prato cheese (semi-hard, Brazil)	DM 517 fat/DM 53 pH 5.2	Brining	10	150, 200 and 250 $g \cdot kg^{-1}$ NaCl	Parallelepiped	Fick (3D) and neural network	1.64, 4.25 and 3	[7]
Romano (hard-type cheese)	DM 535 fat/DM 38	Brining	20	160 $g \cdot kg^{-1}$ NaCl	Slab	Fick (1D)	2.54–3.35	[35]

continued on next page

Table II. Continued.

Cheese	Composition dry matter (DM) (g·kg^{-1}), fat/DM (g·100 g^{-1}) and pH	Brining and/or ripening conditions			Geometry	Model	Effective diffusion coefficient (D_{eff}) (× 10^{-10} m^2·s^{-1})	Refs.
		Process considered	Temperature (°C)	Brine composition				
Sbrinz (hard-type cheese)	DM 650 fat/DM 48	Brining and ripening	Brining at 12 °C (4 days) and diffusion at 7, 11, 15 and 20 °C	200 g·kg^{-1} NaCl	Touching semi-infinite cylinders (after the brining step)	Fick (1D)	1.06 (± 0.15) to 1.88 (± 0.27) (temp. coef.: 0.063 × 10^{-10} m^2·s^{-1}·°C^{-1})	[29]
Cheddar (hard-type cheese)	DM 650	Ripening	10	–	Slab	Fick (1D)	1.16	[86]
Emmental (hard-type cheese)	DM 600 fat/DM 48	Brining	4–18	250 g·kg^{-1} NaCl; 0.3 g·kg^{-1} $CaCl_2$ pH 5.4	Infinite cylinder	Fick (1D)	0.62–2.22	[60]
Model cheese (Gouda style)	DM 580–630 fat/DM ~ 50	Ripening RH 87%	13	–	Slab	Fick (1D)	2.3	[28]
Model cheese (Gouda style)	DM 533, 566 and 638 fat/DM 62, 50, 22 and 12 pH 4.9–5.6	Brining	12.6	130–310 g·kg^{-1} NaCl; 15 g·kg^{-1} $CaCl_2$	Flat cylindrical shape	Fick (1D)	~ 2.3 1.16–3.24 (temp. coef.: 0.12 × 10^{-10} m^2·s^{-1}·°C^{-1})	[26]
Model cheese	DM 370 and 440 fat/DM 20 and 40 pH 6.2 and 6.5 0.5 and 1.5 g·100 g^{-1} NaCl	Release of NaCl from the cheese into water	13	Water	Infinite cylinder	Fick (1D)	2.74–5.1 (± 0.01)	[46]
			15	Artificial saliva		Fick (1D)	2.81–3.43	[23]

continued on next page

Table II. Continued.

Cheese	Composition dry matter (DM) ($g \cdot kg^{-1}$), fat/DM ($g \cdot 100\ g^{-1}$) and pH	Brining and/or ripening conditions			Geometry	Model	Effective diffusion coefficient (D_{eff}) ($\times 10^{-10}\ m^2 \cdot s^{-1}$)	Refs.
		Process considered	Temperature (°C)	Brine composition				
Solute: water								
White cheese (semi-hard, Turkey)	DM 450 fat/DM 42 pH 5.3	Brining	4, 12.5 and 20	150–200 $g \cdot kg^{-1}$ NaCl	Finite slab	Fick (1D)	15% brine: 1.96–3.64; 20% brine: 1.69–3.09	[76]
Solutes: NaCl and water								
Fresh cheese Pasteurized cow and goat milk	No data	Brining	5, 15 and 20	280 $g \cdot L^{-1}$ NaCl; 15 $g \cdot L^{-1}$ $CaCl_2$	Cylinder and parallelepiped	Fick (1D)	Water: 5.71, 8.83 and 9.99; NaCl: 3.56, 8.26 and 9.17	[70]
Mahon cheese (soft-type cheese, Spain)	DM 244	Ripening RH 85%	12	280 $g \cdot L^{-1}$ NaCl; 15 $g \cdot L^{-1}$ $CaCl_2$	Parallelepiped	Fick (3D)	Water: 0.078; NaCl: 5.3	[71]
Gouda (semi-hard cheese)	DM 565 fat/DM 53	Brining	20	170 $g \cdot kg^{-1}$ NaCl	Slab	Maxwell-Stefan (1D)	$D^{SM}_{salt-cheese}$ = 0.0027 – 0.014 from the core to the edge of the cheese	[61]
Solutes: NaCl and KCl								
Fynbo cheese (semi-hard, Turkey)	DM 470 fat/DM 29.6–36.2	Brining	12	100 $g \cdot L^{-1}$ NaCl; 100 $g \cdot L^{-1}$ KCl; 15 $g \cdot L^{-1}$ $CaCl_2$	Diffusion cell	Fick (1D)	NaCl: 4.14; KCl: 3.91	[89]
Prato cheese (semi-hard, Brazil)	DM 540 fat/DM 52.8	Brining	10	146 $g \cdot L^{-1}$ NaCl; 50.6 $g \cdot L^{-1}$ KCl; 5 $g \cdot L^{-1}$ $CaCl_2$	Parallelepiped	Fick (1D)	NaCl: 2.6; KCl: 2.77	[8]

continued on next page

Table II. Continued.

Cheese	Composition dry matter (DM) ($g \cdot kg^{-1}$), fat/DM ($g \cdot 100\ g^{-1}$) and pH	Brining and/or ripening conditions			Geometry	Model	Effective diffusion coefficient (D_{eff}) ($\times 10^{-10}\ m^2 \cdot s^{-1}$)	Refs.
		Process considered	Temperature (°C)	Brine composition				
Prato cheese (semi-hard, Brazil)	DM 540 fat/DM 52.8	Brining	10	146 $g \cdot L^{-1}$ NaCl; 50.6 $g \cdot L^{-1}$ KCl; 5 $g \cdot L^{-1}$ $CaCl_2$	Parallelepiped	Fick (3D)	NaCl: 2.8; KCl: 2.94	[10]
Other solutes								
Lactose in small curd cottage cheese	No available information	Washing	25	Demineralized water pH 4.5 (H_3PO_4)	Sphere	Fick (1D)	3.8	[11]
Lactose in Skimmed Quark cheese (Soft-type cheese, Germany)	No available information	–	4	–	Touching semi-infinite cylinders	Fick (1D)	1.37 (± 0.13)	[85]
Sucrose in milk	Fat 15 $g \cdot kg^{-1}$	Contact with 15 $g \cdot 100\ g^{-1}$ agar gel	20–24 (room temperature)	–	Touching semi-infinite cylinders	Fick (1D)	*Initial gel sucrose concentration* C_{s0} 787 $g \cdot L^{-1}$: 1.9, C_{s0} 515 $g \cdot L^{-1}$: 2.6, C_{s0} 279 $g \cdot L^{-1}$: 3.9	[81]
Lactic acid and NaCl in Pategras	DM 544 fat/DM 43 Lactic acid 13 $g \cdot kg^{-1}$	Ripening RH 90%	13	200 $g \cdot kg^{-1}$ NaCl; 5 $g \cdot kg^{-1}$ $CaCl_2$	Finite slab	Fick (1D) multicomponent diffusion	NaCl: 3.2 lactic acid: ~ 1	[25]

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Table II. Continued.

Cheese	Composition dry matter (DM) ($g \cdot kg^{-1}$), fat/DM ($g \cdot 100\ g^{-1}$) and pH	Brining and/or ripening conditions			Geometry	Model	Effective diffusion coefficient (D_{eff}) ($\times 10^{-10}\ m^2 \cdot s^{-1}$)	Refs.
		Process considered	Temperature (°C)	Brine composition				
Potassium sorbate in American processed cheese	DM 600 fat/DM 45	Brining	Room temperature	250 $g \cdot L^{-1}$ potassium sorbate solutions	Cubes (finite slab)	Fick (1D)	1.31	[40]
Potassium sorbate in Mozzarella	DM ~ 500 fat/DM 45						0.674	
Aroma compounds in model cheese: diacetyl, heptan-2-one, and ethyl hexanoate	DM 370 fat/DM 20 and 40 pH 6.2 1.5 $g \cdot 100\ g^{-1}$ NaCl	Release of aroma compounds in the air	13	–	VASK	Fick (1D)	Diacetyl: 0.04; heptan-2-one: 0.2–0.12; ethyl hexanoate: 0.18–0.07	[47]

the Stefan-Maxwell diffusion coefficients. The model successfully predicted independent shrinkage arising from an excess of outgoing diffusion of water over the incoming diffusion of salt. Their model also indicated that there was a large interaction between salt and the cheese matrix, which caused a significant reduction in the diffusion of salt into cheese. Further work is required to interpret the Stefan-Maxwell diffusion coefficients from a physical point of view.

4. CHARACTERISTIC VALUES OF EFFECTIVE DIFFUSION COEFFICIENTS IN CHEESE

Extensive data on diffusion coefficients in cheese are available in the literature, but cover a large range of values. This is undoubtedly due to the complexity and diversity in cheese structure and composition. This variability depends on the cheese type and origin, as well as on various methods of determination which are not always fully explicit, nor justified [50].

4.1. Salt and moisture transfer

Most of the published studies concerning mass transfer phenomena during cheese production deal with the salting and ripening processes. After moulding, cheese is placed in brine and a net movement of Na^+ and Cl^- ions, from the brine into the cheese, results from the osmotic pressure difference between the cheese moisture and the brine. Consequently, moisture diffuses throughout the cheese matrix to restore osmotic pressure equilibrium [34]. The amount of salt retained and water removed from the cheese depend, mostly, on brine concentration and brining time [32]. Salt diffusive migration in cheese usually occurs slowly. For example, salt equilibration times for cheese range from about 1–2 weeks in soft cheese to several months in semi-hard cheese type. In Parmesan cheese, which represents an extreme case, salt equilibrium is only attained after about 10 months [64]. For the controlled manufacture of these products, it is therefore important to know the factors influencing salt penetration and to be able to predict the diffusion rates. This implies the knowledge of the apparent diffusion coefficient of salt and its dependence on factors such as temperature and brine concentration.

Water and NaCl diffusion transport processes in and out of the cheese matrix during classical brining and ripening are most of the time described using the second Fick's law, considering the diffusion coefficient as constant. This diffusion coefficient represents the NaCl effective diffusion coefficient when considering the cheese matrix and NaCl as the two components of the binary diffusion system [52]. For NaCl, the effective diffusion coefficient D_{eff} varies from $1–5.5 \times 10^{-10}$ $m^2 \cdot s^{-1}$ depending on cheese, compared to 1.16×10^{-9} $m^2 \cdot s^{-1}$ for the diffusion coefficient of NaCl in pure water at temperatures around 12.5 °C (Tab. II). Temperature has a strong effect on the effective diffusion coefficient of NaCl in some cheese types, which can increase up to 9.2×10^{-10} $m^2 \cdot s^{-1}$ at 20 °C during the brining of Fresh cheese [70].

This increase was attributed by Geurts et al. [27] to an increase in true diffusion and to some effect on diffusion-interfering factors. For them, the temperature increase could lead to a possible decrease in the viscosity of the cheese moisture fraction and to a modification of the amount of protein-bound water, which could result in an increase of the relative pore width of the protein matrix. The acceleration of the mass transfer rate with the temperature is not so important in semi-hard and hard-type cheeses, with effective diffusion coefficients up to $2–4 \times 10^{-10}$ $m^2 \cdot s^{-1}$ at 20 °C in cheese like Romano [35], White cheese [75], Sbrinz [29] or Emmental [60]. Indeed, moisture content is much inferior in

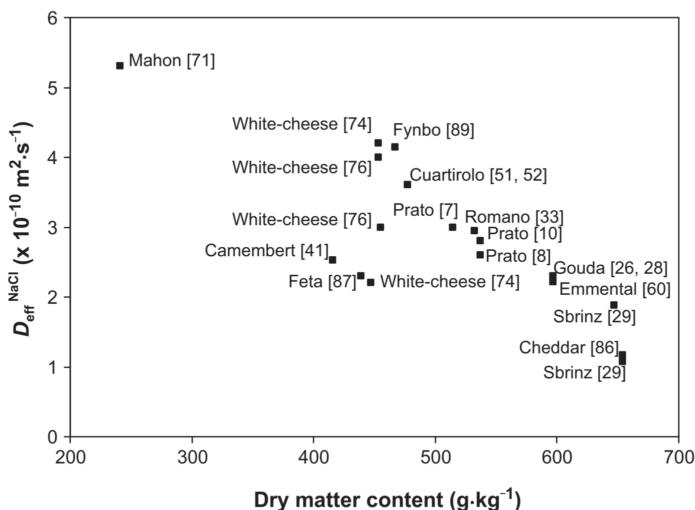

Figure 2. Effective diffusion coefficient of salt versus dry matter content in different cheese types.

semi-hard and hard-type cheeses than in soft- or fresh-type cheeses. Diffusion-interfering effects, which mainly depend on water and protein-bound contents, are then probably much less marked in hard-type cheeses than in soft-type cheeses when the temperature increases.

The factors affecting the rate of salt diffusion in cheese during salting have already been investigated in detail by Geurts et al. [27], Guinee [31, 32] and Guinee and Fox [33–37]. These factors are (i) the concentration gradient across the different zones of cheese, which has a major effect on the level of salt absorption by a cheese during salting, but scarcely affects the rate of salt diffusion; (ii) the ripening temperature and (iii) the cheese composition (fat, protein and moisture). It is difficult to establish the individual effect of each component on the salt diffusion rate because strong interactions exist between them, depending on the cheese microstructure. Data on NaCl effective diffusion coefficients reported on Table II were subjected to statistical analysis by the multiple linear regression (MLR) procedure in Excel®. MLR analysis provides an equation that can be used to predict D_{eff} of salt in cheese matrices, function of parameters such as composition (dry matter (DM) and fat on dry matter ratio (Fat/DM)), temperature (T) and brine composition if available. Each parameter was first centred and reduced to minimize the impact of data order of magnitude. The best equation obtained for D_{eff} of salt was

$$D_{eff} = 3.39 - 1.25 \times \mathrm{DM} + 0.24 \times \mathrm{fat}/\mathrm{DM} - 0.14 \times T. \qquad (18)$$

A highly significant ($P < 0.001$) coefficient of multiple determination (R^2) of 0.75 for this model indicated that D_{eff} can be estimated using these parameters. Fat/DM, T and brine composition parameters were not significant ($P < 0.1$). DM was the only significant parameter ($P < 0.001$), meaning that effective diffusion coefficients of salt solutes can be accurately predicted in cheese matrices knowing their dry matter composition (Fig. 2).

Floury et al. [23] and Lauverjat [46] were first to study the release of salt in the mouth during food chewing according to

the composition of model cheese matrices. The release of salt from the cheese into artificial saliva was mathematically modelled as an effective diffusion process with Fick's second law. The variation in the effective diffusion coefficient of salt according to the cheese matrix compositions was linked to their structural and textural properties. Effective diffusion coefficients were included between 2.7 and 5.1×10^{-10} $m^2 \cdot s^{-1}$ at 13–15 °C depending on the matrix composition (Tab. II). These values were of the same order of magnitude as published diffusion coefficients that were measured during the brining of real cheeses of same dry matter and fat content (Fig. 2).

Table II shows that literature on water diffusion in cheese during brining and ripening is not so abundant. Effective moisture diffusion coefficients in cheese have been reported by Luna and Chavez [53] for Gouda cheese, Turhan and Gunasekaran [75] for White cheese and Simal et al. [69, 70] for Mahon and Fresh cheeses. During the salting of cheese in brine, salt and moisture gradients develop from the surface to the core [53]. The ripening process implies water losses due to dehydration of the cheese and salt migration to achieve an almost uniform salt distribution, which is an important factor in cheese ripening [90]. Notice that the values of effective diffusion coefficients of water considerably vary depending on cheese type, and more particularly on the experimental method that was employed to model moisture transfer (Tab. II). It is then difficult to link those values to cheese composition.

During the brining and ripening of cheese, not only is the water content in cheese reduced and the salt concentration increased but, for example, the lactic acid concentration is also modified. Detection of lactic acid in the brine proves that this solute is able to diffuse from the cheese into the brine [48]. Other solutes than salt and water, like lactic acid or small peptides for example, are of crucial importance for the final quality of the cheese and its preservation. However, diffusion properties of those components were almost not modelled. In the following paragraphs, we give a complete review of the mass transfer properties of these other small solutes in cheese matrices, like lactose, additives and metabolites.

4.2. Transfer of other solutes

Publications concerning the diffusion of small solutes in cheese matrices, except from salt and moisture, are scarce (Tab. II). They deal with the diffusion of whey components such as lactose or sucrose [11, 81, 85], lactic acid [25] and potassium sorbate [40]. One recent study also deals with the diffusion properties of aroma compounds in model cheese matrices of different compositions [47]. Only one research team has published results about mass transfer phenomena of metabolites resulting from biological activities in cheese during brining or ripening [2–4, 72].

4.2.1. Transfer of whey components

Warin et al. [81] modelled the effective diffusion coefficient of sugar in agar gel/milk bilayer system in order to mimic the sucrose and lactose transfer between a dairy product and a fruit layer. The system was modelled with a liquid milk phase on the top of a gel containing agar, citric acid and different concentrations of sucrose. Average disaccharide concentrations at different locations were determined for the system after different diffusion times. Average disaccharide concentrations in each slice of agar gel were deduced from total solids after subtracting agar content and from total solids after subtracting protein and fat contents in the milk phase. Experimental data were fitted to Fick's second law with separate effective diffusion coefficients of sugar in the milk and in the agar gel phases. As sucrose and lactose have the same molecular weight and a similar structure, the authors made the hypothesis that their diffusion properties

were identical. Experimental values of effective diffusion coefficients in milk and agar gel obtained at room temperature (22 °C) were compared to a correlation reported by Hallström et al. [39] for sucrose diffusivity concentration dependence in aqueous solution at the same temperature:

$$\log D_s = -8.271 - 9.2x_s, \quad (19)$$

with D_s the effective diffusion coefficient of lactose and sucrose ($m^2 \cdot s^{-1}$) and x_s the mole fraction of sucrose. For Warin et al. [81], as the effective diffusivity of sucrose in the agar gel and milk phases could be estimated using a correlation usually employed for the calculation of diffusion coefficients in aqueous solutions, there was neither exclusion effect due to the porosity of the agar phase, nor obstruction effect due to tortuosity of the gel, on the disaccharide diffusion properties. This confirms results showing an effective diffusion coefficient of sucrose in 1.5% agar membranes identical to that in water [49]. With regard to the milk phase, similarly, they concluded that there were no exclusion or obstruction effects of milk proteins on the effective diffusion coefficient of disaccharide solutes.

This study led to interesting results with regard to mass transfer properties of sugar in liquid and low-concentrated matrices. However, it gave no information on effective coefficients of such solutes in structured solid matrices like cheeses.

Bressan et al. [11] modelled the diffusion of whey components (rich in lactose) from small curd cottage cheese particles during their washing process. They considered the diffusion of solutes as isothermal (25 °C) in a porous network with several refinements to account for the whey on curd surfaces. Three geometrical approximations (slab, cube and sphere) for small curd cottage cheese particles were examined using Fick's second law. It was assumed that there was no chemical reaction in the system and no convective mass transfer in the pores. The term "whey components" was used by the authors to take solutes from low molecular salts to whey proteins into account in the model. One solution to the problem of presenting all solids in a single pseudocomponent was to use a lumped parameter model [6]. The model also included a correction for the whey introduced into the washing system on the surface of the curd or entrained among cheese particles.

Bressan et al. [11] concluded that diffusion from a spherical cheese particle considering whey entrained in curd interstices by capillary forces was an acceptable basis for a mass transfer model. According to them, the model yielded to an effective diffusion coefficient of expected magnitude for lactose, i.e. 3–4 × 10^{-10} $m^2 \cdot s^{-1}$ at 25 °C (Tab. II). The diffusion coefficient of lactose at infinite dilution in water at 25 °C is 5.2 × 10^{-10} $m^2 \cdot s^{-1}$ [54]. The effective lactose diffusion coefficient in the cheese is smaller than the value for infinitely diluted solution, mainly due to the sterical hindrance to the random movement of lactose by the cheese matrix.

Wilde et al. [85] have also studied matrix effects on the diffusion rates of lactose in a soft-type cheese (Quark cheese) and several milk acid gels of different dry matter contents. A two-chamber diffusion tube was used to determine the effective diffusion coefficient of lactose. The product enriched with lactose was introduced into one of the two cylinders and the product with the original lactose content into the other to ensure the concentration difference required for diffusion. The concentration of the diffusing lactose was measured in each slice of 1 mm thickness using both a high pressure liquid chromatography (HPLC) analysis and enzymatic test kits. The model of one-dimensional infinite media with a constant cross-section based on Fick's second law of diffusion for time-dependent diffusion process was verified with regard to the effective diffusion coefficient of lactose

in viscous milk products. The effective diffusion coefficient D_{eff} obtained from lactose concentration profiles at 4 °C in skimmed Quark cheese (dry matter 180 $g \cdot kg^{-1}$) was $1.37 \pm 0.13 \times 10^{-10}$ $m^2 \cdot s^{-1}$. In the milk acid gels, D_{eff} showed a linear decline from 1.7 to 0.3 $\times 10^{-10}$ $m^2 \cdot s^{-1}$ as the dry matter of the product increased from 110 to 210 $g \cdot kg^{-1}$. The effective lactose diffusion coefficient in skimmed Quark cheese was higher than the value observed in milk acid gels with the same dry matter content (180 $g \cdot kg^{-1}$). Indeed, Quark cheese is a suspension of coagulated casein particles that are dispersed in a milk whey phase. Lactose diffusion may then mainly take place in the liquid whey phase. Pure diffusion of lactose molecules here is probably slowed down by the dispersed casein particles. Indeed, the structure of milk acid gels gets built up directly in the chamber, resulting in a homogeneous protein network that causes a higher diffusion resistance for lactose molecules. For Wilde et al. [85], the slope of the straight line could characterize the matrix resistance to lactose diffusion.

Although these studies revealed interesting results on the diffusion properties of lactose in dairy matrices, we are still quite far from the microstructure of traditional cheeses from soft- to hard-type cheeses for which dry matter contents are superior to 350 $g \cdot kg^{-1}$. We could not find any published studies concerning lactose diffusion in such solid matrices.

4.2.2. *Transfer of food additives*

Potassium sorbate is widely used in processed cheese as a natural preservative. Effective diffusion coefficient of potassium sorbate in American processed and Mozzarella cheeses was determined by Han and Floros [40]. American processed cheese is an emulsion of ingredients such as milk, whey, milk fat, milk protein concentrate, whey protein concentrate and salt, which does not meet the legal definition of cheese itself. American processed cheese and Mozzarella cheeses had a maximum moisture of 400 and 480–510 $g \cdot kg^{-1}$ and a minimum milk fat of 270 and 39–42 $g \cdot kg^{-1}$. To determine the effective diffusion coefficient D_{eff}, the concentration of potassium sorbate in sliced cheese was measured as a function of the distance from the cheese surface. D_{eff} was calculated by non-linear regression with experimental data based on Fick's second law. D_{eff} of potassium sorbate through American processed cheese was 1.31×10^{-10} $m^2 \cdot s^{-1}$ and for Mozzarella cheese 6.74×10^{-11} $m^2 \cdot s^{-1}$. American processed cheese, because of a higher ratio of moisture-to-fat than the one of Mozzarella cheese (Tab. II), enables the fastest diffusion of water-soluble components. For Han and Floros [40], knowledge of the effective diffusion coefficient of potassium sorbate allows one to accurately estimate the concentration of this preservative agent inside and at the surface, function of time. It will then be possible to predict the preservation time of the product, which corresponds to a residual concentration of potassium sorbate above the critical fungistatic level inside and at the surface of the product [40].

4.2.3. *Transfer of aroma compounds*

Lauverjat et al. [47] estimated the effective diffusion coefficients of three aroma compounds (diacetyl, heptan-2-one and ethyl hexanoate) in model cheese differing by their composition (Tab. II). They tested two experimental methods: the classical diffusion cell method and the volatile air stripping kinetic (VASK) method. The VASK method is based on the measurement of the aroma compound's gaseous concentration above a layer of product when a gaseous flow rate is applied. Aroma compound's concentration is then measured in-line using a high sensitivity proton transfer reaction-mass spectrometer. This method is much faster than the classical diffusion cell method,

but it is dedicated to the volatile compounds released from the product. Comparing the values obtained for two model cheeses differing by their fat on dry matter ratios, the known effect of fat content on aroma mobility was mainly observed for the two hydrophobic compounds (heptan-2-one and ethyl hexanoate). When the fat on dry matter content increased from 20% to 40%, the effective diffusion coefficients showed a 45% decrease for heptan-2-one and a 60% decrease for ethyl hexanoate (Tab. II).

4.2.4. Transfer of metabolites

Aldarf et al. [2], Stephan et al. [71], Aldarf et al. [3] and Amrane et al. [4] modelled – independently – the diffusion of lactate, glutamate and ammonium in relation either to the growth of *Geotrichum candidum* or to the growth of *Penicillium camembertii* at the surface of model matrix (agarose) simulating Camembert cheese. The main purpose of these papers was to study the mechanisms of diffusion and to propose a theoretical approach that could be subsequently applied to curd during ripening for its monitoring and control. The assimilation of lactic acid by *G. candidum* (and *P. camembertii*) growing at the surface of the curd induced a concentration gradient, which results in the diffusion of this metabolite from the core to the rind. In a similar way, ammonium production at the surface of the curd induced a diffusion of this metabolite from the rind to the core. These diffusion mechanisms appeared therefore as the main factors in soft cheese ripening.

These authors developed a diffusion/reaction model in which the diffusion of lactic acid from the bottom of the gel to the upper surface, or that of glutamate and ammonium from the upper surface to the bottom of the gel, is induced by their respective consumption and production at the surface of the gel due to fungal growth. Growth kinetics were described using the widespread Verlhust model [58], and both substrate consumption and ammonium production were considered to be linked to growth. The experimental diffusion gradients of substrates (lactate and glutamate) and ammonium recorded during *G. candidum* growth were fitted to the Fick's second law using Crank's solution [17]. Effective diffusion coefficients were deduced from the experimental concentration gradients. Values of $4.63 \pm 0.34 \times 10^{-10}$ $m^2 \cdot s^{-1}$ for lactate, 6.48×10^{-10} $m^2 \cdot s^{-1}$ for glutamate and $9.26 \pm 0.58 \times 10^{-10}$ $m^2 \cdot s^{-1}$ for ammonium were found, regardless of the pH of the experiment. For lactate and ammonium components, the effective diffusion coefficients found in 2% agarose were, respectively, 57% and 64% of their value in pure water.

This result clearly showed that agarose gel slowed down the diffusion rates of lactate and ammonium components. The diffusion/reaction model fitted with the experimental data until the end of growth, except with regard to ammonium concentration gradients during *G. candidum* growth on peptone-lactate-based medium. Of course, the diffusion/reaction model has to be considered as a preliminary step, which has to be followed by a similar work on real dairy model media, more precisely a lactic curd, in order to better understand the mechanism of curd neutralization, responsible for the development of texture.

5. ALTERNATIVE METHODS APPLICABLE TO CHEESE

Concentration profiles can also be considered on a microscopic scale using a representative molecule, or probe molecule, which can be easily characterized using a specific technique [15]. Recent advances in non-invasive, continuous techniques of measurement, e.g. magnetic resonance imaging (MRI), NMR or FRAP, now allow the use of higher space and time resolutions (Tab. I). Indeed, using radioactively labelled or fluorescent molecules, it is possible to measure the rate

of diffusion of one component in a multicomponent system. What is involved is an interchange of labelled and unlabelled molecules, while the total amount of that molecule, labelled and unlabelled, is constant throughout the system [15]. The transport of molecules is essentially caused by intermolecular collisions (Brownian motions). As a consequence, no mass flow occurs and a diffusion coefficient called "self-diffusion coefficient" is measured [18].

5.1. Nuclear magnetic resonance

The pulsed field gradient NMR (PFG-NMR) technique is a powerful tool that can be used to measure polymer self-diffusion coefficients in suspensions and gels. It is a non-destructive and non-invasive way to measure the self-diffusion coefficient of small molecules by detecting the proton mobility [16]. In a PFG-NMR experiment, the observation time can vary from few milliseconds up to several seconds. Depending on the observation time, the magnitude of the diffusion coefficients obtained at different observation scales enables one to discriminate the different transport mechanisms. For example, if the self-diffusion is independent of the observation time for a porous system, then the system exhibits no restriction to diffusion.

In 1983, Callaghan et al. [13] compared water self-diffusion in Cheddar and Swiss-type cheeses. Their results have shown that water molecules were not confined in water droplets, but had the freedom to move over distances much longer than the fat droplet sizes. The magnitude of the diffusion coefficients was consistent with a migration along the surface of the protein chains. According to Mariette et al. [55], water diffusion in casein systems can be explained by two diffusion pathways: one around and the other through the casein micelles. The obstruction effect on water diffusion was related to local restrictions at the casein micelle surface and explained the absence of any effect of casein gelation by rennet. Moreover, Metais et al. [56] showed that the water self-diffusion coefficients in casein matrices could not be simply explained by the water content only. When caseins, fat globules and soluble fractions are mixed in order to obtain cheese models, the effect of each constituent should be determined to accurately explain the water self-diffusion. They also showed that the two obstruction effects, relative to fat globules and casein micelles, seemed to be independent. This result was in agreement with the observation of Geurts et al. [27], despite the fact that the measurement methods and the diffusing molecules considered were different.

Colsenet et al. [16] used PFG-NMR spectroscopy to study the diffusion of molecular probes (polyethylene glycols (PEG)) in casein suspensions and casein gels, in order to determine the effects of probe molecular size, casein concentrations and rennet coagulation. A more complex behaviour was observed for PEG molecules than for water. First of all, a strong dependency of diffusion on probe size was observed, both in casein suspensions and in casein gels: as the PEG size increased, the self-diffusion coefficient was reduced. This effect was more pronounced for high casein concentrations than for low casein concentrations: the larger the PEG size, the greater the obstruction to diffusion. Second, the formation of a rennet gel resulted in an enhanced self-diffusion coefficient for the largest probes.

The main drawback of this technique is the high cost of the material. Its main difficulty for the scientists is to establish the physical link between this self-diffusion coefficient measured by PFG-NMR and the values of the effective diffusion coefficient estimated in complex matrices with more classical methods. Moreover, it is restricted to the study of mass transfer phenomena of solutes which present spectral properties easily discernable from spectral data of the matrix components. The application of this

technique to solutes like small peptides or proteins naturally present in cheese is thus hardly possible.

5.2. Magnetic resonance imaging

Other promising non-destructive approach to measure diffusion properties of salt and water in food products is MRI. ^{23}Na-MRI is based on the paramagnetic properties of the naturally occurring ^{23}Na isotope, which makes it detectable in strong magnetic fields [79]. Within the past decade, ^{23}Na-MRI has proved to be a reliable method for quantitative and qualitative assessment of salt in various foods such as fermented soy paste (Miso), pickled cucumbers and plum seeds [42], snow crab [59] and pork meat [30, 63]. Besides being non-destructive, this method has the advantage of being easily supplemented by other relevant measurements such as sodium profiles and diffusion-weighted imaging, simply by changing the acquisition parameters. Diffusion-weighted imaging allows the visualization of changes in microscopic water molecule motion (Brownian motion) and quantitative measures of diffusion properties of water in food structures like muscle tissues [79]. For Vestergaard et al. [78], the ^{23}Na-MRI methodology is still under intense investigation around the world because the problem of sodium being partly "invisible" (a certain percentage of the Na^+ is not detected) has not been solved yet.

MRI has also been used to visualize water distribution in one, two or three directions during the drying, rehydration, freezing and thawing of various fruits and vegetables [65, 66]. Indeed, loss of proton mobility during phase transitions results in a decrease in signal intensity. Kuo et al. [45] applied this technique to study the formation of ice during freezing of pasta filata and non-pasta filata Mozzarella cheeses, the spatial redistribution of water T_2 relaxation time and the changes of water self-diffusion coefficient within unfrozen and frozen-stored cheese samples. Images of water spin number density and water T_2 relaxation time were obtained using spin-echo imaging pulse sequence. The water self-diffusion coefficient was measured by PFG spin-echo technique. They measured a significant change in T_2 and D values of water following freezing-thawing. The D values of the frozen-stored pasta filata Mozzarella cheese samples were higher than those for the unfrozen samples. Such a difference was not observed for the non-pasta filata Mozzarella cheese samples. These results were attributed to the microstructure differences between the two cheeses.

Despite the advantage of being a very precise non-destructive analytical technique, MRI presents some inherent difficulties, like a complex calibration and data handling work, errors in the determination of the physical boundaries and possible low signal-to-noise ratios [24]. Moreover, the conventional MRI techniques are typically designed for component with high molecular mobility, for which the water T_2 relaxation times are rather long (> ms). Such techniques are then insensitive to molecules with low mobility, for which the transverse relaxation times are very short (< ms). Therefore, limitations of conventional MRI have hampered its application to a major class of food systems, i.e., where mobility of water is restricted because of its strong association with the matrix [62].

5.3. Fluorescence recovery after photobleaching

Within the last 30 years, FRAP has become an important and versatile technique to study the dynamics in various systems, such as living cells, membranes and other biological environments [14]. In polymer physics, the photobleaching methods are employed to investigate diffusion in macromolecular systems, particularly in networks. Although the technique is relatively old, its application to study endogenous

intracellular proteins in living cells is relatively recent [14]. A review of the fundamentals of FRAP and several examples of its applications is given by Meyvis et al. [57]. Its principle is to irreversibly photobleach a certain region within a fluorescently labelled sample by irradiation with a short intense light pulse. Immediately after bleaching, a highly attenuated light beam is used to measure the recovery of fluorescence inside the bleached area as a result of diffusional exchange of bleached fluorophores by unbleached molecules from the surroundings. The analysis of this process yields information about the diffusion coefficient and the fraction of mobile species.

In a common FRAP experiment, only the rate of recovery of the fluorescence intensity within some preselected area is measured. Performing the experiment in a confocal laser scanning microscope (CLSM) reveals the same information with high spatial resolution [68]. To measure the mobility of a fluorescent molecule such as green fluorescent protein, images of the fluorescently labelled cell are collected over time, while the fluorescent and photobleached molecules redistribute until equilibrium is reached. By plotting the relationship between fluorescence intensity and time, the mobility of the fluorescent proteins can be directly measured [14]. The most commonly used approach to describe the mobility of molecules during FRAP experiments is to assume the spatiotemporal dynamics of these molecules to be diffusive in nature. Under this assumption, the kinetic parameter that measures the rate of movement is the effective diffusion coefficient, determined with Fick's diffusion model. This microscopic, non-destructive and slightly invasive technique, in which the probe concentration remains micromolar, originates from mobility studies in biological membranes [5]. It was then extended to other fields, mostly for liquid or highly hydrated systems, in which diffusion follows the Stokes-Einstein law [44]. It covers a wide range of apparent diffusion coefficients, from 10^{-20} to 10^{-9} $m^2 \cdot s^{-1}$ [43].

In spite of its interest and its simplicity to be implemented, the FRAP technique has not been used yet for the determination of solute diffusion coefficients in dairy matrices. Indeed, to be able to use this method, the migrating molecule has to be fluorescent or labelled with a fluorescent probe. This is not the case of small solutes such as NaCl or water. For bigger molecules, it is necessary to find a fluorescent probe with a great affinity for the diffusing solute to be labelled or with similar size and physicochemical properties in order to simulate the targeted molecule. Moreover, this method seems difficult to adapt to complex and opaque matrices like cheese.

6. CONCLUSION

Mass transfer of solutes in cheese is essential for the ripening process and the final quality of the cheese. Numerous studies have been reported on the transfer of salt in different cheese types during the brining and ripening processes. Some of them also take the simultaneous counterflow of water into account, even if modelling moisture transfer seemed to be more complicated. Effective diffusion coefficients of salt and moisture in different cheese types and compositions have been reported in this review. Regardless of the cheese origin, its type (soft, semi-hard or hard) and its composition (dry matter, fat and pH), the effective diffusion coefficients of salt ranged between 1 and 5.3×10^{-10} $m^2 \cdot s^{-1}$ at around 10–15 °C. A significant linear relationship between dry matter content of the matrix and effective diffusion coefficient of salt was statistically observed. However, these values should be considered cautiously because their comparison is difficult. Indeed, there are very large discrepancies of approaches used to determine solute mass transfer properties and of

the experimental conditions employed. For example, if diffusion properties are obtained using the concentration profile method with an invasive method to follow the migrating molecule concentration, spatial resolution is generally quite low and the results are not precise enough.

Very few papers are dealing with the mass transfer properties of other small solutes in cheese. However, modelling the effective diffusion coefficient of cheese minor components, such as lactose and biological metabolites, substrates and products of the enzymatic activity of immobilized colonies, seems essential for the control and the optimization of cheese ripening. Indeed, migration rates of those solutes are probably the limiting step during the ripening stage. The knowledge of the migration rates appears to be essential for the full understanding of cheese ripening.

Alternative methods considered as non-destructive, such as MRI, NMR or FRAP techniques, are currently developed to measure the self-diffusion coefficient of solutes in heterogeneous matrices. Thanks to their high space resolution, these techniques make it possible to obtain concentration profiles of the migrating solute with a good precision and to avoid problems due to sample variability. However, they are still difficult to apply to complex and heterogeneous media like cheese (Tab. I). Further research is necessary to adapt those promising methods to the determination of mass transfer properties of a wide variety of small solutes in complex heterogeneous matrices like cheese or other real food media.

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1. Diffusion of solutes in cheese

1.1. State of the art (Review: Floury *et al.,* 2010a)

1.2. Solutes diffusion and cheese macro- and microstructure

The review in the previous paragraph summarizes the methods available and used to assess solutes diffusion in dairy products, issued from varied technologies (soft cheeses, semi hard, etc…). By this way, some diffusion coefficients were provided in the literature. However, it is not known to which extent and how the composition and molecular organization of the cheese components within the matrix, i.e. the structure of the network, can affect the diffusion process of small solutes. Floury *et al.,* 2010a showed that a linear relationship was statistically proved between the dry matter and the effective diffusion coefficients of salt in dairy products. An important role of structure and texture on salt mobility and perception was found in a model dairy product (Lauverjat *et al*., 2009; Panouillé *et al*., 2011). Roca *et al.,* (2008) outlines the significant impact of assumptions made for moisture transport predictive models for coefficient diffusion (D) determination depending on the structure and hygroscopicity of food products.

Many properties of cheese such as cheese texture and flavour are determined by spatial arrangement of components including: the casein particles that form a protein matrix, the fat globules, dispersed water and minerals (Everett & Auty, 2008). The arrangement of these components on the micron scale in known as the microstructure of cheese (Ong *et al.,* 2010). In general, composition gives only limited information regarding the physical state, structure (micro and macrostructure) or functional properties of foods (Aguilera, 2005).

Rheological properties of cheese are of considerable importance since they affect many aspects in cheese quality including its texture and eating quality. It can reflect the presence of heterogeneities such as curd granule junctions, cracks, and fissures (Fox *et al.,* 2000) and can used to assess cheese macrostructure.

Quite few studies dealt with the relationship between food structure and solutes diffusion.

Because of their homogeneous and uniform composition and structure and well known physical and chemical properties, gelatin provide a suitable food ingredient to modify food texture or even it could be utilized itself as a food model (Wilson & Brown, 1997). Gelatin has been previously used to induce changes in food microstructure notably in yoghurt and acidic milk gels (Fiszman *et al.,* 1999; Supavititpatana *et al.*, 2008).

On the market, there are cheese products to which gelatin has been added. These products include various types of soft cheese (Camembert type) and some hard cheese. A suitable addition of gelatin is in the range of 0.1-5.0% by weight, based on the total weight of the finished cheese. The advantages of use the gelatin in cheese manufacturing (specially in hard cheese) are the possibility of producing cheese of lower fat content, more resilient consistency, better and more full-flavored taste, more rapid ripening process and the higher yield (Hagerman, 1995).

2. Nisin: a biological tool for food bio- preservation

2.1. Nisin, a bacteriocin of lactic acid bacteria

Microbes produce an extraordinary array of microbial defense systems. These include broad-spectrum classical antibiotics, metabolic products, such as lactic acid, lytic agents such as lysozyme, and numerous types of protein exotoxins, and bacteriocins, which are defined as biologically active protein moieties with a bactericidal mode of action (Heng *et al.,* 2007)

Both gram-positive and gram-negative bacteria produce bacteriocins. Bacteriocins are proteinaceous antibacterial compounds which constitute a heterologous subgroup of ribosomally synthesized antimicrobial peptides. In general, these substances are cationic peptides that display hydrophobic or amphiphilic properties and the bacterial membrane is in most cases the target for their activity (De Vuyst & Vandamme, 1994).

Bacteriocins produced by lactic acid bacteria (LAB) are a heterogeneous group of anti-bacterial proteins differing in spectrum of activity, mode of action, molecular mass, genetic origin and biochemical properties (Abee *et al.,* 1995). They can be classified according to their biochemical and genetic characteristics as follow (Klaenhammer, 1993; Nes *et al.,* 1996; Cotter *et al.,* 2005):

Class I (Lantibiotics): Small (<5 KDa) heat-stable peptides acting on the membrane structure; they are extensively modified after translation, resulting in the formation of characteristic thioether amino acids lanthionine (Lan) and methyllanthionine (MeLan). These arise via a two-step process: firstly, gene-encoded serine and threonine are subjected to enzymatic dehydration to give rise to dehydroalanine (Dha) and dehydrobutyrine (Dhb), respectively. Second, thiol group from neighbouring cysteines attack the double bond of Dha and Dhb yielding either Lan or MeLan, respectively. This condensation between two neighbouring residues results in the formation of covalently closed ring within the formerly linear peptide, conferring both structure and functionality.

Subclass I a: These bacteriocins are relatively elongated, flexible and positively charged peptides. They generally act by forming pores in the cytoplasmic membrane of the sensitive species. Nisin is a member of this group.

Subclass I b: These bacteriocins are globular, more rigid in structure and negatively charged or have no net charge. They act by interfering with essential enzymatic reactions of sensitive bacteria. Mersacidin is a member of this group.

Class Π: The molecular mass of this group is <10 KDa, heat stable, do not contain Lan residues. This class contains 3 subclasses:

Class Π a: Antilesterial pediocin-like bacteriocin. These bacteriocins were found to induce leakage of K+, amino acids, and other low-molecular-weight molecules from sensitive cells.

Class Π b: Two-peptide bacteriocin (ex: plantaricin EF). These types of bacteriocins consist of two different modified peptides, both of which must be present in about equal amounts in

order for these bacteriocins to exert optimal antimicrobial activity. These bacteriocins render the membrane of the target cells to various small molecules.

Class Π c: Other bacteriocins.

Class Ш: It includes bacteriocins with molecular mass >30 KDa (ex: helveticin J)

Bacteriocins produced by LAB offer several desirable properties that make them suitable for food preservation: 1) they are generally recognized as safe substance, 2) they are not active and nontoxic on eukaryotic cells, 3) they become inactive by digestive proteases, having little influence on the gut microbiota, 4) are usually pH and heat-tolerant, 5) they have a relatively broad antimicrobial spectrum, against many food-borne pathogenic and spoilage bacteria, 6) they show a bactericidal mode of action, usually acting on the bacterial cytoplasmic membrane and 7) their genetic determinants are usually plasmid-encoded, facilitating genetic manipulation. Foods can be supplemented with *ex situ* produced bacteriocin preparations, or by inoculation with the bacteriocin-producer strain under conditions the favor bacteriocin production *in situ* (Galvez *et al.,* 2007).

To date nisin is the only bacteriocin that has been approved by the World Health Organization (since 1969) for use as a food preservative. Its structure was elucidated as late as 1971 by Gross and Morell (Figure 1) (Cotter *et al.,* 2005).

Nisin is produced by some strains of *Lactococcus lactis*. It is formed by 34 amino acids (3500 Da) of which 13 have been posttranslationally modified. These modifications include the dehydration of serine and threonine, resulting in three dehydroalanine and five dehydrobutyrine residues. Five of these dehydro residues are subsequently linked to the sulfhydryl groups of the five cysteines residues present in pre-nisin Z, resulting in the formation of lanthionine rings (Gross & Morell, 1971).

Nisin exists as two variants (A and Z), which differ by a single amino-acid substituting histidine at position 27 in nisin A and asparagin in nisin Z. This structural modification has no

effect on the antimicrobial activity, but gives nisin Z higher solubility and diffusion characteristics compared with nisin A, which are important characteristics for food applications (de Vos *et al.,* 1993; Parada *et al.,* 2007). Nisin is a flexible cationic antimicrobial protein due to the presence of three lysine residues (at positions 12, 22, and 34) and one histidine at position 31. It has an amphiphilic character with a C-terminal portion and a rather hydrophilic N-terminal rather hydrophobic (Breukink & de Kruijff, 1999).

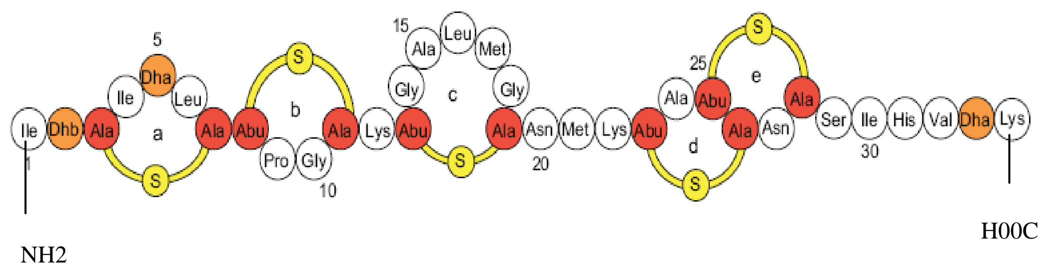

Figure 1: primary stricter of nisin. The lanthionine characteristic residues (Ala-s-Ala) and β-methyllanthionine (Abu-s-Ala) that from the lanthionine rings are colored in red; the dehydrated amino acids Dhb (dehydrobutyrine) and Dha, (dehydroalanine) are colored in orange (Breukink, 2006)

Nisin is ribosomally synthesized. Its biosynthesis occurs during the exponential growth phase and completely stops when cells enter stationary phase growth (de Vos *et al.,* 1993)

2.2. Nisin chemical properties

The notable properties of nisin are the effect of pH on the molecule solubility and stability, which both increase dramatically as the pH is lowered. In aqueous solution, nisin is so stable at pH 2 that it can be autoclaved from 115°C to 121°C without inactivation (Hurst, 1981). In the pH range of 5 to 7, nisin becomes progressively less stable to heating and significant losses in activity are to be expected when heated at elevated temperature (Delves-Broughton *et al.,* 1996). Its solubility dropped sharply and continuously from 57 mg/mL at pH 2 to about 1.5 mg/mL at pH 6, and to 0.25 mg/mL at pH 8.5 (Liu & Hansen, 1990). Nisin is unstable and becomes inactivated at higher pH (Hurst, 1981).

Nisin is inactivated by chymotrypsin and by nisinase, which is a nisin inactivating enzyme produced by some *Bacillus* spp. (like *B. cereus* and *B. circulans*) and *Streptococcus thermophilus* INIA 463. Nisinase is dihydroalaninereductase enzyme which is active against nisin and subtilin (Ralhan *et al.,* 1978; Garde *et al.,* 2004). On the other hand, nisin is resistant to pronase and trypsin under acidic conditions (Gross & Morell, 1971).

2.3. Nisin mode of action

Nisin affects the cytoplasmic membrane of susceptible bacteria and is able to form short-life pores in the membrane (Ruhr & Sahl, 1985; Sahl, 1985; Sahl & Bierbaum, 1998). This leads to an efflux of small molecules (potassium and amino acids) and dissipation of the membrane potential, stopping all cellular biosyntheses (Breukink, 2006).

Because of its positive charge, nisin require anionic phospholipids for membrane binding and pore formation (Driessen *et al.,* 1995; Demel *et al.,* 1996). Lipid II is anionic phospholipids in the bacterial wall. Despite its major role in the bacterial life cycle, it is a minor component of the bacterial cytoplasmic membrane (Breukink, 2006). The specificity of the nisin-lipid II interaction results in high-level activity of nisin, it is indeed enhanced by a factor of 10^3 when lipid II is available for targeted pore formation (Wiedemann *et al.,* 2001). The two N-terminal rings (rings a and b, Figure 2) of nisin form a binding pocket, also called the pyrophosphate cage that envelops the pyrophosphate moieties of the lipid intermediates of the cell wall biosynthesis like a base-ball glove. This binding pocket is stabilized by hydrogen bonds (Hsu *et al.,* 2004). After binding to membrane, the C-terminal part of nisin is able to insert to the membrane, oligomerized and form a pore that contains eight nisin molecules and four molecules of lipid II (Breukink, 2006).

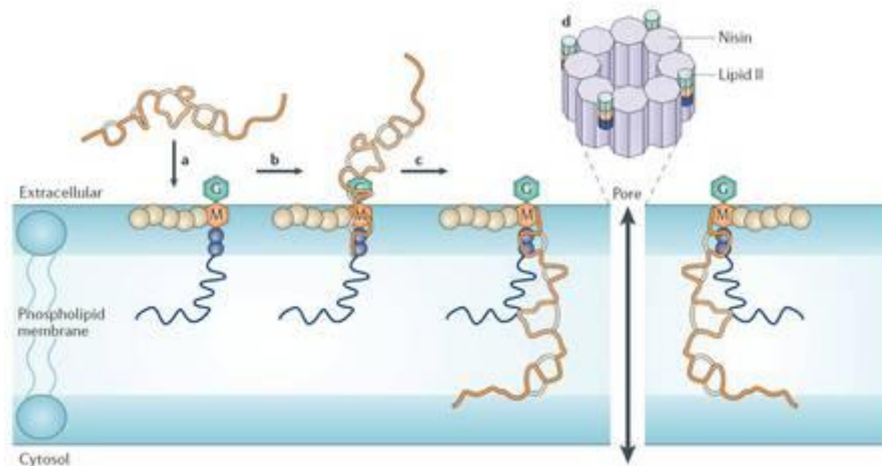

Figure 2: Nisin mode of action of pore-forming (Breukink, 2006). Nisin first reaches the bacterial plasma membrane (a), where it binds to Lipid II via two of its N-terminal rings (b). This is then followed by pore formation (c), which involves a stable transmembrane orientation of nisin. During the pore formation process, four other nisin molecules are recruited to form the pore complex with 8:4 (nisin over Lipid II) stochiometry, generating a 2 nm-diameter pore (d).

The presence of lipid II in such membrane enhances the ability of nisin to attack the membrane. In this case, the lifetime of the pores increases from milliseconds to approximately 6 seconds, and also the pore size increases from approximately 1.0 nm to 2.0-2.5 nm (Wiedemann *et al.,* 2001).

2.4. Nisin applications

Consumers have been consistently concerned about possible adverse health effects from the presence of chemical additives in their foods. As a result, consumers are drawn to natural and “fresher” foods with no chemical preservatives added. This perception, coupled with the increasing demand for minimally processed foods with long shelf life and convenience, has stimulated research interest in finding natural but effective preservatives. Bacteriocins are recognized as safe and as a natural biopreservatives. They are degraded by the proteases in the gastro-intestinal tract. They are useful as a primary hurdle for controlling food-born pathogens (Cleveland *et al.,* 2001). The most widely studied and commercially available bacteriocin is nisin.

Nisin is active against most gram-positive bacteria including *Lactococci*, *Bacilli*, *Micrococci*, *Staphylococcus aureus*, *Listeria monocytogenes* and *Clostridium botulinum* but shows little or no activity against gram negative bacteria, yeast or moulds (Hurst, 1981). However, another antibiotic activity of nisin is the inhibition of the outgrowth of spores. Nisin's dehydroalanine in position 5 has been reported to be involved in this inhibitory activity (Morris *et al.,* 1984).

Such an antimicrobial spectrum has resulted in nisin being as a commercial preservative in products which by their nature can not be fully sterilized but only pasteurized during their production (Delves-Broughton *et al.,* 1996)

Applications of nisin have mainly been developed for dairy products, meat products, fish and canned vegetable (Vandenbergh, 1993). In dairy products, the use of nisin as a preservative tool can be considered via two different ways: nisin could be added to the products by introducing nisin-producing and nisin-resistance or tolerant cultures through the cheese-making process as a starter cultures or adjunct culture. In this manner, the principal interest lies in the fact that it is then not necessary to label the nisin molecule as a food additive on cheese packages. However, convenient environmental conditions and processing factors, such as pH and water activity, are required to stimulate nisin production and activity (Sobrino-López & Martín-Belloso, 2008). This way of nisin addition is compatible with the growing consumer demand for safe food, with less chemical additives. Direct addition of nisin powder to the milk before the production of cheese made is the other way to use nisin as a food preservative. In this case, the preservative has to be labeled on the product (Plockova *et al.,* 1996). Moreover, this way of adding nisin to cheese is costly and has drawbacks, including lower activity, stability and bioavailability (Roberts & Zottola, 1993).

The beneficial effect of including nisin-producing starters in the cheese fermentation has been noted by a number of workers. One of the most striking effects is the prevention of late gas blowing in the cheese which is caused by the outgrowth of clostridia spores. This has been

reported as a particular problem in number of hard and semi-hard Dutch and Swiss cheeses, mainly due to the outgrowth of spores of *Clostridium butyricum* and *Clostridium tyrobutyricum* (Gould, 1995). Nisin also reduces the thermal resistance of *Bacillus* spores in a nutrient-rich chocolate milk model system (Beard *et al.,* 1999), and prevents spores germination of *Bacillus* in heat-treated cream (90°C for 15 min) stored at 8°C (Nissen *et al.,* 2001).

Utilization of nisin in dairy product can be done alone or in combination with thermal treatments or non-thermal treatments like other antimicrobial substances, high pressure (HP), or high-intensity pulsed electric fields (HIPEF), in order to improve their efficiency by synergistic effects.

Another interesting way of using nisin as a food preservative tool was recently developed. It consists in producing anti-microbial edible films incorporating nisin. These films were proved to inhibit *Listeria innocua*, *Listeria monocytogenes* and *Staphylococcus aureus* due to nisin desorption from the film and its diffusion in food products (Coma *et al.,* 2001).

Nisin *in situ* effectiveness in food product is based on both nisin diffusion throughout food materials and keeping its activity during shelf-life of the product. This event is influenced by several parameters including food composition, food physio-chemical properties, temperature of storage. However, the diffusivity of this kind of solutes such as nisin or other peptides in food or gel systems has not been extensively studied (Carnet-Ripoche *et al.,* 2006) and will be presented in paragraph 3.

2.5. Nisin *In situ* activity and quantification

Agar diffusion method is the most widely used method to determine nisin activity as well as for the quantification of nisin, due its simplicity and cost-effectiveness. This method is based on the measurement of the inhibition zone produced in nisin sensitive strain entrapped

in poured Petri dishes. The size of this zone is affected by many factors, such as the nisin sensitive strain used, the amount of agar and the pre-diffusion step. *Lactobacillus sake* was found to be the more nisin sensitive species compared with *Micrococcus luteus* and *Brochothrix thermosphact* (Pongtharangkul & Demirci, 2004).

However, poor accuracy of this assay limits the interpretation of results (Bouksaim *et al.,* 1998). It is also time consuming and laborious, requiring preparation and cooling of plates, boring of test wells in agar and manual measuring of the inhibition zones after 24-48h of incubation (Tramer & Fowler, 1964). Results depend largely on human ability and judgment and the suggested precision cannot be obtained when the inhibition zone is unclear or not perfectly circular (Parente *et al.,* 1995).

Another method was proposed (Raheem & Saris, 2009) using the nisin producing strain L*actococcus lactis* LAC309. The amount of nisin produced in Nigerian Wara cheese was measured by a nisin bioassay in which nisin induces the production of green fluorescent protein. Nisin-induced fluorescence was measured in cheese aqueous phase at 485 nm (excitation) and at 538 nm (emission) and was detected in terms of relative fluorescence units (RFU). The results in RFU were extrapolated to standard levels of Sigma commercial nisin stock solutions to obtain the amount of nisin in Wara cheese curd.

The nisin activity was quantified in many cheeses, mainly by the agar diffusion method. The results have been summarized in the Table (1).

Table 1: Literature review of nisin *in situ* activity in different cheese types.

Cheese type	Nisin producing strain and its inoculation level	Target strain and its inoculation level	Lost (log $_{10}$ cfu/mL) in the target strain	Corresponding Nisin *in situ* activity	Ripening conditions	Notes	reference
Camembert	*Lactococcus lactis ssp lactis* (2%)	*Listeria monocytogenes*V7 (10^5 cfu/mL)	1.57 ,after 24h 3.30, after 2weeks	700 IU/g after 9h	11°C 85-95% RH 6 weeks	+ 2% spores of *Pencillium camemberti* Milk fat=2.8%	(Maisnier-Patin *et al.*, 1992)
Manchego (Spanish raw ewes milk cheese)	*lactococcus lactis* ESI 515 (1%)	*Listeria innocua* (10^5 cfu/mL)	1.43, after 24h. 4.62,after 60 days	13.1 mm (24h) 11.5 mm (60 days)	12°C 60 days	pH= 5.07 (day 1) pH= 5.02 (day 60)	(Rodríguez *et al.*, 1998)
Cheddar	*Lc lactis subsp. lactis biovar. Diacetylactis UL 719*	*Listeria innocua* ATCC 33090 (10^5-10^6 cfu/mL)	1.5 after production 0.93±0.5 after 6 months	540±20.1 IU/mL after 14h	7°C 6 months	+ *Lc lactis ssp. cremoris KB* and *Lc lactis ssp. lactis KB* (as a cheese starter) Cheese composition after manufacture (%): Moisture: 37.46±0.31 Fat: 31.50±0..10 Protein: 23.47±0.33 pH=5.2±0.10	(Benech *et al.*, 2002a)
Cheddar	*Lc lactis subsp. lactis biovar. diacetylactis UL 719* (10^7-10^8 cfu/mL)	*Listeria innocua* (10^5-10^6 cfu/mL)	3.5±0.3 after 6 months	After 6 months, 12% of initial nisin (300±15 IU/g) was remains active	7°C 6 months	Milk fat content 3.4%	(Benech *et al.*, 2002b)
Vidiago (semi hard farmhouse Spanish cheese)	*lactococcus lactis ssp. lactis* IPLA 729 (1%)	*Clostridium tyrobutyricum* CECT 4011 (1.2 *x* 10^6 cfu/mL)	2.97 after 30 days	1600 AU/mL after 24h and was maintained until 15 days	12°C 90% RH 30days	-Target cells were injected in the curd by a sterile syringe -Cheese composition at day 1: Fat: 57.19 ± 2.06	(Rilla *et al.*, 2003)

						Protein: 42.11±0.5 pH=5.7±0.10	
Wara (Nigerian soft cheese)	*lactococcus lactis* LAC309 (8.0 *x* 10^5 cfu/mL)	*Bacillus licheniformis 553/1* (7.0 *x* 10^2 cfu/mL)	1.32 after 3 days	28.8, 34.6 and 37.4 IU/mg in the first, second and third day, respectively	30°C 3 days	This kind of cheese is stored in its whey Fat= 13.1% Protein =15.0% pH=6.3	(Raheem & Saris, 2009)
Ultra-filtrated model cheese	*Lactococcus lactis ATCC* 11454 (10^7-10^8 cfu/g)	*Listeria monocytogenes* ATCC 19117 (10^5cfu/g)	1.80,after 7 days				(Mirdamadi *et al.,* 2010)
Gouda	*Lc lactis subsp. lactis biovar. diacetylactis UL 719* (0.6%)	---	---	256 after pressing 512 IU/g after 6 weeks		Cheese composition after pressing (%): 38.03±2.08 moisture Fat: 29.75±1.77 Salt: 2.18±0.18 Protein: 26.23 pH= 5.28±0.03	(Bouksaim *et al.,* 2000)

2.6. Factors affecting nisin efficiency as a food preservative

Comparisons of data obtained in culture media with those obtained in food systems reveal that the efficacy of bacteriocins is often much lower in the later (Schillinger *et al.,* 1996). Factors that may influence the recovery and efficiency of bacteriocins in foods are related to food components, and conditions that destabilize the biological activity, like proteolytic degradation or oxidation (Daeschel, 1993; Murray & Richard, 1997; Gänzle *et al.,* 1999). Reduced activity of bacteriocins in foods was shown for nisin with high fat content (Bell & de Lacy, 1986; Jung *et al.,* 1992; Davies *et al.,* 1999). Nisin may also adsorb to proteins in the food matrix by ionic or hydrophobic bonds. These kinds of interactions and their effect on the inhibition efficiency have been less studied than the influence of fat, but Goff *et al.,* (1996) and Murray & Richard (1997) demonstrated that protein binding may cause a significant reduction in free bacteriocin in foods. Addition of casein reduced the activity of nisin in synthetic media (Gänzle *et al.*, 1999).

Nisin production and stability in a food matrix is also affected by several factors such as the producer lactococci strain, nutrient composition, pH, temperature, adsorption of nisin onto the producer cells or onto food component or enzymatic degradation (Parente *et al.,* 1995; Schillinger *et al.,* 1996). Among the factors influencing the effectiveness of bacteriocins as antimicrobials in food systems, factors influencing bacteriocin production are of most importance when using bacteriocinogenic cultures (Figure 3).

Homogenization of milk or cheese slurries was found to reduce nisin efficacy against *Listeria monocytogenes* comparing with non homogenized milk (Bhatti *et al.,* 2004). It is known the homogenization decrease average diameter of fat globules and increase their numbers and surface area, which results in increment of the nisin adsorption on fat surface and hence decreased its overall antimicrobial activity. Some activity could be recovered by adding 0.2 v/v% of the Tween 80 emulsifier and to a lesser extent by adding lecithin (Glass & Johnson, 2004).

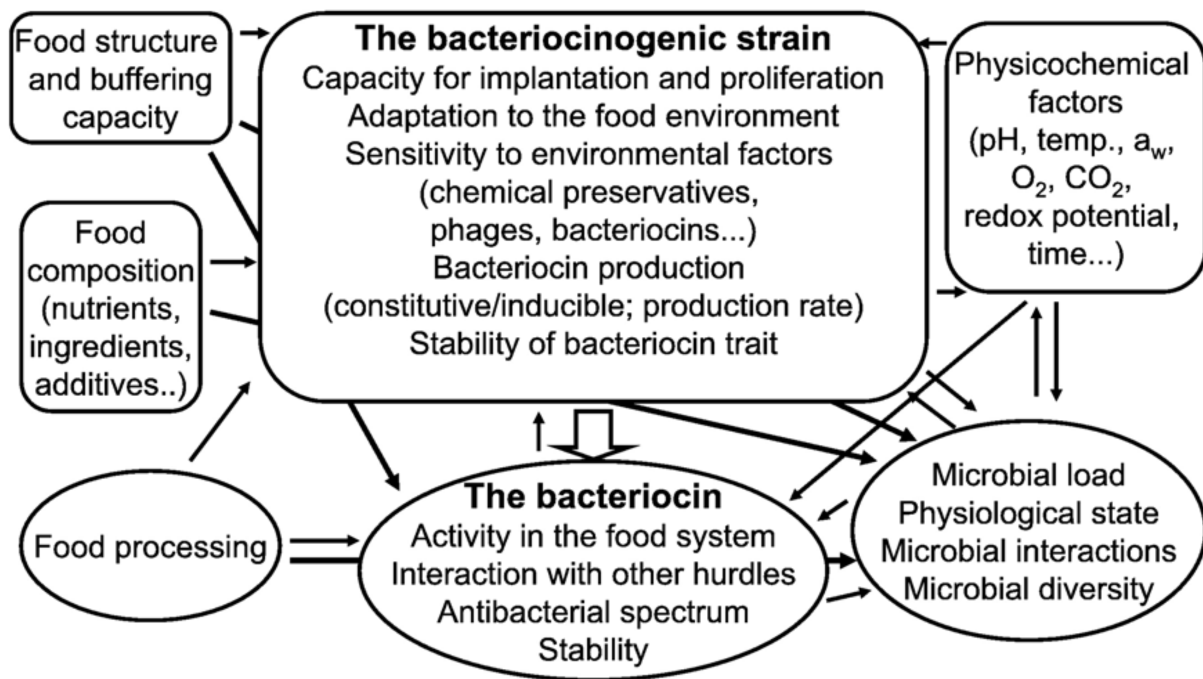

Figure 3: Influence of different factors on the efficacy of *in situ* bacteriocin production for biopreservation (Galvez *et al.,* 2007).

The main problem with quantitative detection of nisin in food matrices, and particularity in dairy products, is its adsorption to both the protein and lipid fractions (Somers & Taylor, 1987; De Vuyst & Vandamme, 1994) due to its strong cationic and hydrophobic character. (Jung *et al.,* 1992) also stated that initial the nisin activity (50 IU/mL) decrease by about 33% when it was added to skim milk and by 88% when added to milk with fat content 12.9%. Nisin has inhibited growth of *Staphylococcus aureus* 196 in the media Brain Heart Infusion (BHI) but this inhibitory action of nisin was not as effective in the presence of whole milk (Jones, 1974).

In non dairy food too, nisin can be quickly adsorbed to proteins in the food matrix (consists of homogenates of cold-smoked salmon, chicken cold cuts and raw chicken), but in general less nisin was adsorbed to proteins at low pH (Aasen *et al.,* 2003).

Nisin and other antimicrobial compounds are effective at inhibiting pathogenic bacteria in food; however, these compounds are often subject to rapid depletion after their initial application and quickly lose their activity. For bacteriocins such as nisin, this depletion

is usually caused by diffusion or inactivation (Delves-Broughton, 2005); Quintavalla and Vicini, 2002; Rose *et al.,* 1999).

3. Diffusion of nisin in solid matrix

3.1 In agarose gel and food-like matrix

Nisin diffusivity was calculated in agarose gels only by taking into account the different factors capable to influence the diffusion, such as temperature, pH and agarose concentration in the gel (Sebti *et al.,* 2004). Fick's second law was validated as a satisfactory mathematical model to assess nisin diffusion in such a model matrix (Carnet-Ripoche *et al.,* 2006).

The experimental procedure used to determine the diffusivity of nisin is illustrated in Figure 4. The cylindrical geometry is usually chosen in order to be able to consider unidirectional mass transfers along the *x*-axis in the product. Before diffusion assay, the gels were coated with paraffin and parafilm layers in order to avoid evaporation of water.

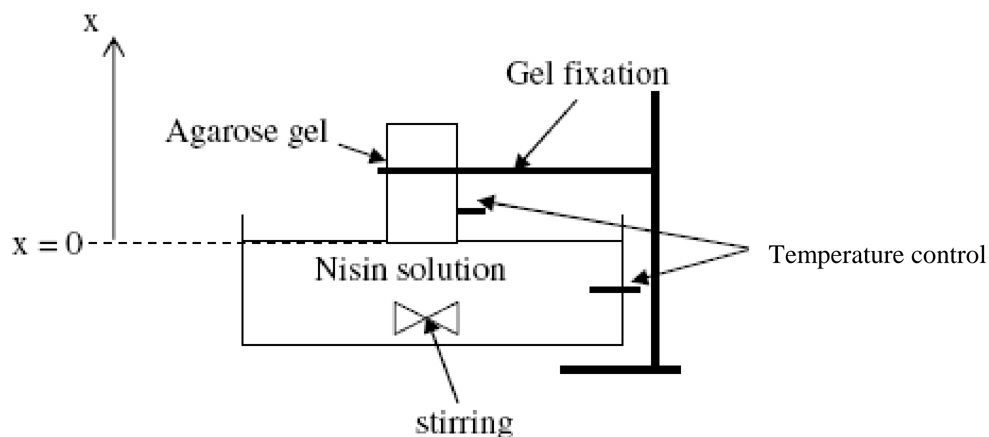

Figure 4 : Experimental procedure of diffusion (Sebti et al., 2004; Carnet-Ripoche et al., 2006)

The agarose gel surface was placed upper a solution of nisin with fixed concentration to assure an interfacial contact. The solution was stirred in order to be able to neglect the external resistance to mass transfer in the liquid boundary layer at the surface of the gel.

At different experimental times, agarose gel cylinders were cut into slices of about 1 mm thickness beginning with the gel side in contact with nisin solution ($x = 0$). Nisin concentration was quantified in each slice either by a colorimetric method BCA (bicinchoninc

acid) in Sebti *et al* (2004) and Carnet-Ripoche *et al* (2006) or by HPLC in Chollet *et al.,* (2008).

Nisin concentrations were then represented *vs* the distance from the surface to the centre of the model gels on the *x*-axis for different contact times between the nisin solution and the gels. At last the nisin concentration profiles were compared to the predicted values obtained from the second Fick's low in order to determine the apparent diffusion coefficient of the nisin in the agarose gel.

Different factors may affect nisin diffusion

i. Temperature

The influence of temperature on nisin diffusivity was evaluated by (Sebti *et al.,* 2004). Five different temperatures ranged between 5.4 and 22.3°C for 6 and 12 days were tested in their agarose model matrix.

Table 2. Apparent nisin diffusion coefficients at different temperatures in 3 w/w% agarose gel cylinders (Sebti *et al*., 2004)

T (°C)	**Time (days)**	**C_{sol}^{*} (µg.ml^{-1})**	**D** (µm^2/s)
5.4	5.92	256	19.2
9.9	6.11	252	35.2
9.8	6.91	372	32.7
10.2	11.60	336	37.4
22.3	5.93	376	81.4

**C_{sol} is nisin concentration in the solution*

Values of Table 2 showed that the nisin diffusivity increased with the temperature and a satisfactory linear relationship (R^2 = 0.98) was obtained between *D* versus 1/T. This approved that the diffusion phenomena satisfied Arrhenius relationship.

ii. Agarose content %

Carnet-Ripoche *et al.,* (2006) and Sebti *et al*., (2004) showed that nisin apparent diffusion coefficient (*D*) decreased when agarose concentrations increased. (Sebti *et al.,* 2004)

compared the obtained nisin diffusion (at 10 Cº for 6 days) in 3 agarose gels containing 3, 6 and 8% agarose. For the 3 and 6% agarose gel, they obtained the same diffusion coefficient (35.2 µm^2/s). The coefficient obtained in 8% agarose gel was 13.1 µm^2/s. Similar results were obtained by (Carnet-Ripoche *et al.,* 2006) with three other agarose gel contents (3.2, 3.9 and 6.7% w/w) in the same conditions. The obtained D values were 42 and 34 µm^2/s for the 3.2 and 3.9 % agarose gel respectively. While D was dropped down to 25 µm^2/s in the 6.7% one.

Carnet-Ripoche *et al* (2006) explained the effect of agarose content as follow: when agarose concentration in gel increased a) the path length for diffusion was certainly increased by the presence of network, so that the diffusion coefficient was reduced. b) The part of solvent (water) and the pore size decreased, so it probably reduced the diffusivity of the molecule in the gel. This last assumption seems more acceptable.

iii. Fat and NaCl contents

The influence of fat content in a model matrix (agarose) on nisin diffusivity was evaluated by Carnet-Ripoche et al (2006). The authors revealed that there was no significant difference between nisin apparent diffusion coefficients in 3% agarose gels with or without hydrogenated coprah oil (from 33 to 100% (w/w in the agarose) of hydrogenated coprah oil (Vegetaline®) incorporated into 3% (w/w) agarose gel before gel preparation. D was 42 µm^2/s in all treatments. For the authors, these results could be attributed to that the amount of nisin which was so high (277- 334 µg nisin/mL) that apart of nisin saturated the fixation sites on Vegetaline®, the other part could diffuse easily. However, Chollet *et al*.,(2008) obtained different result when they examined the effect of fat and salt level on nisin recovery in cheese-like gels with 3% agarose and 2 different studied levels of anhydrous milk fat% and NaCl % w/w (5 and 30; 0.5 and 2, respectively) and Emmental cheese slurries. In their study, no significant differences were noticed according to the level of NaCl, while increasing anhydrous milk fat concentration in agarose gels caused nisin concentration to drop by a

factor of 1.6, and also gave rise to a significant decrease in nisin bioactivity. The binding of nisin to fat seemed to limit its activity.

To prolong nisin efficacy, nisin has been incorporated into packaging films and coatings (Bi *et al.*, 2011). Some results concerning nisin diffusion from the pachaging films into food matrixes were published.

3.2 In packaging films

Many food products can be subjected to contamination by undesirable microbes such as fungi, yeasts and bacteria (Hotchkiss, 1997). In order to prevent or impede such contamination, novel packaging technologies are continually being developed to prolong the shelf-life and improve the safety or sensory properties of fresh foods (Ahvenainen, 2003). A more recent and advanced class of food packaging system is known as "active packaging"(Hotchkiss, 1997).The active packaging has been defined as ''a type of packaging that changes the condition of the packaging to extend shelf-life or improve safety or sensory properties while maintaining the quality of the food''(Quintavalla & Vicini, 2002).

To determine nisin diffusion in these active packing, a thin film was immersed in water. Nisin concentration into water over time is estimated. The mass transfer was modeled thanks to the Fick's second law. Many similar aspects were found between nisin diffusion in active packaging and in agarose gels. Temperature was found to have the same effect on nisin diffusion in both packaging films and agarose gels. Teerakam *et al.,* (2002) showed that nisin diffusion coefficients (D) were increased when temperature was elevated. Diffusion was calculated in different protein films [cost corn zein (CCZ), heat-pressed corn zein (HPCZ), cast wheat gluten (CWG), and heat-pressed wheat gluten (HPWG)] at several temperatures from 5 to 45°C. D values ranged from 5.6 to 1123 x 10^{-5}µm^2/s. The same trend was also obtained by Dawson *at al*., (2003). It can be noticed that the rate of diffusion is much lower in

polymeric films, and even more in proteinic ones, than in model food systems like agarose gels.

Also, an inverse relationship was found between nisin diffusion and the concentration of its constituent in active packaging as well as in agarose gels. Buonocore *et al*., (2003) investigated the release kinetics of antimicrobial agents (including nisin) from crosslinked polyvinylalcohol (PVOH) film into water at ambient temperature (25°C) and under moderate stirring. The tested films had different % of PVOH 0.077; 0.77, 2.00 and 7.70 % w/w. The obtained *D* values were 86.10; 62.40; 31.60 and 3.01 x 10^{-3} $\mu m^2/s$, respectively.

To conclude, until now the diffusion of nisin has only been studied in model matrix containing agarose and various fat and salt contents. The main parameter acting on the apparent diffusion coefficient of nisin is the temperature, the diffusivity increasing dramatically with the temperature. Milk fat and milk protein contents had a negative effect on nisin diffusivity in the agarose gels. Published data usually evaluate the apparent diffusion coefficient of nisin by fitting experimental concentration profiles of nisin to the Fick's second law, the simplest mass transfer model. Even if this model implies much simplifying assumptions, it allows getting good approximation of the apparent diffusion coefficient of this molecule in food matrix of defined composition, structure and temperature.

No data concerning nisin diffusivity in dairy products like cheese is available in the literature. The only studies that introduce mass transfer of nisin concern the release of active compounds from packaging films. Also, almost no data is available concerning the influence of cheese rheology and microstructure on peptide (like the nisin) diffusion. This missing information is very important to better understand cheese ripening which is a slow and expensive process that is not fully predictable or controllable. Consequently, there are economic and possibly technological incentives in this research.

Nisin efficacy is strongly affected by the physico-chemical properties of food and cheese matrix. Several strategies to enhance the LAB bacteriocin; and nisin; activity have been tried. Liposome-encapsulated nisin was tested in milk fermentation (Laridi *et al.,* 2003) and in the ripening of *Lactobacillus*-containing cheddar cheese (Benech *et al.,* 2003). The stability and entrapment of nisin in liposomes has been studied (Taylor *et al.,* 2007; Were *et al.,* 2003), and the remaining hurdle for a liposome strategy is to realize controlled release. Recently, the ability of carbohydrate nanoparticle-stabilized emulsion to prolong the efficacy of bacteriocin against food pathogens was investigated in BHI agar (Bi *et al.,* 2011). However, part of these methods was effective, but the utilised methods were rather complicated especially if implicated in a large scale for an industrial application.

Chapter 1

Nisin diffusion in UF model cheese

La quantification de la nisine par ELISA permet la modélisation du coefficient diffusion de la nisine dans un fromage modèle

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Samar Aly[a,b], Juliane Floury[a,b], Marie-Hélène Famelart[a,b], Marie-Noëlle Madec[a,b], Didier Dupont[a,b], Yann Le Gouar[a,b], Sylvie Lortal[a,b], Sophie Jeanson[a,b]

[a]INRA, UMR1253, Science et technologie du lait et d’œufs, F-35000 Rennes, France

[b]Agrocampus Ouest, UMR1253, Science et technologie du lait et d’œufs, F-35000 Rennes, France

Résumé

La diffusion des petits solutés dans le fromage est un phénomène essentiel pour les réactions enzymatiques impliquées dans les processus d’affinage. Cependant, la plupart des connaissances concernent la diffusion du sel, et aucune sur la diffusion des peptides et des protéines. La nisine, une bactériocine produite par *Lactococcus lactis*, est un peptide de 34 acides aminés. Sa diffusion a été étudiée dans une matrice fromage modèle, réalisée à partir d’un rétentat d’ultrafiltration sans matière grasse. La méthode du profil de concentration et une quantification par ELISA, spécifiquement développée dans ce travail pour quantifier la nisine en fromage, ont été mises en œuvre pour déterminer le coefficient de diffusion de ce peptide dans la matrice UF. Le moyen de ce coefficient déterminé est de 49.5 µm²/s (n=2); cette valeur représente la première estimation du coefficient de diffusion d’un peptide dans une matrice fromage. Quand 10% (v/v) de gélatine sont incorporées au rétentat, à extrait sec final identique, le moyen de ce coefficient de diffusion a diminue à 34.4 µm²/s (n=2). Les deux matrices étudiées présentent des caractéristiques rhéologiques, et microstructurales (mises en évidence en microscopie confocale), clairement différentes ce qui soutient l’hypothèse que la composition et l’organisation du réseau protéique influencent de fait la diffusion des petits solutés tels que les peptides.

Mots clés : Nisine / Diffusion / Modélisation / ELISA / Fromage / Microstructure

Nisin quantification by ELISA allows the modeling of its apparent diffusion coefficient in model cheeses

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Samar Aly[a,b], Juliane Floury[a,b], Marie-Hélène Famelart[a,b], Marie-Noëlle Madec[a,b], Didier Dupont[a,b], Yann Le Gouar[a,b], Sylvie Lortal[a,b], Sophie Jeanson[a,b]

[a]INRA, UMR1253, Science and Technology of Milk and Eggs, F-35000 Rennes, France

[b]Agrocampus Ouest, UMR 1253, 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 immune sorbent 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% (w/w) 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 like peptides.

Key words: 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 also are entrapped within the curd and develop as bacterial colonies with a 3D 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, i.e. 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 only been determined 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). UF milk retentate was used as a model cheese, as previously described *(14)*. This non-fat UF model cheese is a homogeneous matrix unlike real cheese and is therefore a better model system as it is more suited towards 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 immune sorbent assay (ELISA) was developed to specifically quantify nisin within the cheese protein network. ELISA was chosen for its accuracy and its sensitivity of detection. It is also a method whose 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 the syneresis in yogurt *(16)*.

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

Material 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 were added. The ultrafiltered (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)*:

$$\text{Denaturation (\%)} = \frac{(NCN_{RR} - NPN_{RR}) - (NCN_{HTR} - NPN_{HTR})}{(NCN_{RR} - NPN_{RR})} x100 \qquad (1)$$

where X_{RR} are the variables for the raw retentate, X_{HTR} are the variables for the heat-treated retentate, NCN is the 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 chymosine agent Maxiren (DSM Food Specialties, France). After molding in plastic

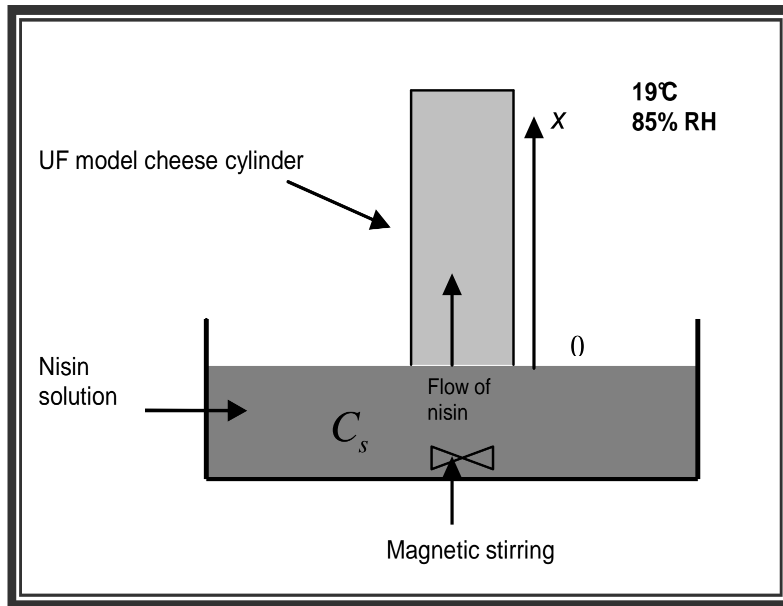

Figure 1: Schematic experimental device for nisin diffusion in two model cheeses in an incubator with controlled temperature and relative humidity.

cylinders, the UF model cheeses were all incubated at 30°C for 1 h for the coagulation process.

In order 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% (v/v), 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) based on 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 in order 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 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 up 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. Both, after 3 days and after 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).

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).

Physico-chemical analysis

Dry matter content and pH

Four cylindrical blocks of each type of cheese (R and R-G) were prepared as described in experimental device for nisin diffusion section. 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 incubation with the nisin solution *(19)*. Both model cheeses had the same final 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 each slice 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 (anti-nisin antibodies) were 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 anti-nisin antibody was added; (vi) a substrate was added and 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 µL/well of 0.5 µg/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 3 times by filling all wells simultaneously with 300 µL 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®, USA). To reduce non-specific binding, the microplates were blocked with 250 µL of 1 % (w/v) porcine gelatin (gelatin from porcine skin, Sigma Aldrich, France). At the same time, serial tenfold dilution 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 µL 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 µL 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, USA). The curve fits were performed by Gen5 data analysis software (BioTek, USA). Both negative and positive controls were added to several microplates in order 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 while 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 based on 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 duplicates.

Determination of apparent diffusion coefficient for nisin

Assuming no convective fluxes, Fick's diffusion equation describing unsteady state mass transfer can be written as follows *(24)*:

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

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:

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

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

The initial and boundary conditions are as follows:

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 a semi-infinite medium.

Then, at $t > 0 \Rightarrow C(0, t) = C_s$ and $C(\infty, t) = 0$

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

The solution of equation (3) is then:

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

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_{exp}) and theoretical model values (C_{model}) of nisin concentrations (mg/kg).

$$Crit = \sum_{i=1}^{N} (C_{\exp} - C_{\text{model}})^2 \quad (5)$$

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 towards 1, it means that 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 previously described, specifically for the rheology experiment. They were cut into 2 cm long slices after coagulation at 30°C for 1 h, and then incubated at 19°C for 2 h. The macrostructure of the cheeses 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 6 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 x 40 magnification (oil immersion) directly on a 2 mm thick slice of model cheese. Nile blue (1% aqueous solution,

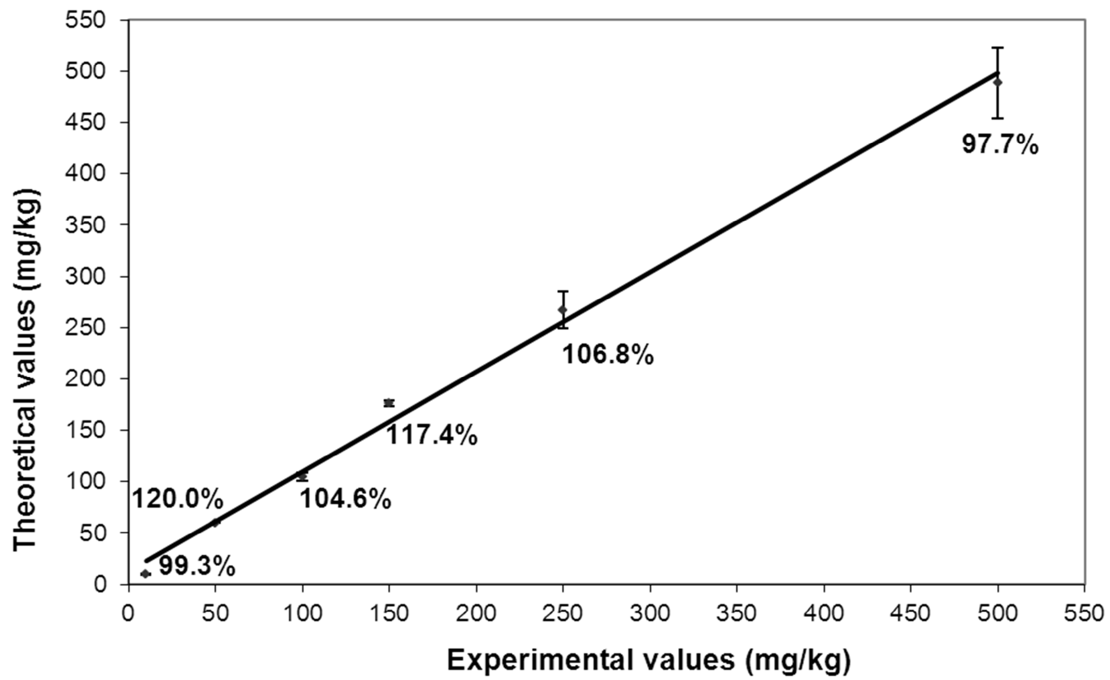

Figure 2: Theoretical *versus* experimentally measured values for nisin concentrations, determined by measuring the percentage of nisin recovered in spiked cheeses by ELISA inhibition (n=4).

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, Germany) before being added to the R-G model cheese. The gelatin solution was firstly diluted twofold 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 (cut off 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 in order to measure the nisin concentration within the linear range of the ELISA calibration curve. The LOD of this assay was 0.626 mg/kg.

The accuracy of the ELISA was estimated based on nisin recovery varying from 98% to 120% with a correlation coefficient R^2 of 0.99 (**Figure 2**). These results correspond to 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

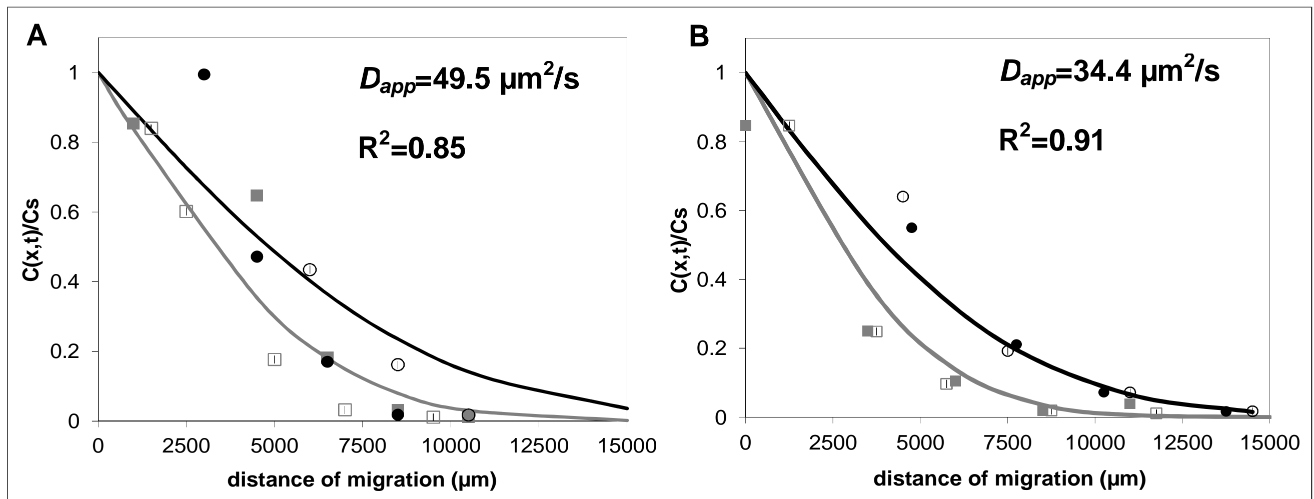

Figure 3: Concentration profiles of nisin in R cheese without gelatin (A) and in R-G cheese with 10% gelatin (B) after 3 (■ and □, grey lines) and 6 (● and ○, black lines) days in contact with a 500 mg/L nisin solution - experimental data for the two repetitions (plain and empty symbols) and theoretical data (continuous line).

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 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 furthest slice after 6 days. The obtained apparent diffusion coefficients for nisin were 49 µm²/s (with R^2=0.79) and 50 µm²/s (with R^2=0.97) with an average diffusion coefficient of 49.5 µm²/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 µm²/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 µm²/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, i.e. 152 times smaller.

When gelatin was incorporated in the R-G cheese (**Figure 3b**), the calculated apparent diffusion coefficients for nisin drastically dropped to 33 µm²/s (with R^2=0.93) and 36 µm²/s (with R^2=0.93) with an average diffusion coefficient of 34.4 µm²/s. The correlation coefficients R^2 were even closer to 1 for this matrix, which also indicated that experimental

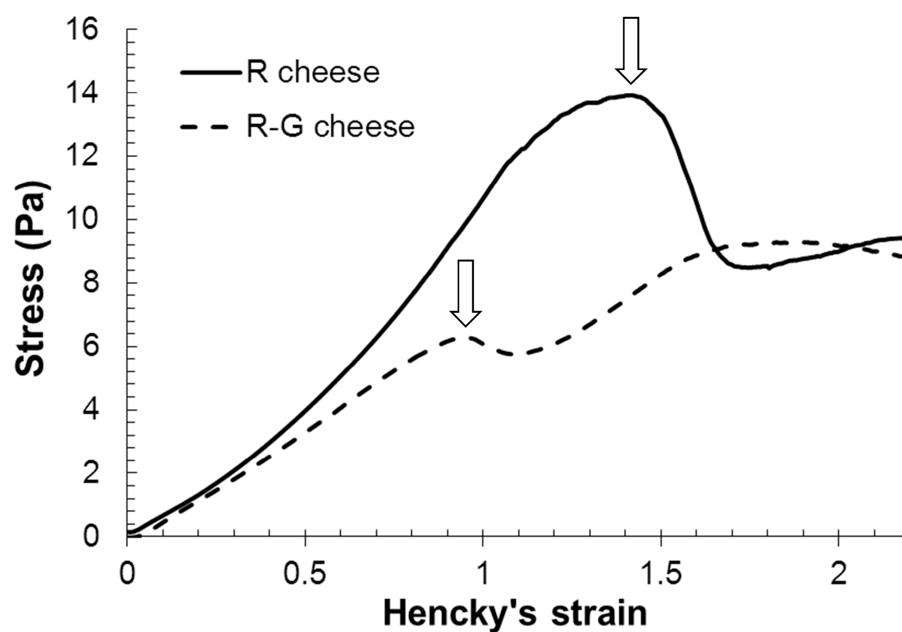

Figure 4: Rheological behavior of the two model cheeses obtained under large strain compression; stress *versus* Hencky strain for R cheese (continuous line) and R-G cheese (dashed line). The arrows show the fracture point.

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 furthest 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 $\mu m^2/s$ 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 physico-chemical 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 their 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**) shows 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 on **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

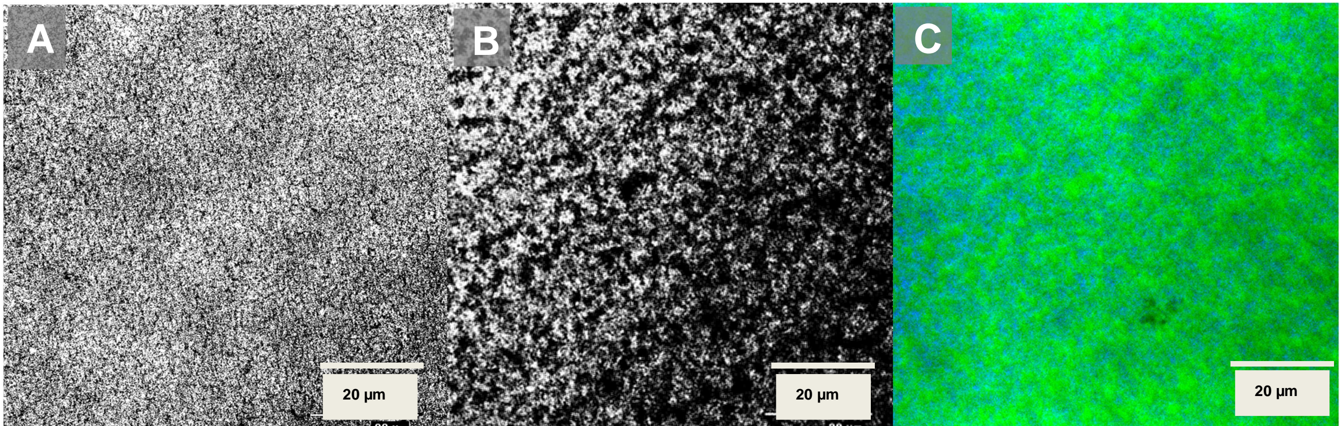

Figure 5: Confocal microscopy images of R cheese (A) and R-G cheese (B and C, prepared with FITC–labeled gelatin). (A) black and white micrograph of R cheese stained with Nile blue, (B) and (C) represent one micrograph (same microscopic field) of R-G cheese made with FITC-labeled gelatin and cheese protein 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.

(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 glucono-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 on **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. **Figures 5b** and **5c** 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, while **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 out 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. These 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 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 varying size and charge (water, salt, lactates, peptides, etc...) within a cheese matrix. However, the profile concentration device can also provide information on the macroscopic resolution. Since tests are rather time-consuming, it is only feasible to compare a limited number of different cheese compositions. Future work

should focus on developing non-destructive and *in situ* techniques on a more microscopic scale.

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Chapter 2

Nisin in situ efficacy in UF model cheese

La concentration de la nisine active ne permet pas de présager de son efficacité dans les matrices fromagères.

ALY Samar, FLOURY Juliane, PIOT Michel, LORTAL Sylvie and JEANSON Sophie

Résumé

La nisine est une bactériocine produite par certaines souches de *Lactococcus lactis.* Elle a un large spectre d'activité contre les bactéries gram-positives et est fréquemment utilisée comme bio-conservateur. *Lactobacillus sake* a été utilisée ici comme espèce cible reflet de l'activité de la nisine Z produite *in situ* en matrice fromage par *Lc. lactis*. Les matrices modèles utilisées ont été réalisées à partir d'un rétentat d'ultrafiltration sans matière grasse, avec 0, 4 et 10% (w/w) de gélatine ajoutée, à extrait sec final identique. L'ajout de la gélatine permet comme le montre des travaux antérieurs (article 1) de changer la microstructure de la matrice et ses caractéristiques diffusionnelles. La quantité de nisine totale était mesurée par le test ELISA précédemment développé, et la quantité de nisine active par la méthode des halos de lyse en boite de Pétri. La mesure de la perte de viabilité de *Lb. sake* était utilisée comme un indicateur indirect de l'efficacité *in situ* de la nisine. La présence de gélatine n'affecte ni la croissance du lactocoque, ni la quantité totale et active de nisine produite. En revanche, la quantité de lactate finale était supérieure et le pH final légèrement plus bas (0.2 upH) dans les fromages avec gélatine. Les cinétiques de pertes de viabilité de *Lb. sake* observées étaient très différentes selon la matrice. Après 24 h, la perte de viabilité variait de 0.33 +/- 0.1 à 3.2 +/- 0.01 $\log_{10}$ cfu/g dans les matrices contenant respectivement 0 et 10% de gélatine. Ces résultats montrent que l'incorporation de gélatine, déjà pratiquée dans certains produits industriels, augmente l'efficacité *in situ* de la nisine. Différentes hypothèses sont discutées, les plus

plausibles semblent être une interaction moindre de la nisine avec la gélatine *versus* la caséine qu'elle remplace, et la légère différence de pH final qui favoriserait la libération de la nisine de la colonie productrice de *L. lactis.* Ces observations démontrent que la concentration de nisine ne permet pas de présager de son efficacité *in situ* dans des matrices alimentaires. La validité de cette observation reste à démontrer dans d'autres matrices alimentaires où la gélatine est utilisée.

The concentration of active nisin does not allow the prediction of its *in situ* efficiency in model cheese.

ALY Samar, FLOURY Juliane, PIOT Michel, LORTAL Sylvie and JEANSON Sophie

Abstract

Nisin is a natural bacteriocin produced by some strains of *Lactococcus lactis*. It has a broad inhibitory effect against gram-positive bacteria. This study investigated how incorporation of gelatin in ultrafiltrated (UF) milk retentate influenced the activity of nisin Z produced *in situ* by a nisin-producing strain. Three different UF model cheeses, containing 0, 4 or 10% of gelatin, were manufactured and their physico-chemical characteristics were analyzed. These model cheeses were inoculated with both the nisin Z producing strain, *Lactococcus lactis* subsp. *lactis* biovar *diacetylactis UL179* and the nisin-sensitive strain *Lactobacillus sake ATCC15521*^{RifR}. Measurement of *Lb. sake* loss of viability was an indirect indicator of nisin *in situ* efficacy. After 24 h, the more gelatin was added, the more *Lb sake* lost viability. *Lb sake* lost from 0.33 ± 0.11 to 3.21 ± 0.01 log_{10} cfu/mL in the model cheeses with 0 and 10% of gelatin, respectively. In addition, the incorporation of gelatin led to a lower pH which may have induced an increase of nisin efficiency. However, neither *Lc lactis* growth nor its nisin production quantified by enzyme-linked immune sorbent assay (ELISA) was affected by the presence of gelatin. Finally, this study demonstrated that the concentration of nisin does not reflect its *in situ* efficiency in complex environment such as model cheese.

1.Introduction:

Nisin is a bacteriocin widely used as food preservative in particular in cheese (Delves-Broughton, 2005; Deegan *et al.*, 2006). It is a 3,5 kDa peptide (34 amino acids), positively charged (Breukink & de Kruijff, 1999), produced by some strains of *Lactococcus lactis* (Delves-Broughton *et al.,* 1996). Nisin exists as two variants (A and Z), which only differ by a single amino-acid. This modification has no effect on the antimicrobial activity but nisin Z has higher solubility, so it is mostly chosen for food applications (de Vos *et al.*, 1993; Parada *et al.*, 2007). Nisin is active against most gram-positive bacteria including *Lactococci*, *Bacilli*, *Micrococci*, *Staphylococcus aureus*, *Listeria monocytogenes* and *Clostridium botulinum* but shows little or no activity against gram-negative bacteria, yeast or moulds (Hurst, 1981). *Lactobacillus sake* is considered to be one of the most non-pathogenic nisin-sensitive species, this is why it is always used for nisin bioassay by agar diffusion method (Pongtharangkul & Demirci, 2004). Nisin affects the cytoplasmic membrane of sensitive bacteria by forming short-life pores in the membrane (Ruhr & Sahl, 1985; Sahl, 1985; Sahl & Bierbaum, 1998) leading to a bactericidal effect. As long as nisin is produced *in situ* by lactic starters, nisin is not considered as a food additive (Galvez *et al.*, 2007)

When a nisin-producing strain is used in cheese manufacture, the main outlines in the literature were: (i) the loss of viability of targeted species in different kinds of cheese such as Camembert cheese (Maisnier-Patin *et al.*, 1992; Rodríguez *et al.*, 1998; Benech *et al.*, 2002a; Benech *et al.*, 2002b; Rilla *et al.*, 2003; Raheem & Saris, 2009; Mirdamadi *et al.*, 2010)with or without testing nisin *in situ* activity, (ii) the immunolocalisation of nisin in the target cell membrane by electron microscopy and the evidence for bursting the sensitive strains (Benech *et al.*, 2002a).

As bacteria, starters or ingenious flora, are immobilized in cheese after renneting whatever the technology used, they grow as colonies and their spatial distribution is an important parameter

to consider in the investigations of bacterial interactions (Jeanson *et al.*, 2011). When produced *in situ* by *Lc. lactis*, nisin has to migrate, from the producing lactococci colonies to the target bacteria colonies, and has also to remain active *in situ* to be efficient in a cheese matrix. The apparent diffusion coefficient for nisin was recently determined for the first time in UF model cheese (Aly *et al*., in press). The modeled value was 49.5 ± 0.3 µm^2/s, which is about 2-10 times smaller than the apparent diffusion coefficient of salt. Regarding the activity of nisin, many physicochemical parameters can affect its activity in real food, like pH, temperature, adsorption on caseins, or fat (Bell & de Lacy, 1986; Jung *et al.*, 1992; Daeschel, 1993; Murray & Richard, 1997; Gänzle *et al.*, 1999; Davies *et al*., 1999).

Gelatin is frequently incorporated in low fat hard cheeses, as well as in some soft cheese types, with a percentage up to 5% of dry matter (Hagerman, 1995). However, gelatin incorporation was found to modify the rheological properties and the microstructure when added to dairy products (Hsieh *et al.*, 1993; Fiszman *et al.*, 1999; Koh *et al*., 2002). As a consequence, the quantity of nisin produced *in situ*, its migration, as well as its activity *in situ*, could also be influenced by the incorporation of gelatin.

The aim of this study was to explore how gelatin could influence the efficacy of nisin produced *in situ* within a cheese matrix. For that purpose, three different UF model cheeses, containing 0, 4 or 10% of gelatin, were developed, and their physico-chemical characteristics were analyzed. They were all inoculated with both a nisin-producing strain (*Lactococcus lactis*) and a nisin-sensitive strain (*Lactobacillus sake*). The level of inoculation was strictly controlled to estimate the distance between colonies (Jeanson *et al.*, 2011). Measurement of *Lb. sake* loss of viability was an indirect indicator of nisin *in situ* efficacy. The influence of the different percentages of incorporated gelatin was then assessed by following the kinetic of viability loss of the nisin-sensitive strain in each matrix. Furthermore, the absolute quantity of nisin produced *in situ* was measured using an inhibition ELISA.

2. Material and Methods

2.1. Bacterial strains and culture conditions

Lactococcus lactis subsp. *lactis* biovar *diacetylactis UL719* (kindly provided by the STELA Dairy Research Center, Laval University, Canada) was used as a nisin Z producer strain (Boukaaim *et al*., 2000). *Lactobacillus sake ATCC15521* (CIRM BIA, INRA STLO, Rennes, France) was used as a nisin-sensitive strain (Boukaaim *et al*., 1998). Stock cultures were maintained at -80°C in M17 + 0.5% lactose (VWR international, Belgium) and MRS (Difco, France) media, respectively, with 50% glycerol.

So that these two strains could be counted individually when grown in co-culture, a rifampicin-resistant mutant, was obtained after incubating the strain *Lb. sake ATCC15521* on MRS + 0.8% (w/v) rifampicin (Sigma Aldrich, Germany). From there, this mutant strain *Lb. sake ATCC15521*^{RifR} was then used as the nisin-sensitive strain and counted on the selective medium MRS + 0.8% (w/v) rifampicin.

2.2. Manufacture of the three model cheeses inoculated with the two strains

Skimmed milk was microfiltrated to remove the indigenous microflora and then ultra-filtrated (UF) according to (Ulve *et al*., 2008) except that no NaCl and no cream were added, giving a non-salty and non-fat UF milk retentate. It was then stored at -20°C. The total proteins of milk were concentrated 4.2 times, and the retentate composition was as follows: 208.5 g/kg dry matter, 146.4 g/kg total nitrogen in which 118.6 g/kg caseins and 26.1 g/kg whey proteins. The pH of retentate was 6.64 ± 0.01.

The UF retentate was heat-treated at 93°C/15 min. 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 matrices during the experiment (Ferron-Baumy *et al.,* 1991*;* Vaziri *et al*., 2010). Indeed, it led to more than 80% denaturation

of whey proteins, which denaturation percentage was calculated as described by Aly *et al* (in press).

As explained before, the influence of gelatin incorporation on *in situ* nisin activity was assessed by manufacturing three different model cheeses differing in their gelatin content (0, 4, and 10%). The heat-treated retentate in which no gelatin was added was used to make the UF model cheese (R) considered as a reference. The two other UF model cheeses were made by replacing with gelatin either 4% (R-G4%) or 10% (R-G10%) of the UF retentate dry matter. To reach these final concentrations of 4% and 10% (v/v) of gelatin in the model cheeses, 18.5% and 46.2% (v/v) of the UF retentate were respectively replaced by a gelatin stock solution made of 210 g/L gelatin in deionized water, pH=6.6 (gelatin type B from bovine skin, Bloom 225, Sigma Aldrich, Germany). All the three model cheeses had the same initial dry matter (21.17 ± 0.12 %) and the same initial pH (6.64 ± 0.01). Even if many manufactures of model cheeses have been performed to measure the growth, only duplicates were presented in the present results.

Lc. lactis UL719 and *Lb. sake ATCC15521*^{RifR} were grown in M17 + 0.5% lactose and MRS, respectively, at 30°C for 24 h, and then separately pre-cultured in sterile reconstituted milk from 10% (w/w) of milk powder (DIFCO, France), at 30°C for 24 h. Subsequently, *Lc. lactis UL719* cells were washed in a sterile citrate solution and the cells were then pelleted by centrifugation at 6000g for 10 min. This washing step was essential to eliminate the nisin already produced by *Lc. lactis* in the overnight milk culture. The *Lc. lactis* washed cells and the *Lb. sake* overnight culture were used to inoculate the model cheeses. They were all inoculated with *Lc. lactis UL719* at $3.39 \times 10^6 \pm 1$ cfu/mL and with *Lb. sake ATCC15521*^{RifR} at $4.72 \times 10^5 \pm 1$ cfu/mL. Then, a chymosine agent Maxiren (DSM Food Specialties, France) was added at a final concentration of 0.03% (v/v) and homogeneously mixed. The renneted mixtures were distributed as 12 mL aliquots in sterile plastic pots (one for each sampling

time). To complete the coagulation step, all cheeses were incubated at 30°C for 1 h, and then at 19°C for 24 h. Sampling times were: immediately after manufacture, after 14 h, 16 h and after 24 h of incubation. Cell enumerations and pH measurement were performed at these time points.

2.3. Physico-chemical characterization of the three model cheeses

Dry matter content

Moisture content was measured in duplicate in the UF model cheeses (IDF, 1993) immediately after coagulation.

Water activity (a_w)

This measurement was performed at 19°C using a water activity meter instrument (Fast lab, GBX, France) as described by Aly *et al.* (in press). Measurements were performed in triplicates after 24 h of incubation at 19°C. Differences between the mean A_w were tested using a Student test, with the T.Test function of Excel software (Microsoft Inc.).

Buffering capacity

Buffering capacity of the heat-treated UF retentate and the heat-treated UF retentate-gelatin mixtures was measured by the titration of 30 g of the sample (two independents samples) from the initial pH to pH=5 using 1N HCL automatically added by 0.15 mL increments each 120 s. Buffering capacity was performed using an automatic system (Titrino Metrohm 702 SM, Switzerland). The buffering index (d*B*/dpH) was calculated using the buffering intensity equation given by Van Slyke (1922). This ratio expresses the relationship between the volume of acid added at each increment (0.15 mL) and the changes in pH and calculated according to equation (1):

$$\frac{dB}{dpH} = \frac{(volume\ of\ acid\ added) \times (normality\ of\ acid)}{(volume\ of\ sample) \times (pH\ change\ produced)} \tag{1}$$

Buffer indices were then plotted against pH values to find the maximum buffering indexes. Means of buffering capacities were statistically compared using a Student test with the T.TEST function of Excel Software (Microsoft Inc.).

Lactate quantification

Concentrations of lactate were determined in the aqueous phase of the model cheeses inoculated with the nisin-producing strain after 24 h incubation at 19°C. The cheese extracts were prepared as detailed for cell enumerations, except that 10 g of cheese were 1/5 diluted in ultra-pure water. Then, the mix was centrifuged at 10 000g for 8 min at 4°C, and the supernatant was further filtrated on 10 kDa cut-off columns (Vivaspin 20,Sartorius, Germany) at 8000g for 20 min.

Separation was carried out, for the filtrated permeate, on ion-exchange chromatography system ICS 3000 (Dionex S.A., France) using the principle of patent Small (Dow Chemicals, USA) with an anion exchange column (Dionex IonPac AS11, 4 x 250 mm), a self-regenerating suppressor Dionex ASRS 300, 4 mm and a conductivity detector. Elution was performed using a gradient of sodium hydroxide from 0.5 to 34.0 mM and the sample signal was finally compared to the signal of the reference range of the lactate.

2.4. Cell enumerations and pH measurements

For the microbial analysis, cheese samples were diluted at 1/10 (w/w) in sterile citrate solution (2%, Carlo-Erba Reagents, France). The mixture was blended for 1 min using a laboratory blender (Waring laboratory science®, Grosseron, France). Serial tenfold dilution was prepared using sterile tryptone water (0.1%, Biokar diagnostics, France). For cell counts, M17 agar + 0.5% lactose was used as a selective media for *Lc. lactis UL719,* aerobically incubated at 30°C for 2 days, while MRS agar with 0.8% (w/v) rifampicin was used as a selective media for *Lb. sake ATCC15521*^{RifR} anaerobically incubated at 30°C for 2 days.

Means of pH values after 14, 16 and 24 h were statistically compared using a Student test with the F.TEST function of Excel Software (Microsoft Inc.).

2.5. Controls for nutritional competition and acidification

The same protocol was performed as previously described but the nisin-producing strain was replaced by a non nisin-producing strain, selected to be from the same species as *Lc. lactis UL719*, i.e. *Lc. lactis* subsp. *lactis* biovar *diacetylactis SRTA2116*, in the co-culture with *Lb sake*. Sampling and samples preparation for cell enumeration were performed as previously described. Growth of both strains and pH were also followed after 0, 14 and 24 h of incubation at 19°C.

2.6. Nisin quantification

To quantify the secreted nisin, repetitions (n=2) of the three model cheeses were made using exactly the same protocol except that *Lb. sake* was not inoculated, because part of the secreted nisin molecules could have been adsorbed on the cell membrane and be not detectable anymore. Cheese samples were prepared either as described before for cell enumeration for the bioassay, adding one step of filtration on 0.2 µm syringe filter (Minisart®, Germany), or as described in Aly *et al* (in press) for ELISA. For both methods, a standard curve allowed quantification.

Nisin agar diffusion bioassay

This method is based on the measurement of the diameter of the inhibition zone when nisin is diffusing in an agar medium inoculated with a nisin-sensitive microorganism (Pongtharangkul and Demiri, 2004). Quantification is based on a standard curve.

Stock nisin solutions at 0.1 and 10 mg/L were concomitantly prepared by adding commercial nisin powder (Shanghai Richem international Co., China) into sterile diluent solution (0.02 N HCl). Standard nisin solutions of 0.1, 0.5, 1, 10, 20 and 30 mg/L were then prepared in the diluent solution in order to draw the standard curve. The standard curve was then plotted

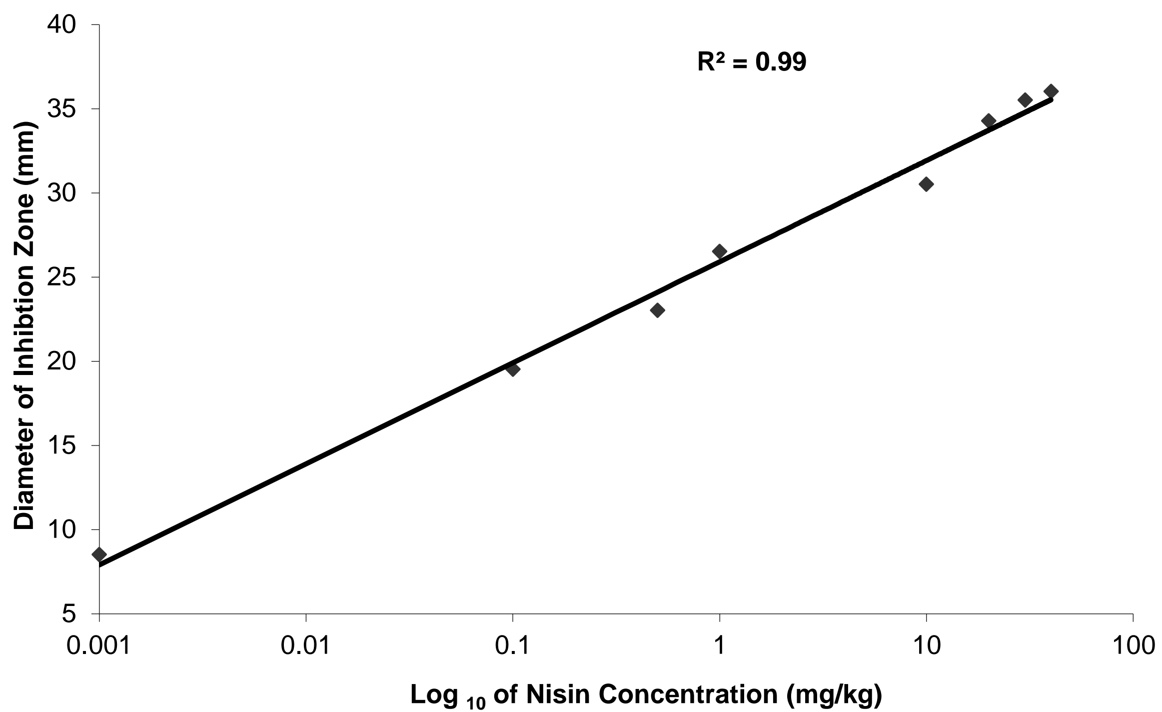

Figure 1: Nisin standard curve, correlating nisin concentration (log_{10} mg/kg) *versus* diameter of inhibition zone (mm)

(Figure 1) and a linear regression was calculated using Excel software (Microsoft Inc.) with a correlation coefficient (R^2=0.99).

Warm MRS agar was inoculated with 0.1% of exponentially growing culture of the nisin-sensitive strain (*Lb. sake ATCC15521*^{RifR}). After MRS solidification, identical wells were done and filled up with 50 µl of either the cheese filtrates, in which the *in situ* nisin was produced, or nisin standard solutions. Diameters of inhibition zones (mm) *versus* $\log_{10}$ nisin concentrations (mg/L) were plotted in order to obtain the standard curve.

Quantitative inhibition ELISA

The ELISA used was the inhibition ELISA described by Aly *et al* (in press) especially developed for nisin quantification in cheese. Briefly, the secreted nisin was extracted from the cheese samples by 1/5 diluting them in citrate water pH=5 and homogenizing them with an Ultra-Turrax T25 (IKA Labortechnik, Staufen, Germany). After centrifugation, serial dilutions were performed with the cheese extract.

A polystyrene microtiter plate (Maxisorp, Nunc®, Denmark) was coated with 100 µL/well of 0.5 µg/mL nisin in 0.1 M sodium carbonate-bicarbonate buffer (pH=9.6). In parallel, serial tenfold dilution of samples (from 1:20 to 1:500) were made in PBS-T (0.05% Tween20 in 0.05 M phosphate buffered saline) and incubated at 37°C for 1 h in test tubes with the same volume of rabbit polyclonal anti-nisin antiserum diluted at 1:1250 in PBS-T (Stanic *et al.,* 2010). After incubation, 100 µL 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 µL of substrate (1 mg/mL tablet of p-nitrophenyl phosphate - KPL, USA - diluted in 1 M diethanolamine) and measured as OD_{405}.

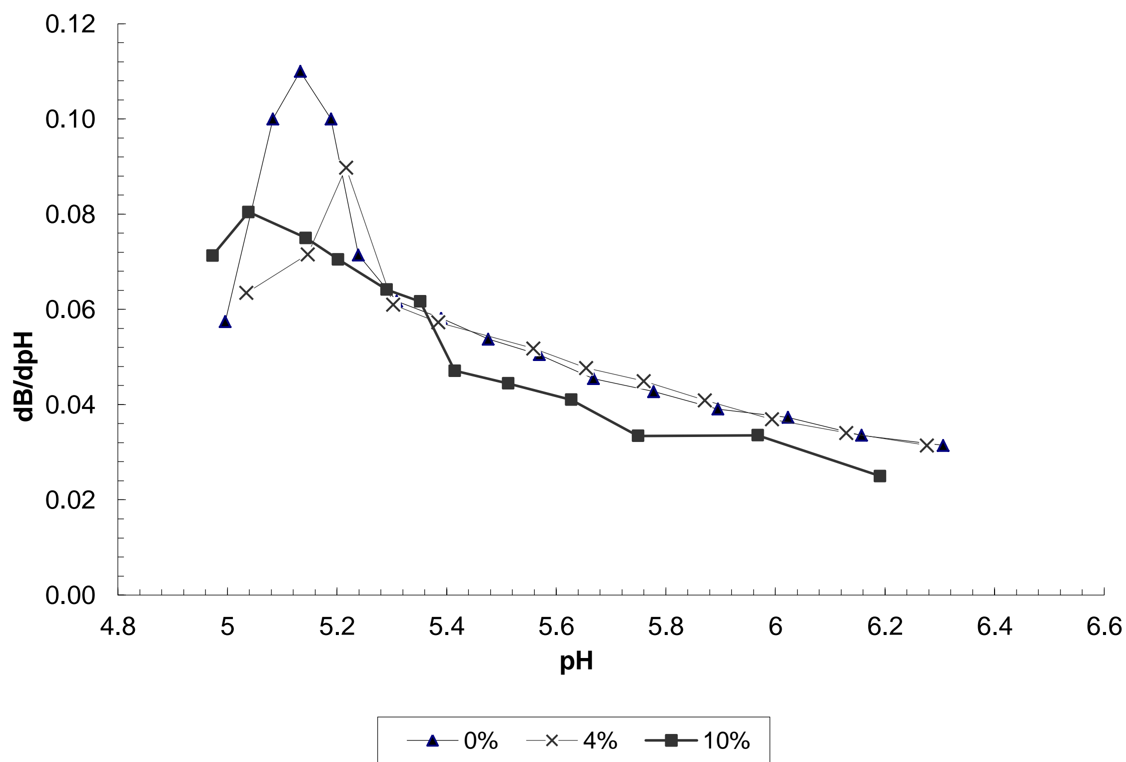

Figure 2: Buffering capacity of the heat treated UF retentate and the heat treated UF retentate-gelatin mixtures, with 4 and 10% of gelatin, from initial pH (about 6.6) to pH about 5.0 with 1N HCl (n=2)

For quantification, a standard curve was prepared from 0.8 to 80 mg/L of exogenous nisin Z (Shanghai Richem international Co., China) added to the UF retentate before manufacture of the spiked model cheeses whose extracts were also prepared as previously described.

All the analytical characteristic of the assay are detailed in Aly *et al* (in press). To summarize, the limit of detection was 0.626 mg/L. It was calculated from the mean of 20 nisin quantification in a negative control (model cheese made without added nisin) plus 3 times the standard deviation (SD) of the mean (mean + 3SD) (Dupont *et al.,* 2007). The accuracy of the assay was estimated from concentration recoveries varying from 98% to 120% with a correlation coefficient of R^2=0.99. It was estimated from a spike-and-recovery experiment adding exogenous nisin Z (Shanghai Richem international Co., China) at known concentrations in specific UF model cheeses.

3. Results:

3.1. Characterization of the three model cheeses

Water activity

No significant difference ($p < 0.05$) could be observed in the water activities (a_w) of the three model chesses after 24 h of incubation at 19°C. The observed values were: 0.993 ± 0.003 for R cheese, 0.990 ± 0.003 for R-G4% cheese and 0.997 ± 0.003 for R-G10% cheese. The presence of gelatin did not modify the a_w when the model cheeses were inoculated with an acidifying strain.

Buffering capacity

In the chosen pH range (from 6.5 to 5.0), the R cheese showed more buffering capacity than the R-G10% cheese did (Figure 2). The means of buffering capacity indices at pH 6.04 ± 0.015 were found statistically different (p<0.05). Maximum buffering capacity was observed around pH 5.10 for treatments with 0 and 4% of gelatin, while for the treatment with 10% of gelatin, the maximum buffering capacity was observed at 5.03. The lower the buffering

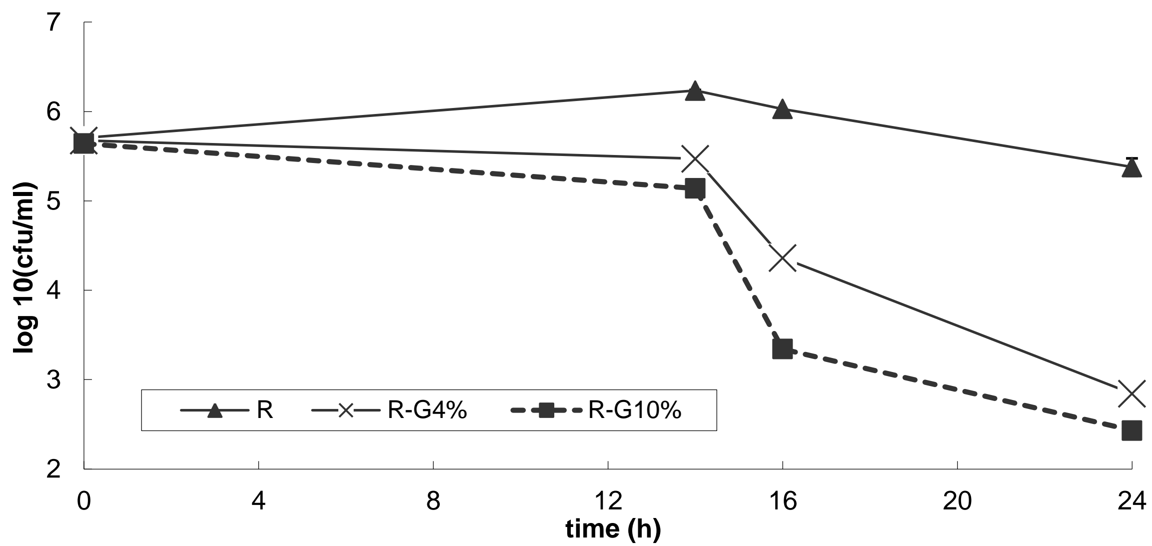

Figure 3: Enumeration of *Lb. sake ATCC 15521*^{RifR} in UF co-culture with the nisin^{+} strain *Lactococcus lactis subsp. Lactis biovar. diacetylactis UL719* in the model cheeses with different percentage of gelatin

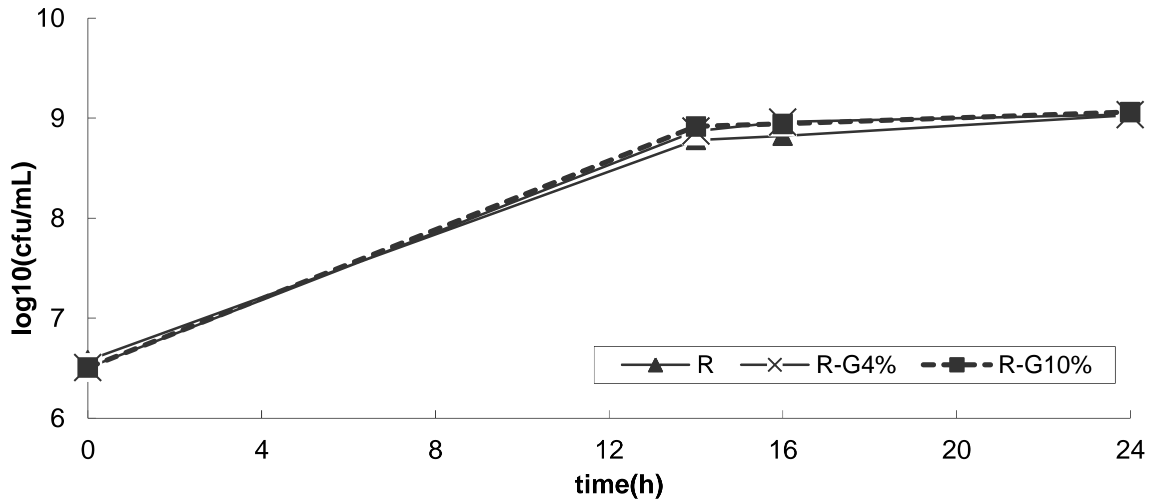

Figure 4: Enumeration of the nisin^{+} strain *Lactococcus lactis subsp. Lactis biovar. diacetylactis UL719* in UF model cheeses in co-culture with *Lb. sake ATCC 15521*^{RifR} with different percentage of gelatin.

capacity is, the higher the reduction in the pH with the same quantity of lactate. Thus, the observed difference in the buffering capacities between R and R-G10% cheeses could explain a difference in the final pH of these two model cheeses.

Lactate concentrations

The production of lactate was positively correlated with the proportion of incorporated gelatin. Lactate concentrations were 343 ± 11 mg/kg in R cheese, 534 ± 10 mg/kg in R-G4% cheese and 622 ± 22 mg/kg in R-G10% cheese. Even if lactose was half diluted from the beginning in R-G10% cheese by incorporation of gelatin, non-negligible quantities of lactose could be measured after 24 h (data not shown).

3.2. *Populations of the two strains and acidification*

Populations of the two strains

Figures 3 and 4 show the populations profiles of the nisin-sensitive strain *Lb. sake ATCC15521*^{RifR} and the nisin-producing strain *Lc. lactis UL719* throughout 24 h at 19°C. *Lb. sake ATCC15521*^{RifR} viability was significantly lowered by an increasing proportion of incorporated gelatin. The more gelatin was incorporated, the faster viability was lost. After 14 h of incubation, *Lb. sake ATCC15521*^{RifR} grew only in R cheese (+0.53 ± 0.023 $\log_{10}$ cfu/mL), while it started to lose viability within 14 h in R-G4% and R-G10% cheeses (-0.20 ± 0.010 and -0.50 ± 0.007 $\log_{10}$ cfu/mL, respectively). The most drastic loss of viability was observed between 14 h and 16 h in R-G4% and R-G10% cheeses, -1.11 and -1.80 $\log_{10}$ cfu/mL, respectively (only -0.21 $\log_{10}$ cfu/mL in R cheese). After 24 h of incubation at 19°C, the nisin-sensitive strain has lost 0.33 ± 0.114 $\log_{10}$ cfu/mL in R cheese, while it has lost 2.83 ± 0.037 and 3.21 ± 0.011 $\log_{10}$ cfu/mL in R-G4% and R-G10% cheeses, respectively.

Figure 4 shows that the growth of *Lc. lactis UL719* followed the same kinetics in the three different model cheeses, meaning that the growth of nisin-producing strain was not affected by the presence of gelatin. *Lc. lactis UL719* grew within 14 h (+2.33 $\log_{10}$ cfu/mL between 0

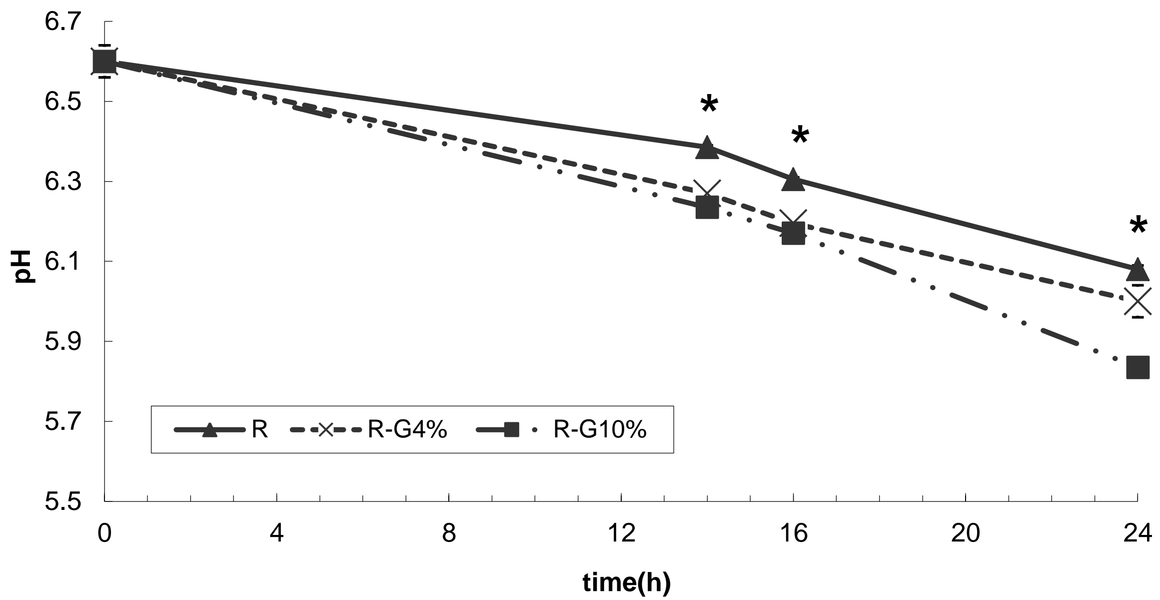

Figure 5: pH of model cheeses with different percentages of gelatin when inoculated with *Lc. lactis UL719* (nisin^{+}) and *Lb. sake ATCC15521*^{RifR}. Statistical differences are indicated by (*) at a threshold of $p<0.05$.

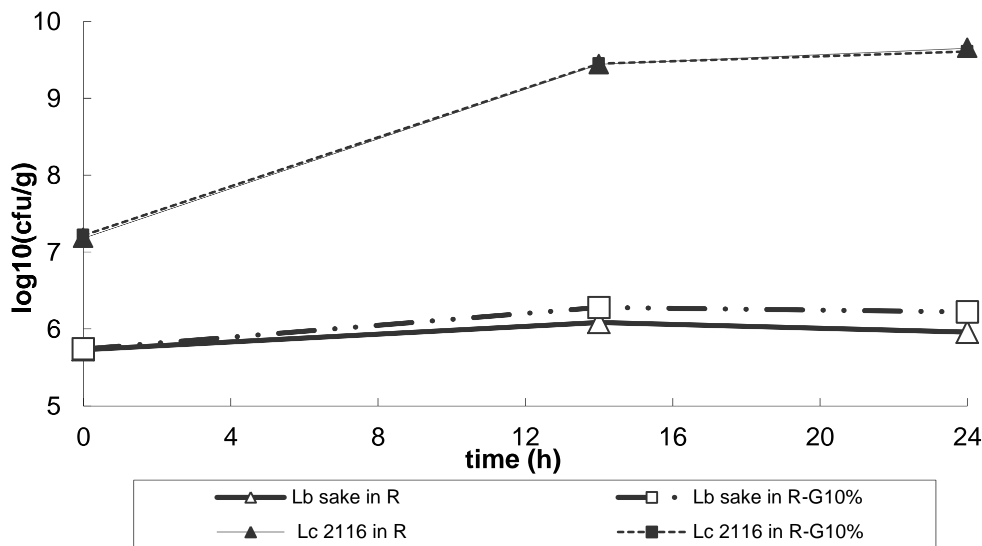

Figure 6: Enumeration of *Lb. sake ATCC15521*^{RifR} and *Lc. lactis* subsp. *lactis* biovar *diacetylactis SRTA 2116* (nisin^{-}) in two model cheeses with 0 and 10% gelatin.

and 14 h), and then remained stable until the end of the experiment (+0.19 log_{10} cfu/mL between 14 h and 24 h).

Acidification

The acidification (expressed as pH reduction) was a result of *Lc. lactis UL719* acidification only (Figure 5). Replacing part of the retentate by gelatin in R-G4% and R-G10% cheeses decreased significantly ($p<0.05$) the pH values compared to R cheese, even if the differences in pH were small. The more gelatin was added, the lower the final pH. The three model cheeses had the same initial pH equal to 6.60 ± 0.040. Then, pH decreased faster, in R-G4% and R-G10% cheeses during 16 h than in the R model cheese. However, between 16 h and 24 h, the acidification in R-G4% cheese slowed down.

3.3. Influence of nutritional competition, acidification and gelatin incorporation on Lb. sake viability

To explain the loss of viability of *Lb. sake ATCC15521*^{RifR} in the presence of a nisin-producing strain, few hypotheses could be drawn: (i) is there a nutritional competition between the *Lb. sake* and the *Lc. lactis* strains? (ii) does the acidification produced by the nisin-producing strain affect the *Lb. sake* viability? and (iii) is there an effect of the presence of gelatin on *Lb. sake* viability?

The nisin non-producing strain *Lc. lactis* subsp. *lactis* biovar *diacetylactys SRTA2116* was chosen to be exactly the same subspecies as *Lc. lactis UL719*, with most likely the same metabolism. When inoculated with *Lc. lactis SRTA2116*, *Lb sake ATCC15521* displayed a stable population within 24 h, with no loss of viability in both R and R-G10% cheeses (Figure 6). The presence of gelatin did not affect the survival of *Lb. sake*. At the same time, populations of *Lc. lactis SRTA2116* were not affected by the presence of the gelatin (Figure 6) with a similar development in both R cheese (+2.45 ± 0.01 log_{10} cfu/mL) and R-G10% cheese (+2.56 ± 0.04 log_{10} cfu/mL). The presence of gelatin did not affect the growth of any of the

Table 1: Quantification of active nisin produced *in situ* by the nisin-producing strain *Lactococcus lactis UL719* in UF model cheeses with different percentage of gelatin using the agar diffusion method (n=4)

Gelatin% in UF model cheeses	Active nisin concentration (mg/kg)			
	After 14h		After 24h	
	Mean	S.D	Mean	S.D
0%	1,45	0,274	2,12	0,402
4%	1,75	0,332	2,57	0,487
10%	1,75	0,332	2,57	0,487

Table 2: Quantification of nisin produced *in situ* by the nisin-producing strain *Lactococcus lactis UL719* in UF model cheeses with percentage of gelatin by ELISA inhibition method (n=4)

Gelatin% in UF model cheeses	Nisin concentration (mg/kg)					
	After 14h		After 16h		After 24h	
	Mean	S.D	Mean	S.D	Mean	S.D
0%	2,55	0,30	3,16	0,11	3,68	0,25
4%	2,45	0,07	3,18	0,14	3,54	0,25
10%	2,44	0,22	3,08	0,04	3,45	0,18

two strains. Furthermore, acidification by *Lc. lactis SRTA2116* of the different matrices was found similar to the acidification of the nisin-producing strain *Lc. lactis UL719* (data not shown). These results confirmed that the loss of viability of *Lb. sake* ATCC15521^{RifR}, in co-culture with *Lc. lactis UL719*, was only due to the bactericidal activity of the secreted nisin.

3.4. Nisin concentrations

Nisin secreted by *Lc. lactis UL719* was quantified using two different methods. The agar diffusion bioassay allows to quantify the concentration of nisin active against *Lb. sake* after its diffusion in an agar medium, whilst inhibition ELISA allows to quantify the concentration of nisin on the basis of the presence of the nisin molecule itself in the cheese extracts, whether it is active or not.

Concentrations of active nisin

The active nisin concentrations were measured in the three model cheeses using the agar diffusion bioassay (Table 1). No significant effect (p<0.05) of the proportion of gelatin incorporated in the matrices was found on the active nisin concentrations, produced *in situ* by the nisin-producing strain *Lc. lactis UL719*. Furthermore, kinetics of nisin production were similar in the three model cheeses throughout time.

Concentrations of secreted nisin

The absolute concentrations of secreted nisin were measured using an inhibition ELISA (Table 2). Concentrations of nisin secreted *in situ* displayed no significant differences (p<0.05) in the three model cheeses, confirming the previous result that nisin production by *Lc. lactis UL719* was neither influenced by the presence nor the quantity of incorporated gelatin.

Discussion

Lb. sake lost viability in a UF cheese when inoculated in co-culture with a nisin-producing strain of *Lc .lactis*. We can conclude, after several controls, that the activity of nisin was effectively responsible of this loss of viability. This bactericidal effect was drastically increased, from 0,3 to 3.2 $\log_{10}$ cfu/mL, when 18% and 46% of the retentate were respectively replaced by gelatin in the model cheeses, maintaining the same initial dry matter. The growth of the nisin-producing *Lactococcus* strain was identical in all cases. However, the final pH was 0,2 pH unit lower in R-G10% cheese in comparison to R cheese. In addition, the final concentration of lactate was higher in the presence of gelatin, suggesting a post-acidification of the *Lactococcus* strain after the exponential growth phase. As *Lb. sake* is known not to be sensitive to acid (Champomier *et al*., 1999; Ammor *et al*., 2005), the reduction of pH cannot be regarded as responsible for the loss of viability. In parallel, ELISA quantification and agar diffusion method showed, respectively, that the absolute concentration of nisin and the concentration of active nisin were the same in the three matrices, independently of the proportion of incorporated gelatin. Thus, three main hypotheses could be raised here to explain our results: (i) the *in situ* efficiency of nisin may be affected by physico-chemical interactions with the milk protein network, (ii) the release of nisin molecules from the producer cells and/or the sensitivity of the target cell membrane could be influenced in the presence of gelatin, and (iii) the migration of the nisin molecules may be faster from the producer strain colonies to the nisin-sensitive ones in the matrices where gelatine was incorporated. It is also possible that these phenomena are cumulative.

The hypothesis of a better *in situ* efficiency of nisin when gelatin is incorporated to replace caseins is supported by the fact that some studies have already showed that nisin could adsorbed to the caseins (Somers & Taylor, 1987; De Vuyst & Vandamme, 1994; Lakamraju *et al*., 1996). In the R model cheese, caseins are twice as much concentrated as in the R-

G10% cheese. Then, if nisin molecules are effectively adsorbed or slowed down by ionic interactions with caseins, they are probably less available for migration, and thus less efficient towards *Lb. sake* cells in R cheese.

Differences in pH values between the three model cheeses (0,2 pH unit between R and R-G10% cheeses) could also explain why *Lb. sake* lost viability significantly faster in the model cheeses where gelatin was present. Indeed, it is known that a decrease in pH enhances the activity of nisin in liquid media. However, Amiali *et al* (1998) showed no difference of activity between pH 5.0 and 5.5 in whey permeate. Moreover, using the agar diffusion bioassay to test the activity of the commercial nisin Z at pH 6.0 and pH 5.8, we observed no differences in nisin activity between these two pH in the agar MRS (data not shown). In contrast, nisin release from the producer cells was shown to be dependent on the pH. At pH 6.0 and above, most of the nisin was adsorbed onto the cell wall of the producer strain while at pH under 6.0, more than 80% of the secreted nisin was released into the medium (Lee & Kim, 1985; Somers & Taylor, 1987; Amiali *et al.,* 1998). Thus, a higher proportion of the secreted nisin could be released in the R-G10% cheese since the final pH was 5.8 instead of 6.0 in the R cheese.

Furthermore, the disruption of the cell membrane by the formation of pores is facilitated by an increase of the membrane potential, $\Delta\psi$ and/or an increase in the pH difference between the internal and the external pH of the membrane, Δ pH, (Alakomi *et al*, 2000*).* Consequently, the lower pH in R-G cheeses could be responsible for the better nisin efficacy than in R cheese. Moreover, the presence of gelatin led to an increased production of lactate, and it was also previously shown that lactate addition improved nisin efficacy in food such as fruit (Dike & Williamf., 2004) or meat-based foods (Long & Phillips, 2003). Many factors can thus explain the better efficiency of nisin in these UF cheeses in presence of gelatin.

Finally, the hypothesis of a faster diffusion is not supported by previous results. The apparent diffusion coefficient for nisin was lower in the UF model cheese with gelatin; it was 34.4 $\mu m^2/s$ in R-G10% cheese, while it was 49.5 $\mu m^2/s$ in R cheese without gelatin (Aly *et al*, in press). However, in the study of Aly *et al* (2011, in press), the apparent diffusion coefficient for nisin was modelled in model cheeses, with and without gelatin, which were not inoculated with microbial strains. In the present work, UF cheeses were inoculated with *Lc. lactis* and thus, the matrices were progressively acidified, most probably leading to different structures of the protein network. Moreover, the distance between colonies could be evaluated to less than 40 µm (Jeanson *et al.,* 2011), which does not support the hypothesis that the diffusion phenomenon could be a limiting factor for the in situ efficiency of nisin on target bacterial colonies.

The concentration of nisin molecules (active or not) quantified by ELISA could only be higher than the concentration of active nisin molecules quantified using the bioassay. The concentration of secreted nisin increased by 2.5 mg/kg during the exponential growth phase (up to 14 h). It increased again by 0.65 mg/kg between 14 and 16 h, and then remained almost stable until 24 h as the *Lc. lactis* strain was in its stationary growth phase. *Lb. sake* displayed a drastic loss of viability between 14 and 16 h when the *Lc. lactis* population and the nisin concentration reached their maximum; but also probably when nisin molecules reached *Lb. sake* cells and formed the membrane pores. Concentrations of active nisin found in the literature are of the same order than our results. Higher concentrations (17.5 mg/L after 9 h) were found *in situ* in Camembert cheese (Maisnier-Patin *et al*., 1992); while lower concentrations (0.71 mg/L after 24 h) were obtained in the Nigerian Wara cheese (Raheem & Saris, 2009). Active nisin concentration could still be measured in Gouda cheese after 6 months (Bouksaim et al., 2000).

The present study showed how the incorporation of gelatin in UF model cheeses increased the efficiency of nisin on its target bacteria, even if gelatin did not influence its production. In the investigated model cheeses, it demonstrates that the concentration of *in situ* active nisin is not directly connected to its efficiency in food matrices. The agar diffusion bioassay or the ELISA quantifications of nisin produced *in situ* did not allow predicting its efficiency on the nisin-sensitive strain. The physico-chemical interactions and/or the bacterial response to the environment, depending on the composition of the matrix, are of key importance on the nisin efficiency in food matrices. These observations may be transferable to any food matrices in which nisin-producing strains are used as food preservatives.

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General Conclusion & Perspectives

General conclusion and Perspectives

In the work undertaken, we integrated technology, microbiology and mathematical approaches to increase the scientific knowledge about nisin diffusion and its *in situ* efficiency in cheese matrices in relation to cheese structure and composition.

The apparent diffusion coefficient for nisin is determined for the first time in a cheese matrix. Since nisin is a positively charged peptide of 3.5 kDa, the results confirm that peptides are able to diffuse in the matrices. Even for small size peptides such as nisin, the composition of the matrix, and thus its microstructure, can influence their apparent diffusion coefficient. This result is of key importance for proteolysis, which takes place during cheese ripening by releasing peptides from caseins, in particular through the action of the cell wall proteases of lactic acid bacteria. As a consequence, these results suggest that the peptides obtained from lactic acid bacteria proteolysis do not accumulate around the colonies and may diffuse within the cheese.

A value of 49.5±0.3 $\mu m^2/s$ was obtained for the apparent diffusion coefficient for nisin in a UF model cheese without gelatin. The apparent diffusion coefficient for nisin is from 2 to 10 times lower than the apparent diffusion coefficient for salt in the literature (100 to 530 $\mu m^2/s$, according to cheese composition and experimental conditions). This can probably be explained by the difference in size of these two solutes. However, like nisin, sodium and chlorides are charged solutes, thus they may generate electrostatic/ionic interactions within the matrix influencing their migration. The literature review (Floury *et al.*, 2010a) shows that it is difficult to take into account ionic interactions with the matrix in the mass transfer modeling. It is also known that the Fick’s approach is not able to differentiate pure diffusion phenomena from solutes interactions. However, it is the easiest model available to experimentally determine the apparent diffusion coefficient for a solute. Finally, this result raises the open question of what is really limiting diffusion of solutes in cheese matrices with

different microstructures, is it only a matter of molecular size or of the molecular charge, or both?

Therefore, a similar value (68±9 µm²/s) was recently obtained in the same model cheese with a 4 kDa FITC-dextran using the imaged-based Fluorescence Recovery After Photobleaching (FRAP) technique (Floury *et al.,* submitted). The FRAP technique presents the great advantage to explore the diffusion properties of solutes in complex media like cheese, *in situ* and on the microscopic scale. Microscopic investigations are particularly relevant and crucial for further understanding of the metabolic activity of immobilized bacteria during the ripening process of cheese. The main limitation of the FRAP technique is that the migrating solute has to be either naturally fluorescent, or labeled with a fluorescent probe. Nevertheless, the FRAP technique is innovative in food science and promising for the investigation of the diffusing properties of ripening metabolites (depending on their size and chemical nature), in cheese matrices of different compositions. Recently, the diffusion coefficient of a 20 kDa FITC-dextran was shown to be three times lower than the one of a 4 kDa using the FRAP technique (Floury *et al.,* submitted; Floury *et al.*, 2010b), proving the major role of the molecular size.

Gelatin incorporation in cheese matrix has modified cheese structure and therefore changed the apparent diffusion coefficient for nisin. Lower nisin diffusion coefficient was found when gelatin was incorporated. The same trend was observed by Floury *et al* (2010b), who studied the diffusion of FITC-dextran molecules (4 and 20 kDa) in the same UF model cheeses with gelatin thanks to the FRAP technique. Once again, the same conclusion can be drawn about the impact of gelatin in the same non-inoculated UF cheese using a different technique and other diffusing solutes.

Knowing (i) the apparent diffusion coefficient for nisin in the cheese matrix, (ii) its concentration thanks to the ELISA, and (iii) the mean theoretical distance between bacterial

colonies (Jeanson *et al*, 2011), it would be interesting to mathematically simulate the order of magnitude of the mass transfer rate for nisin, between a nisin-producing colony to a nisin-sensitive colony.

Besides the “mechanical” migration of nisin in a model cheese, it was of course very important to explore its *in situ* “biological” activity on a target bacterium in model cheese. *Lactobacillus sake* was already known to be a very nisin-sensitive species, and was thus an accurate indicator of the *in situ* activity of nisin. The concentration of the nisin produced by the nisin-producing strain of *Lactococcus lactis* has to be measured to conclude about the relation between concentration and activity. The inhibition ELISA, especially developed to quantify nisin within a cheese matrix, can now be applied to any cheese or dairy matrix. ELISA showed to be a sensitive, repetitive and accurate method to assess nisin concentration within a proteic network. This technique measures the absolute concentration of nisin, either active or inactive, whilst the agar diffusion method measures only the quantity of active nisin. Both techniques are complementary and give the information about of the active nisin in proportion of secreted nisin. The most important conclusion is that, despite the same concentration of secreted nisin, in which the same proportion of active nisin, the composition of the cheese matrices drastically modifies the *in situ* efficiency of nisin towards *Lb. sake*. The incorporation of gelatin, probably by slightly accelerating the acidification, accelerated drastically the loss of viability of *Lb. sake*. Even if the differences in pH were small, it may have influenced the whole microbial system, the nisin release from the nisin-producing strain as well as its bactericidal effect on the target strain. Furthermore, gelatin incorporation most likely led to changes in the nisin interactions with the cheese matrix, especially by decreasing the amount of caseins in the model cheese, and as a result, by decreasing the amount of nisin adsorbed on caseins. In conclusion, the interactions between the immobilized bacteria and the cheese matrix are very complex. To investigate separately the different phenomena (physico-

chemical and biological phenomena) does not give an accurate knowledge of the whole system. Finally, the strategy of using a couple of strains, like a nisin-producing strain and nisin-sensitive one, can now be used in any cheese matrix or even any other food matrix to test the *in situ* efficiency of nisin. This method gives a true vision about nisin *in situ* activity and its interaction within the matrix, depending on its composition and microstructure.

In general, the investigation on the microscopic scale of the biochemical and microbial phenomena will first need further developments of *in situ* and non-destructive techniques but will allow a better understanding of the *in situ* interactions between the bacteria, or bacterial colony, and the cheese matrix.

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Annexes

Development of the new methods used in this thesis

Development of the UF model cheese:

As there is no available data in the literature about nisin or peptide diffusion in cheese matrix, we made the choice to use a simple model cheese without adding salt or milk fat to simplify the modelisation to obtain the order of magnitude for the first time.

In addition, milk ultrafitrate (UF retentate) was chosen as the raw material for the manufacture the model cheeses to ensure their reproducibility (kilos of UF retentate were stored at -20°C), to provide homogeneous matrices as well as to avoid the draining step.

To be able to study the diffusion phenomena, we had to avoid cheese syneresis. To achieve that goal, several treatments were tried including: different percentage of rennet (0.01 and 0.03%), different incubation temperatures (15, 19 and 24°C) and different levels of heat-treatments. The best conditions were: the use of rennet at 0.03%, the use of 19°C as in incubation temperature and the heat-treatment at 93°C / 10 min (then cooling in ice-water for 2 min).

Development of ELISA inhibition method:

There were two key steps in the development of ELISA inhibition method:

1. **Nisin Extraction:**

   The first challenge was to optimize the nisin extraction from model cheeses. Several buffers were tried to optimize nisin extraction from our UF model cheeses. These buffers were: pure water, nisin buffer (pH=2), citrate water (pH=7) and acidified citrate water (pH=5). The best results were obtained when using the acidified citrate water to extract the nisin from the model cheeses. Samples were homogenized by Ultra-Turrax 25 for 2 min at 8500 rpm. The extract was then centrifuged at 6000g for 10 min. The supernatant was stored in aliquots at -80°C until analysis.

2. Quantification:

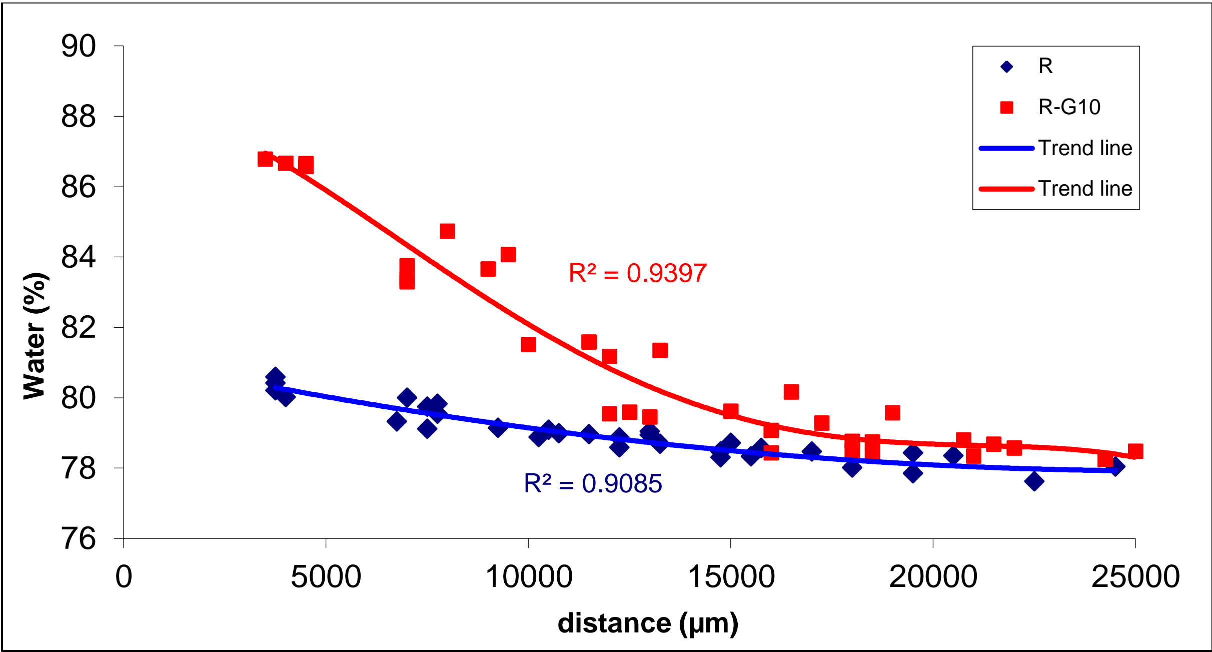
The other challenge was to optimize the standard curve for quantification. Two different type of standard curve could be made (1) with pure nisin solutions or (2) with spiked cheese with known concentrations of nisin. With the last one we obtained the highest accuracy (from 98 to 120%). Finally, the standard curve was made using spiked cheese with nisin final concentrations of 0.8, 1.6, 8, 16, 80 mg/L of the UF retentate.

Development of the microbiological strategy:

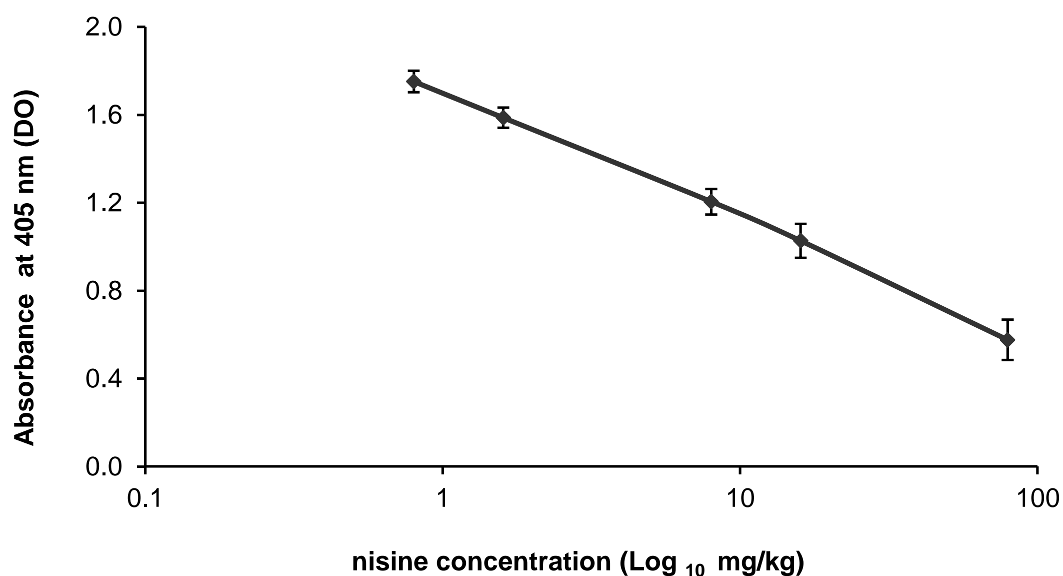
When we started optimizing the co-culture conditions, we did not wash the cells of the producing strain (*Lactococcus lactis* subsp. *lactis* biovar *diacetylactis UL719*) before inoculation within the model cheese in co-culture with the sensitive strain (*Lactobacillus sake ATCC15521*). Without this essential step, the sensitive strain lost its viability just after few minutes. After several attempts, we realized that this fast loss of viability was due to the nisin already produced in the milk pre-culture of the *Lactococcus lactis* strain. The washing step was essential to be sure that the loss of viability of the sensitive strain was only due to the nisin produced *in situ* in the model cheeses.

The pellet of the nisin producing cells was washed with citrate water performed after centrifugation of at 6000g for 10 min at 18°C. After centrifugation, the washed cells were then diluted in fresh sterile milk and inoculated in the manufacturing of the UF model cheeses.

The data not shown



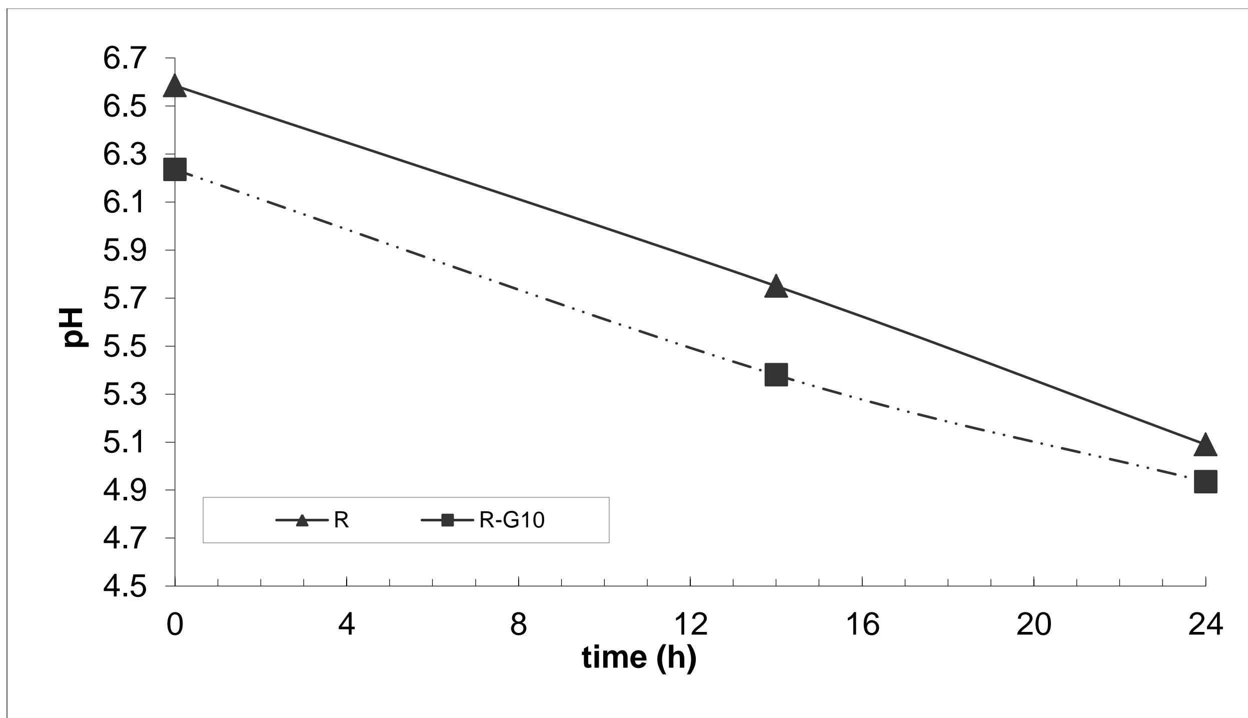
Evolution of water content in the slices of the model cheese cylinders during migration of nisin.



Calibration curve of the optimized ELISA for nisin quantification. The curve is drawn from the means ± standard deviation of five assays run on 5 different days. The curves were prepared using duplicate for each standard concentration, with $R^2 \geq 0.99$ (total n=25).

Lactose and lactate concentrations (mg/kg) in the UF model cheeses with different percentage of gelatin as inoculated by *Lactococcus lactis* subsp. *lactis* biovar *diacetylactis UL719* (n=4) after 24 hours of incubation at 19°C

Gelatin% in the UF model cheeses	**Lactose**		**Lactate**	
	Mean	**S.D**	**Mean**	**S.D**
0%	6752,90	342,87	342,87	10,73
4%	6237,65	534,11	534,11	9,98
10%	3536,80	622,43	622,43	22.41



Acidification (pH) of the UF model cheeses with different % of gelatin, inoculated with *Lactococcus lactis* subsp. *lactis* biovar *diacetylactis SRTA2116* (nisin^{-}) and *Lactobacillus sake ATCC15521*^{RifR}.

Means of the diameter of the inhibition zone (mm) representing the activity of the commercial Z-nisin using the in the agar diffusion method (n=3), at different pH.

pH	nisin concentration (mg/L)					
	0.1		1		10	
	Mean	S.D	Mean	S.D	Mean	S.D
5.8	19.00[a]	0.00	27.67[b]	0.47	35.17[c]	0.24
5.9	18.17[a]	0.24	27.00[b]	0.00	35.15[c]	0.25
6.0	18.67[a]	0.47	27.33[b]	0.47	35.33[c]	0.23

The same letters indicate no significant difference between values at $p<0.05$

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Spatial Distribution of Bacterial Colonies in a Model Cheese[▽]

S. Jeanson,[1,2]* J. Chadœuf,[3] M. N. Madec,[1,2] S. Aly,[1,2] J. Floury,[1,2] T. F. Brocklehurst,[4] and S. Lortal[1,2]

INRA, UMR1253, F-35000 Rennes, France[1]; Agrocampus Ouest, UMR1253, F-35000 Rennes, France[2]; INRA, UR546 BioSP, F-84914 Avignon, France[3]; and Institute of Food Research, Norwich Research Park, Norwich NR4 7UA, United Kingdom[4]

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In most ripened cheeses, bacteria are responsible for the ripening process. Immobilized in the cheese matrix, they grow as colonies. Therefore, their distribution as well as the distance between them are of major importance for ripening steps since metabolites diffuse within the cheese matrix. No data are available to date about the spatial distribution of bacterial colonies in cheese. This is the first study to model the distribution of bacterial colonies in a food-type matrix using nondestructive techniques. We compared (i) the mean theoretical three-dimensional (3D) distances between colonies calculated on the basis of inoculation levels and considering colony distribution to be random and (ii) experimental measurements using confocal microscopy photographs of fluorescent colonies of a *Lactococcus lactis* strain producing green fluorescent protein (GFP) inoculated, at different levels, into a model cheese made by ultrafiltration (UF). Enumerations showed that the final numbers of cells were identical whatever the inoculation level (10^4 to 10^7 CFU/g). Bacterial colonies were shown to be randomly distributed, fitting Poisson's model. The initial inoculation level strongly influenced the mean distances between colonies (from 25 μm to 250 μm) and also their mean diameters. The lower the inoculation level, the larger the colonies were and the further away from each other. Multiplying the inoculation level by 50 multiplied the interfacial area of exchange with the cheese matrix by 7 for the same cell biomass. We finally suggested that final cell numbers should be discussed together with inoculation levels to take into account the distribution and, consequently, the interfacial area of colonies, which can have a significant influence on the cheese-ripening process on a microscopic scale.

During cheese making, regardless of the cheese type, bacteria are immobilized in the curd during the coagulation step. It is generally accepted that 90% of the bacteria present in the milk are retained, trapped in the curd, while only 10% are lost in the whey during draining (16). In cheeses made by ultrafiltration (UF), the draining step is absent, and 100% of the cells are then retained in the curd. In any case, after immobilization by coagulation, each inoculated bacterial cell is assumed to grow, generating a colony inside the curd. Colonies are then spread within the cheese curd, and they interact with the cheese matrix during ripening. Consequently, the ripening process must take place on a microscopic scale around colonies. Only studies showing microscopic examinations of bacterial colonies in cheese either by electronic microscopy (24) or, more recently, by confocal laser scanning microscopy (7, 19) have been reported.

The ripening process (proteolysis, lipolysis, amino acid catabolism, and the production of organic acids, etc.) relies on the metabolic activities of bacterial colonies, leading to the formation of flavors and textures of cheese (11, 25). So far, ripening has always been described with average processes on the cheese scale with destructive techniques like grinding (5, 12, 23) or slicing (10), and microgradients of nutrients and metabolites are thus assumed to occur between colonies in the cheese matrix. Ripening process kinetics should then depend not only on the activities of colonies but also on the spatial organization of colonies inside the matrix. Currently, there are no quantitative data about the spatial distribution of bacterial colonies within a cheese matrix or any other food-like matrix. Our hypothesis is that the distance between colonies is a crucial parameter to understand cheese ripening. Our hypothesis is based on (i) that the distribution of colonies will change the distribution of bacterial enzymes in the cheese matrix and (ii) that interactions between colonies will be modulated by the distance between them, as metabolites must diffuse from one colony to its neighbor.

The distribution of immobilized bacteria in food has been described on a macroscopic scale both for minced meat, by grinding meat samples (27), and for Cheddar cheese blocks, by cutting cheese sections (29), using destructive techniques. Maps of the average cell numbers for each neighbor section were then drawn to describe the macroscopic distribution of bacteria in Cheddar cheese. In minced meat, theoretical Poisson and gamma Poisson distributions were fitted to experimental data in order to determine how many steps of grinding were necessary to obtain a random distribution of pathogens. It was finally not clear which one of these two models of distribution was the most accurate for this objective (35). In cheese, bacteria are immobilized after a long stirring step and the coagulation step. The spatial repartition of colonies should then depend on the distribution of bacterial cells at the end of the immobilization step, on the spatial distribution of nutrients, and on the interactions between colonies. If bacteria are not well mixed in milk before being immobilized or if the matrix is not homogeneous, so that some regions are favorable for bacterial growth (a high concentration of nutrients, for example) or so that bacteria cannot grow in one of the components of the matrix (the fat phase, for example), then an aggregative distribution, for example, a Neyman-Scott distribution (9),

* Corresponding author. Mailing address: INRA-UMR STLO, 65 Rue de Saint-Brieuc, 35042 Rennes Cedex, France. Phone: 33 (0) 223 485 337. Fax: 33 (0) 223 485 350. E-mail: sophie.jeanson@rennes.inra.fr.
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would be expected. If colonies compete for nutrients very early, not all immobilized cells would have developed as a colony, and a regular repartition of colonies is then expected, such as a Gibbs model distribution (9). Finally, in a homogeneous matrix with an excess of nutrients (lactose and proteins, etc.), no interaction is suspected, at least at the beginning of development, and a complete random distribution is then expected, such as a Poisson distribution (9).

In 1995 (3, 40), the growth of bacterial colonies started to be described for model systems (gelatin or agar media) by measuring the colony surface in transparent medium. The immobilization of two species of pathogenic bacteria, growing as colonies in a solid-cheese-like medium, was shown to decrease the growth rates in comparison with those for liquid milk cultures (32). Therefore, the predictive models of growth in liquid are generally inaccurate for immobilized bacteria, as was shown previously for several bacterial species by Wilson et al. (39). Mean theoretical distances between colonies according to their inoculation level were calculated based on the hypothesis that they were randomly distributed (34). The surface of colonies grown in agar was positively correlated to the mean theoretical distance between colonies.

The present study is the first one to experimentally assess bacterial colony distribution/size in a solid-food matrix using a model system (gel cassette) and nondestructive techniques. The objective of the present work was to provide, for the first time, quantitative experimental data regarding the distribution of bacterial colonies in cheese, depending on the level of inoculation. An optical distortion was revealed by the experimental data with confocal microscopy and was taken into account in the mathematical treatment. Theoretical calculations were first performed and then experimentally validated by using stacked photographs taken with a confocal microscope and statistical image analysis. Because cheese matrices made by UF are homogeneous matrices, with high concentrations of lactose, we tested a random distribution of colonies.

MATERIALS AND METHODS

Strain and growth conditions. A *Lactococcus lactis* strain producing green fluorescent protein (GFP) was chosen to visualize lactococcal colony distribution in a cheese matrix. *L. lactis* subsp. *cremoris* MG1363 (38) carrying plasmid pJIM2246:*gfp* was provided by Marie-Pierre Chapot-Chartier (Micalis, INRA, Jouy-en-Josas, France). Briefly, it was obtained by transferring the transcriptional fusion of the *ldhL* promoter with *gfp* (described by Gory et al. [13]) in the pJIM2246 vector (28). This allows the constitutive expression of GFP under the transcriptional control of the *Lactobacillus sakei* promoter of the lactate dehydrogenase gene (*ldh*). The emission of GFP is then linked to the metabolically active state of the cells. *L. lactis* MG1363(pJIM2246:*gfp*) was stored at −80°C in 15% (vol/vol) glycerol and was first precultured twice in M17 (Difco, Becton Dickinson, Le Pont de Claix, France) supplemented with 0.5% glucose plus 10 μg/ml of chloramphenicol and incubated overnight at 30°C. Because the MG1363 strain has been cured of all its plasmids (38), this strain is a lactose-negative (lac^-) and proteinase-negative ($prtP^-$) strain.

The preculture grown overnight was used to inoculate the ultrafiltration (UF) retentate to targeted inoculation levels of 10^4 CFU/g, 10^5 CFU/g, 10^6 CFU/g, and 10^7 CFU/g. The initial inoculation levels in the gel cassettes were finally measured at 2.1×10^4 CFU/g, 2.0×10^5 CFU/g, 1.6×10^6 CFU/g, and 9.2×10^6 CFU/g by plating enumerations on M17 plates incubated for 48 h at 30°C.

Microfiltration and ultrafiltration of milk. The UF retentate was produced from microfiltrated milk to remove the indigenous microflora as described previously by Ulvé et al. (37), except that no NaCl and no cream were added, giving a nonsalty and nonfatty retentate. Briefly, microfiltration pilot equipment was used with skimmed milk heated at 50°C. It was equipped with 19 P1940 units (Pall-Exekia, Bazet, France) and 4.6 m^2 of Sterilox mineral membranes (0.8-μm pore size). The microfiltrate was then ultrafiltrated using pilot equipment (TIA, Bollene, France) equipped with 13.6 m^2 of mineral membranes with a molecular mass cutoff of 8 kDa (Tami, France).

The total proteins of milk were concentrated 4.2 times, and the retentate composition was as follows: 208.5 g/kg dry matter, 146.4 g/kg total nitrogen, 27.8 g/kg noncasein nitrogen, and 1.73 g/kg nonprotein nitrogen. The pH was 6.64 (±0.01). The UF retentate obtained was stored at −20°C in sterile plastic bottles.

Model cheese making. The UF retentate (35 ml per gel cassette) was thawed at 4°C overnight and then at 48°C 20 min before use.

The UF retentate was stirred, heated up to 93°C for 15 min, and immediately placed into melting ice for 3 min with manual stirring. The temperature dropped to 30°C.

The coagulant agent Maxiren 180 (DSM Food Specialties, France) was 1/10 diluted in sterile water and immediately added at a final concentration of 0.03% into the UF retentate. After inoculation of the strain, the mixture was manually and vigorously stirred for more than 2 min to reach the best possible homogenization.

Preparation of gel cassettes. The gel cassette system (2, 4) was used, as it allows nondestructive microscopic examinations. The cassette is constituted with an acetate frame 2 mm thick with an open window of 10 cm by 10 cm sealed within a sleeve of polyvinyl chloride (PVC) that is 15 μm thick and gas permeable. The whole system was autoclaved at 110°C for 15 min.

About 35 ml of the above-described mixture (retentate plus coagulant agent plus strain) was slowly poured into the gel cassette by the aid of a 50-ml syringe from the top of the frame inside the PVC sleeve. The gel cassette was then vertically incubated at 30°C for 1 h, clamped within a supporting frame that had a Perspex front to prevent the distortion of the cassette. This ensured that the thickness of the coagulated retentate within had a regular thickness of 2 mm. After 1 h, the clamps were removed, and the gel cassettes were incubated horizontally (with air access on both sides) at 19°C for 3 days to avoid syneresis. Before microscopic examination, the gel cassettes were stored at +4°C in order to increase oxygen dissolution in the retentate and improve the GFP fluorescence efficiency.

Confocal microscopy. The microstructural analysis was performed by using an Eclipse-TE2000-C1si inverted microscope (Nikon, Champigny-sur-Marne, France), allowing confocal laser scanning microscopy (CLSM). Confocal experiments were performed by using an argon laser operating at a 488-nm excitation wavelength (emission was detected between 500 and 530 nm). The gel cassettes were examined directly under the confocal microscope without any specific preparation. For all gel cassettes, a lens with a ×10 magnification was used without immersion. The optical field was 1,300 by 1,300 μm. For the gel cassettes inoculated at 1.6×10^6 and 9.2×10^6 CFU/g, a lens with a ×20 magnification was also used, with an optical field of 636 by 636 μm and oil immersion. With the ×10 magnification, small colonies could not be detected. GFP fluorescence was excited with a 488-nm laser fixed at 10% intensity. The detector rate at 515 nm ranged from 6.9 to 7.5 in order to optimize the detection of colonies among the autofluorescence of the UF retentate.

Stacks of photographs were taken under the microscope from the surface of the gel cassette through the PVC film into its depth (100 to 200 μm deep) by 2-μm steps (50 to 100 photographs per stack). At least 12 stacks were taken for each gel cassette by scanning the surface of the 10- by 10-cm gel cassette, leading to a total of about 7,000 photographs.

Calculations of mean theoretical distances. For calculations of mean theoretical distances, we did not use any experimental data.

Based on the assumption that the colonies were evenly distributed at a given density of colonies, i.e., the mean number of colonies per unit volume, mean three-dimensional (3D) distances (d_3) were calculated from center to center with the following equations:

$$d_3(\lambda) = \frac{1}{\sqrt[3]{\lambda}} d_3 \quad (1)$$

$$d_3 = 4\pi \int_{u>0} u^3 e^{-\frac{4}{3}\pi u^3} du \quad (2)$$

The first equation can be understood as a scale change: if the length unit is multiplied by a constant (a), then the distance between neighboring colonies is divided by a, while the number of colonies per unit volume is multiplied by a^3. The second equation comes from the fact that the probability of finding no particle inside the sphere of the radius (u) is as follows:

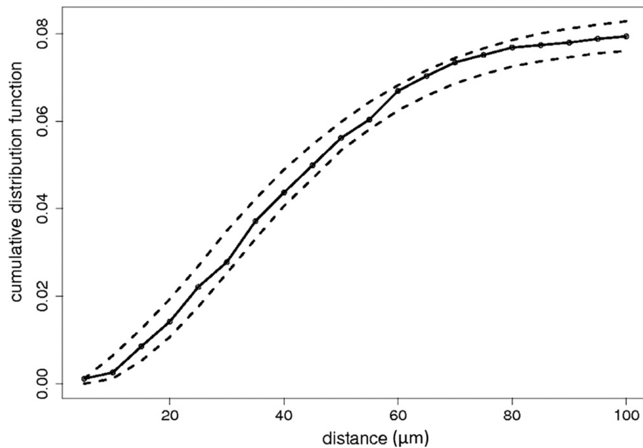

FIG. 1. Test of random repartition of colony sections. Black curve, cumulative distribution function of the distance between a colony and its nearest neighbor; dashed curves, individual confidence band at the 5% level.

$$e^{-(4/3)\pi u^3} \quad (3)$$

for a Poisson point process of intensity 1 (31).

Image analysis of experimental photographs. (i) Evaluation of the optical distortion to be introduced into the mathematical model. Fluorescent polystyrene beads (spheres) with a 15-μm diameter (FluoSpheres; Invitrogen, Cergy-Pontoise, France) were inoculated into the cheese and observed under the same conditions in order to clarify the distortion of colonies observed with the confocal microscope (see Results, and for further details, see the Appendix).

(ii) Selection of bacterial colonies among autofluorescent components. Colonies appeared on each photograph as fluorescent components, i.e., groups of connected pixels with a higher intensity than that of their surroundings (see the Appendix). However, cheese matrix autofluorescence generated random noise, with pixels of high intensity randomly spread in the image, so some fluorescent components of high intensity could also be due to autofluorescence. To optimize the detection of bacterial colonies, we chose photographs at a level maximizing the contrast between colonies and background.

(a) Step 1: selection of analyzed photographs from stacks. Two photographs per stack were thus chosen. The selected photographs were separated by 30 μm so that they could be considered an independent repetition, since colonies could not intercept both photographs. More than 120 photographs were all analyzed as ".tif" files with R software (26).

(b) Step 2: detection of fluorescent components. We applied an intensity threshold at an intensity level of 0.1 so that pixels under this threshold were considered background. This threshold was performed since choosing images with a high level of contrast was not sufficient to suppress random noise due to autofluorescence. The 0.1 threshold was chosen so that all components larger than 2 pixels were removed. We then selected fluorescent components of more than 3 pixels to potentially be colonies. Components with fewer than 3 pixels were considered to be autofluorescence. We estimated the intensity distribution, "*g*," of one pixel using all these fluorescent components with fewer than 3 pixels.

(c) Step 3: extraction of colonies among fluorescent components. Colony components differ from autofluorescent components by the fact that the intensity of each one of their constitutive pixels should be higher than that of the autofluorescent ones and more homogeneous between pixels (see Fig. A1 in the Appendix). We then tested if a component of "*j*" pixels was a colony by testing if its total intensity (defined as the sum of the intensities of its pixels) was greater than the expected value under the autofluorescence intensity distribution, "*g*," at the 1% level. This threshold was validated by visually and/or manually confirming several hundred detected components as being real colonies.

(iii) Test of complete random spatial distribution of colonies. As colonies were assumed to be independently uniformly and randomly spread in space, we tested the random spatial distribution, which is an essential prerequisite to estimate colony radius and colony density in cheese. The estimation is presented below in the next paragraph. The assumption of a random uniform distribution of colony centers is fulfilled as soon as bacteria are initially randomly spread in the cheese matrix by the stirring step before coagulation and because colonies are assumed to develop independently from each other. If this assumption is fulfilled, then the centers of colony sections are randomly uniformly spread in the section plane. This consists of testing (9) whether the cumulative distribution function of the distance between a colony section center and its nearest neighbor lies within its confidence band (Fig. 1). This confidence band was obtained by randomly redistributing the centers of the colony section within their sections (9).

(iv) Estimation of colony diameter probability density and colony density. Bacterial colonies in the cheese matrix are modeled as a Boolean model (22), supposing that (i) colony centers are Poisson distributed and (ii) colonies are spheres with a random independent radius, *r*. This model is characterized by the density of colonies (λ) and the probability density of the radius, $a(r)$. Fluorescent colonies observed in space under the confocal microscope appeared to be deformed. They followed a Boolean model with the same mean number of colony centers, but deformed colonies were modeled by ellipsoids of axis length ($2r$, $2r$, and $2kr$), where k is the anisotropy ratio (see Results) and $2r$ is the colony diameter (17).

The sections of colonies in confocal photographs were then modeled as the Boolean process of disks obtained by the intersection of this Boolean model of ellipsoids with a horizontal plane. Its mean number of disk centers per unit area and the probability density of the disk radius as functions of k, λ, and $a(r)$ are given in the Appendix.

Estimations of λ and $a(r)$ were performed by the method of maximum of likelihood (6), after modeling $a(r)$ as a step function, the definition of which is as follows:

$$a(r) = \sum_{i \geq 0} a_i 1_{[\delta i \leq r < \delta(i+1)]} \quad (4)$$

Confidence bands were obtained by block bootstrap (18).

RESULTS

Theoretical spatial distribution of colonies by mathematical calculation. In order to visualize how the theoretical distribution

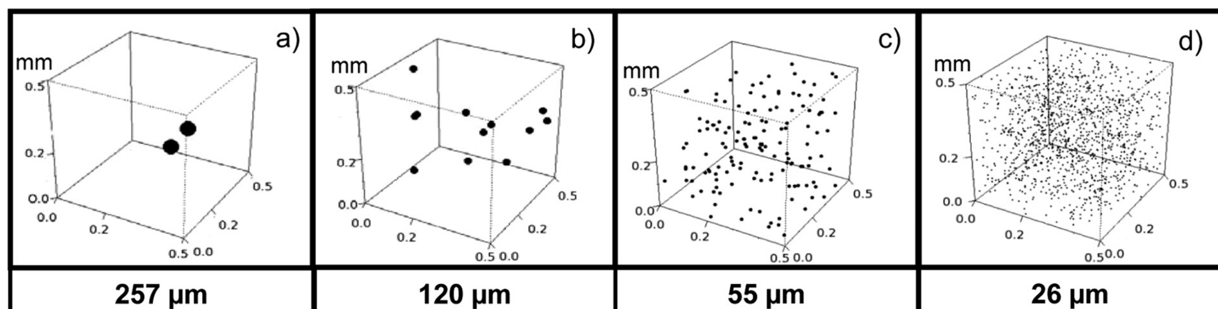

FIG. 2. Theoretical distribution of bacterial colonies in a volume (0.5 by 0.5 by 0.5 mm), such as a piece of cheese, assuming that they are evenly distributed at 10^4 CFU/cm^3 (a), 10^5 CFU/cm^3 (b), 10^6 CFU/cm^3 (c), and 10^7 CFU/cm^3 (d), and associated mean 3D theoretical distances to the nearest neighbor colony.

TABLE 1. Experimental data[a]

Inoculation level (CFU/g)	Observed values from image analysis[b]		Values calculated from observed values	
	Observed density[c] (λ) (colonies/cm^3)	Avg diam (2*r*) (μm) (±σ)	3D distance (d_3) (μm)	Interfacial area (*S*) (cm^2/cm^3)
2.1×10^4	NA	NA		
2.0×10^5	0.90×10^5	10.4 (2.6)	123	1.32
1.6×10^6	1.38×10^6	5.2 (1.6)	50	5.19
9.6×10^6	4.45×10^6	4.0 (0.5)	34	9.49

[a] Shown are enumerations, average densities of colonies per cm^3 (λ) and average diameters (2*r*) computed from the photographs using the mathematical model, average 3D distances (d_3) between the nearest neighbor colonies, and interfacial areas (*S*) of colonies calculated from λ and 2*r*, respectively.
[b] NA, not analyzed because of too few colonies in each photograph.
[c] A value of 1 cm^3 can be considered 1 g in retentate cheese.

of colonies in a volume should be according to the inoculation level, we performed theoretical calculations and representations.

Figure 2 shows the theoretical distribution of colonies in a constant volume, a cube of cheese, for example, when cells are inoculated at 10^4 to 10^7 CFU/g. This 3D visualization brings to light the short distance between colonies when cells are inoculated at 10^7 CFU/g in comparison with an inoculation level of 10^4 CFU/g. The mean distance, $d_3(\lambda)$, from a colony center to the center of its nearest neighbor colony can be calculated (Fig. 2) based on the hypotheses that (i) all inoculated cells grow independently, each leading to a colony, and (ii) inoculated cells are randomly spread, fitting a Poisson distribution.

In a constant volume, depending on the level of inoculation, two colonies or hundreds of colonies can occur, giving a completely different environment in the food matrix. When the inoculation level was increased 1,000 times (from 10^4 to 10^7 CFU/g) the mean theoretical 3D distance decreased 10 times (Fig. 2) down to 26 μm, which is a very short distance between colonies. Space without bacterial activity was widely available when only two colonies occurred, while the space was completely covered by bacterial activity when hundreds of colonies occurred.

Distribution and size of lactococcal colonies in a model cheese depend on the level of inoculation determined by confocal microscopy examinations. We then wanted to check if colonies were truly randomly spread in the model cheese and if mean experimental distances between colonies fitted the mean theoretical ones.

Both the initial inoculation levels and the final numbers of cells after a 3-day incubation period at 19°C were measured by plating enumerations (Table 1). All the gel cassettes reached the same final number of cells, which was $(5.0 \pm 1.6) \times 10^8$ CFU/g, as well as the same final pH (pH 6.39 ± 0.01) whatever the initial inoculation level. These results have been confirmed by several preliminary experiments (data not shown).

Figure 3 shows the fluorescence emission produced by a strain producing GFP grown as colonies throughout a non-

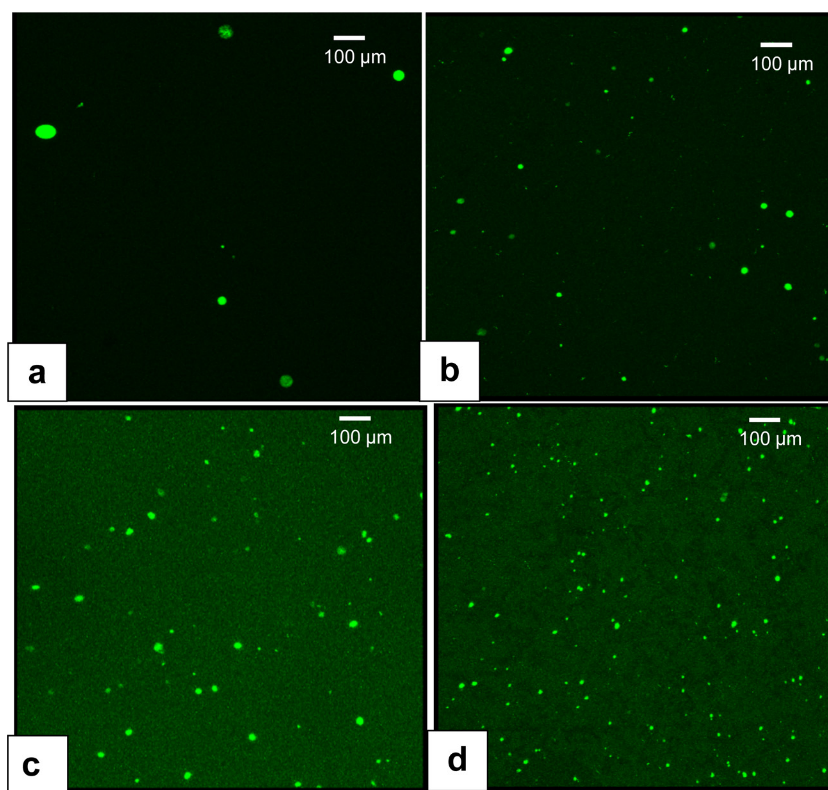

FIG. 3. Compilations in depth of stacked photographs (examples from 60 stacks) taken by confocal microscopy of gel cassettes filled with coagulated UF milk retentate and inoculated with a *Lactococcus* strain producing GFP at 2.1×10^4 (a), 2×10^5 (b), 1.6×10^6 (c), and 9.6×10^6 CFU/g (d), representing bacterial colonies grown in a 3D volume of a 120- to 150-μm depth by 1.3 by 1.3 mm.

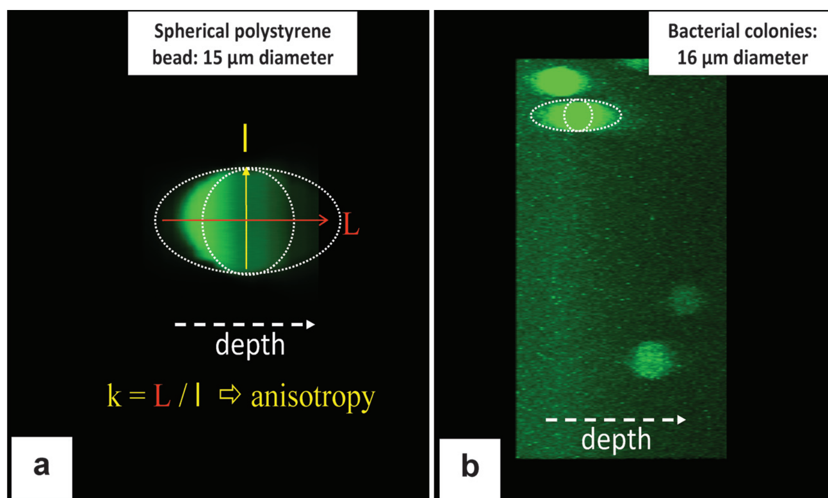

FIG. 4. Microscopic examination in the depth of the UF retentate (*z* compilation of tiled photographs) of a 15-µm fluorescent polystyrene bead (a) and bacterial colonies (b).

transparent matrix, such as milk UF retentate coagulated in gel cassettes. The inoculation level was a major influence on both the distribution of colonies and the mean diameter of colonies in the model cheese (Fig. 3). The observation of fluorescent colonies in the 7,000 photographs of the model cheese brought to light the correlation between the increasing number of colonies in a constant volume and the increasing inoculation level. It is also obvious that the mean size of colonies inside the model cheese increased when the number of colonies decreased and, at the same time, when the distance between colonies increased.

Spatial distribution of colonies in model cheese according to the inoculation level by statistical image analysis. The three highest levels of inoculation (2×10^5, 1.6×10^6, and 9.2×10^6 CFU/g) were statistically analyzed for one or two magnifications (120 photographs analyzed), while the inoculation level of 2.1×10^4 CFU/g had too few colonies in each photograph for statistics to be performed on them (Fig. 3).

Colonies were assumed to be independently spread out because inoculated cells were mixed as homogeneously as possible in the UF retentate. After selecting the fluorescent colonies among the background of the natural fluorescence of the retentate (see Materials and Methods), the hypothesis that colony centers followed a Poisson point process was not rejected at the 5% level for the three analyzed inoculation levels. We then concluded that colonies are randomly distributed in this nonfat model cheese.

On the basis of this hypothesis, both the density of colonies, λ, and the mean nearest 3D distances, d_3 (λ), between the neighbor colonies were computed (Table 1), taking into account the ellipsoid shape of colonies under the microscope by integrating the anisotropy ratio, k, into the calculation of the number of colonies per area (colonies in each photography). The initial inoculation levels measured by plating were always higher than the computed colony densities for the same inoculated cheese matrices (Table 1). This could be due to the low sensitivity of the enumeration technique (1). The computed colony densities may also have been underestimated because of the threshold values fixed for the intensity and size of the colonies. Hence, some small colonies may have been eliminated as background even if we visually and/or manually checked that the selection of colonies by the image analysis was accurate. However, even if the initial inoculation levels measured by plating were higher than the computed colony densities, both values were similar (maximum factor of 2). We can then conclude that all the inoculated cells gave rise to a colony in the cheese made by UF.

When the inoculation level was increased 50 times (inoculation level from 2.0×10^5 to 9.6×10^6 CFU/g), the mean diameters decreased 2.6 times. Table 1 confirms that colonies inoculated at a level between 10^6 and 5×10^6 CFU/g, which is the usual inoculation level in cheese making, are very close to each other (30 to 50 µm), confirming theoretical calculations. If the interfacial area (S) is the total surface (cm^2) of all the colonies per unit volume (cm^3), it corresponds to the total exchange surface between the bacterial colonies and the cheese matrix. The interfacial area was then multiplied by 7.2-fold (in cm^2/cm^3), with a 50-fold increase of the inoculation level. For the same increase in the inoculation level, the interfacial area, S, was multiplied with a much higher coefficient than the mean colony diameters, which means that the level of inoculation had a dramatic influence on the surface area of colonies in contact with the cheese matrix.

These statistical analyses could be performed only because we took into account in the mathematical model the optical distortion that we experienced by observing colonies under a confocal microscope.

Measurements of optical distortion. The assumption that colony sections can be represented by disks was tested by regressing the square root of the colony section areas on the colony section perimeters and testing that they are proportional with the parameter $1/2\sqrt{\pi}$. We accepted this assumption at the level of 5% in the x and y planes and rejected this assumption in the x and z, or y and z, planes (Fig. 4). If colonies are most likely spherical in 3D renderings, they are observed as ellipsoids on the z axis. This raises the question of the quantification of colony volume and distribution using measurements with image analyses by confocal microscopy. As the polysty-

rene beads also appeared as ellipsoids in the *z* axis, we confirmed that the ellipsoid image was an optical distortion (Fig. 4). Consequently, sections of colonies could be detected on photographs at depths where they were not actually present, which could lead to an overestimation of the number of colony sections in each photograph and then to an overestimation of the density of colonies, if not taken into account.

We then described the confocal observation of colonies as ellipsoids of axis length ($2r$, $2r$, and $2kr$), where k is the anisotropy ratio. The ratio k was calculated by the mean ratio of 30 random colonies at each inoculation level. The ratio k was constant (4.11 ± 0.3) whatever the size, the volume, and the density of colonies, confirming that the optical effect was probably due to the field depth of the lens independent of the observed object.

DISCUSSION

Bacteria immobilized in cheese grow as colonies, and they are mostly responsible for the ripening process. Ripening must then take place on a microscopic scale, between colonies, depending on the distance between colonies.

In this work, the spatial distribution of bacteria in cheese was described for the first time, using a real food matrix, in the form of a model cheese made by UF and a *Lactococcus* strain producing GFP. Bacterial colonies were shown to be randomly distributed. Quantitative data were provided regarding distances between colonies and sizes of colonies, depending on the level of inoculation, which had a crucial impact on spatial colony distribution. Mean distances calculated from the image analysis of experimental data fitted perfectly the theoretical calculations. At high levels of inoculation, colonies were extremely close to each other in the third dimension, since mean distances of 25 to 30 μm (Fig. 2 and Table 1) were obtained. Furthermore, the parameter that increased the most when the level of inoculation increased was the interfacial area, *S*.

In terms of methodology, we developed a nondestructive and *in situ* approach to investigate the spatial distribution of bacterial colonies in cheese on a microscopic scale. The gel cassette is a perfect tool to study immobilized bacterial colonies (20, 30, 33). In the present paper, for the first time, these gel cassettes were successfully adapted to a model food matrix instead of gelatin or agar medium. In the developed methodology, we also showed that the fluorescence emission from a strain producing GFP is sufficient enough to avoid any additional staining before confocal observations are made. This is the first time that a strain of *Lactococcus* expressing GFP has been observed under the microscope in a nontransparent food matrix, such as this model UF cheese matrix. We proved that it was possible to quantify fluorescence. This work demonstrates an optical effect on the *z* axis when bacterial colonies are observed, likely due to the confocal lens giving them an ellipsoid appearance. Such an effect should be taken into consideration if colony density has to be estimated from stacks and image analysis. The *Lactococcus* strain was a lactose-negative and proteinase-negative mutant, which most likely explains why the final cell number was limited to 10^8 CFU/g and did not reach the usual maximum level, which is around 10^9 CFU/g for a lactose-positive and proteinase-positive (lac$^+$/prtP$^+$) strain (15, 37), with no loss of viability before 7 days. Further assays using that strain should first reintroduce the lac/prtP plasmid. Nevertheless, we are convinced that the lower maximum population of this strain did not affect the main conclusions of the present study.

The inoculation level influenced the mean size of the colonies. As the same final number of cells was reached (10^8 to 10^9 CFU/g) regardless of the inoculation level, if the level of inoculation was 10^4 CFU/g, colonies should grow to 10^4 to 10^5 cells each, while if the inoculation level was 10^7 CFU/g, colonies should grow to only 10 to 100 cells each. Of course, these final numbers of cells per colony do not lead to the same size and/or surface of the colony. Recently, the measured surface of bacterial colonies was shown to be linearly correlated to the number of CFU/ml (14, 30). Therefore, the final number of cells indicates neither the number/distribution of active colonies in the matrix nor the interfacial area between colonies and the surface. A growing colony may be constituted of cells in different physiological states. Cells in the exponential growth phase were shown previously to grow at the outer layer of the colony (21), in contact with the matrix. This outer layer is thus most likely highly metabolically active. We can therefore assume that the larger the interfacial area, *S*, the higher the bacterial activity on the food matrix (ripening processes). From this point of view, increasing the interfacial area, *S*, by more than 7 times when cells are inoculated at 9.6×10^6 CFU/g instead of 2×10^5 CFU/g should accelerate ripening process kinetics. If the interfacial area, *S*, is wide at high inoculation levels, the mean distance between colonies is short, and bacterial activity is widespread in the cheese matrix. This is the first study to introduce this concept in food microbiology, although it is widely used in physicochemistry and medicine. We now think that the final number of cells in the cheese must be discussed together with the inoculation level, as the latter is more indicative of the distribution and the mean size of the colonies than is the final number of cells.

Metabolites from the ripening processes, such as nutrients and aroma precursors, must be diffusing into the matrix between bacterial colonies. Some metabolites were shown previously to be responsible for synergism and antagonism in immobilized bacterial cocultures (36). It is therefore very important to quantify these distances, as they also influence the interaction between bacterial colonies of the same species or of different species, such as lactic acid bacteria and pathogens (34) or ripening species in cheese. Very little data are available regarding the diffusion of small solutes in cheeses (10). Whatever the diffusion rate, the closer the colonies are, the less the matrix microstructure influences the diffusion between colonies.

Further work should investigate the microenvironment around colonies depending on their size and the composition of the cheese matrix. The spatial distribution of colonies should now be assessed in real cheeses as a factor influencing cheese-ripening kinetics. The addition of fat will create a heterogeneous matrix with two phases. Colonies may not be evenly distributed, as it was previously shown that bacterial colonies were located at the fat-protein interface (8, 19), and these were not spherical. The interfacial area would also be increased because of their shape. The Boolean process will then not be homogeneous. Modeling must take into account

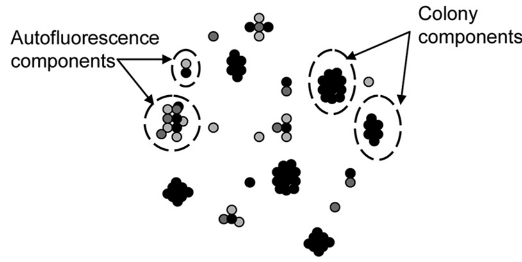

FIG. A1. Schematic representation of a confocal photograph with fluorescent components (groups of connected pixels) from bacterial colonies and from autofluorescence of the cheese matrix (background).

this nonhomogeneity by using, for example, inhomogeneous Boolean models directed by the phase structure.

The microenvironment of colonies can now be investigated *in situ* on a microscopic scale, monitoring different cheese-making and ripening processes or bacterial interactions within cheese matrices.

APPENDIX

To estimate the density of bacterial colonies on a photograph, we first differentiated the colony sections from the background and then estimated the density of colonies using a geometrical model of colonies.

Detecting colony sections. Colonies were detected by fluorescence and were characterized by groups of high-intensity pixels. However, the UF retentate within the gel cassettes was autofluorescent. This autofluorescence constituted the background. Therefore, we first thresholded it at intensity level of 0.1 so that pixels under this threshold were considered background. We then extracted the connected components composed of the remaining segments. These connected components were either colonies or groups of background fluorescent pixels.

The area distribution of these components was then expected to be a mixture of a sharply decreasing distribution and a unimodal distribution, whereas the distribution of the mean component intensity of small components was expected to be very variable due to the high variability of background pixels.

First, assuming that background pixels were spread at random on the photograph with independent fluorescence intensities, the area distribution of background-connected components was expected to be continuously decreasing, with a large variance of the mean fluorescence values for small components and a small variance for large components, even if the expected value of these mean values should be constant.

Second, supposing that bacterial colonies grew independently in a homogeneous medium, their section distribution was expected to be of a unimodal volume. The expectation of their mean fluorescence value was expected to be independent of the area of the section and larger than the expected area of the background components (Fig. A1).

Bacterial colonies were then estimated as components larger than an area threshold estimated as the value under which the sharp decrease of the area component distribution could no longer be detected and for which the *P* value of the mean fluorescence was lower than 0.01 under the assumption that it was a group of independent background pixels. This *P* value for each component was computed by using the fluorescence of components of one pixel as background pixels.

Estimating colony density and radius distribution. (i) Modeling of the colonies observed by confocal microscopy. We confirmed that the colonies were observed with the confocal microscope as ellipsoids, where the larger axis was in the z axis of the depth, the two other axes (x and y) were equal, and the ratio between axes (anisotropy) was constant. We tested whether colonies are spherical on the x and y axes. Being detected by their fluorescence, colonies that did not physically intercept a photograph at a given depth, but were near enough, could be optically detected on this photograph because of the ellipsoid shape in the z axis. We denote k the anisotropy ratio of the larger axis, z, and another axis (x or y) so that a colony of radius r is seen as an ellipsoid of axis lengths $2kR$, $2R$, and $2R$.

The following formulas are direct extensions of formulas obtained previously by Kok (17) for spheres and oblate ellipsoids.

(ii) Estimating the probability density of the colony radius. We assumed that if the colony distribution is random in the two-dimensional (2D) plane (on each photograph), it is also random in the 3D volume.

Therefore, the radius distribution of colonies has to be calculated on a 2D examination plane (i.e., one photograph of a stack). The observed section of a colony detected in the photograph and of radius R is a disk whose radius r on the photograph is random, and its probability density is as follows:

$$p(r) = \frac{r}{R^2}\frac{1}{\sqrt{1-\frac{r^2}{R^2}}} \quad \text{(A1)}$$

If colony radii are random and $a(R)$ is the probability density of their radius, the radius r of a colony section on the photograph is random, and its probability density equates to the following:

$$p(r) = \frac{r}{\int_0^\infty Ra(R)dR}\int_r^\infty \frac{a(R)}{\sqrt{1-\frac{r^2}{R^2}}}\frac{dR}{R} \quad \text{(A2)}$$

The probability density of the colony radii is then estimated by the maximum of likelihood (6), maximizing the probability, $\prod_{i=1}^{n} p(r_i)$, to observe the radii, $r_1, \ldots r_n$, observed for the colony sections.

(iii) Estimating the mean number of colonies per unit volume. The colonies growing from single bacteria uniformly and independently spread out in the model cheese matrix. The colony centers are modeled as a Poisson point process. Let λ be the mean number of colony centers per unit volume. The colony sections on an examined photograph follow a Poisson process with the mean number per unit area, μ, equating to the following:

$$\mu = 2k\lambda \int_0^\infty Ra(R)dR \quad \text{(A3)}$$

The mean number of colony sections per unit volume was then estimated by replacing the probability density of the colony radius by its estimation, replacing k by the mean of the measured values estimated directly for 30 independent colonies of various volumes and by replacing μ by an estimate equal to the number of observed disks divided by the total area of the sections.

Confidence intervals were obtained by block bootstrapping (18) of the observed sections, with a block being a quarter of a section.

(iv) Computing the distribution of the mean nearest distance between colony centers. With colony centers being Poisson distributed, the probability density of the distance (z) from one center to the center of its nearest neighbor depends only on the density of colonies per unit volume, with a probability density as follows:

$$p(z) = 4\pi \lambda z^2 \exp\left(-\frac{4}{3}\pi\lambda z^3\right) \quad \text{(A4)}$$

Similarly, the probability density of the distance from the center of one colony section to the center of its nearest neighbor on the plane is equal to the following:

$$p(z) = 2\pi \mu z \exp(-4\pi\mu z^2) \quad \text{(A5)}$$

This depends only on μ, so it is a function of both λ and the colony radius distribution.

(v) Computing the interfacial area, *S*, per unit volume. With the mean number of colonies per unit volume being λ and the radius probability density of the colonies being $a(R)$, the mean interfacial area of the colonies per unit volume is as follows:

$$S = 4\pi\lambda \int_0^\infty R^2 a(R)dR \qquad \text{(A6)}$$

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Valorization and Formation

Results Valorization and training courses

- **Publications in international journals references:**

1. Juliane Floury, Sophie Jeanson, Samar Aly and Sylvie Lortal (2010): Determination of the diffusion coefficients of small solutes in cheese: A review. *Journal of dairy Science and technology*. 90 ,477–508.
2. Sophie Jeanson, Joël Chadoeuf, Marie-Noëlle Madec, Samar Aly, Juliane Floury, Tim Brocklehurst and Sylvie Lortal (2011)**:**Spatial Distribution of Bacterial Colonies in a Model Cheese. *Applied and Environmental Microbiology*. 77(4), 1493–1500.
3. Samar Aly, Juliane Floury, Marie-Hélène Famelart, Marie-Noëlle Madec, Didier Dupont, Yann Le Gouar, Sylvie Lortal and Sophie Jeanson (2011): Nisin quantification by ELISA allows the modeling of nisin apparent diffusion coefficient in model cheeses. *Journal of Agricultural and Food Chemistry*. In press.
4. Samar Aly, Juliane Floury Michel Piot, Sylvie Lortal and Sophie Jeanson (2011) Nisin efficiency in UF cheeses is increased by gelatin incorporation. On way for submission.

- **Conferences and Seminars:**

1. **IDF world Dairy Summit, United Dairy World 2009, Berlin-Germany**: 20-24 September 2009. Samar Aly, Juliane Floury, Sylvie Lortal and Sophie Jeanson. “Is diffusion of nisin in a model cheese modified by differences in the cheese matrix microstructure?” (Poster).
2. **Biopolymers 2010, Food matrices: Construction, Destructuring, Sensory and Nutritional properties, Le Croisic-France**: 1-3 December 2010. Samar Aly, Sophie Jeanson, Marie-Noelle Madec, Marie-Hélène Famelart, Sylvie Lortal and Juliane Floury. “Modelling Nisin diffusion in UF model cheese systems with regarding their composition”. (Poster).
3. **Second Edition of Doctorama:**18 September 2008, Rennes

Oral presentation of thesis topic during the day Doctorama organized by PhD students of UMR STLO.

4. **Reflexive®:** Linguistics and Research Practices. Inter-department interdisciplinary seminars INRA. Ronce les Bains du 29 mars au 3 april 2009.

- **Followed Training courses**

1. French langue courses proposed by INRA STLO, Rennes in June and July 2008. 12 hours including 8 courses, 1.5 hours each.
2. English langue courses offers by school VAS University Rennes 2 from 27 January to 10 March 2009 (18 hours) in 6 modules, 3 hours each.
3. Industrial visit (Evialis) March 5, 2009 with the School of VAS
4. Training "IST MISTER" INRA = Control the Scientific and Technical Information in Research:

   Axis 1: Optimized his search for scientific and technical information, February 4, 2009, 7h.

   Axis 2: Managing the bibliography, 05 - February 2009, 7h.

   Axe 3: Communicating the results of his research, March 13, 2009, 7h.
5. Work shop of Gel Cassette, Paris :18 and 19 may 2009
6. Microbiology lectures, Academic year 2009-2010, semesters 5 and 6 (January-February), course for the agricultural engineering. 26 hours (13 lessons, 2 hours each).
7. Introduction and statistical training in R software (INRA Rennes) March 16, 2010, 8h.
8. Formation "After the Thesis" proposed by the school VAS. On 2 June, 2010, 8h.
9. Formation "Writing for the Web", INRA Rennes. 03 and 04 February 2011. 14 hours
10. Industrial visit (Lactalis) June 8, 2011 with the School of VAS.