COMMUNAUTE FRANCAISE DE BELGIQUE

ACADEMIE UNIVERSITAIRE WALLONIE-EUROPE

FACULTE UNIVERSITAIRE DES SCIENCES AGRONOMIQUES DE GEMBLOUX

Isolement d'une bactérie lactique produisant de la sakacin G et utilisation sur des matrices alimentaires

Carine DORTU

Dissertation originale présentée en vue de l'obtention du grade de docteur en sciences agronomiques et ingénierie biologique

Promoteur : Pr. Philippe Thonart

2008

COMMUNAUTE FRANCAISE DE BELGIQUE

ACADEMIE UNIVERSITAIRE WALLONIE-EUROPE

FACULTE UNIVERSITAIRE DES SCIENCES AGRONOMIQUES DE GEMBLOUX

Isolement d'une bactérie lactique produisant de la sakacin G et utilisation sur des matrices alimentaires

Carine DORTU

Dissertation originale présentée en vue de l'obtention du grade de docteur en sciences agronomiques et ingénierie biologique

Promoteur : Pr. Philippe Thonart

2008

Dortu Carine. (2008). Isolement d'une bactérie lactique produisant de la sakacin G et utilisation sur des matrices alimentaires. Gembloux Faculté Universitaire des Sciences Agronomiques, 135 p., 10 tabl., 17 fig.

Résumé : L'objectif de ce travail a été de caractériser et d'étudier les facteurs influençant la production et l'application de la sakacin G produite par *Lactobacillus sakei* CWBI-B1365. Au terme d'une première sélection, quatre bactéries lactiques produisant des bactériocines ont été isolées. Les gènes de structure de certaines bactériocines ont été identifiés dans le génome de ces souches par amplification à l'aide d'amorces spécifiques. *Lactobacillus sakei* CWBI-B1365 a été ensuite plus particulièrement étudiée. La sakacin G a été identifée comme la seule bactériocine produite par cette souche. Le séquençage d'une partie des gènes impliqués dans sa production a permis d'identifier une organisation génétique originale. Le pH, la température, la source de carbone utilisée et sa concentration ainsi que la quantité d'extrait de viande influencent fortement la production de sakacin G. Celle-ci est par ailleurs produite et a une activité antilisteria dans de la viande de bœuf. Dans de la viande de volaille, l'application de *Lb. sakei* CWBI-B1365 en combinaison avec *Lb. curvatus* CWBI-B28, une souche productrice de sakacin P, est nécessaire pour obtenir une inhibition de la croissance du pathogène.

Dortu Carine. (2008). Isolation of a sakacin G producing lactic acid bacteria and use in food matrix. (Thèse de doctorat in French and English). Gembloux, Belgium

Gembloux Agricultural University, 135 p., 10 tabl., 17 fig.

Summary : The objective of this work was to characterize and study the factors affecting the production and the application of sakacin G produced by *Lactobacillus sakei* CWBI-B1365. After a first screening, four bacteriocin producing lactic acid bacteria were selected. The structural genes for some of the bacteriocins were identified in the genome of these strains by amplification using specific PCR primers. *Lb. sakei* CWBI-B1365 has then been specifically studied. Sakacin G was identified as the only bacteriocin produced. Sequencing of a part of the sakacin G gene clusters involved in the bacteriocin production allowed to identify an original genetic organization. The pH, the temperature, the carbon source used and its concentration, as well as the meat extract quantity, strongly influence sakacin G production. Sakacin G was produced and showed antilisterial activity in beef. To obtain antilisterial activity in poultry meat, it was necessary to combine *Lb. sakei* CWBI-B1365 and *Lb. curvatus* CWBI-B28, a sakacin P producer.

REMERCIEMENT

Au terme de ce travail, mes remerciements vont tout d'abord au Prof. P. Thonart qui m'a permis de réaliser cette thèse au sein du Centre Wallon de Biologie Industriel. Ses conseils judicieux m'ont permis de mener à bien ce travail.

Ensuite, je tiens à remercier le Prof. W. Holzapfel et le Dr. C. Franz pour leur accueil lors du stage de six mois que j'ai pu réaliser dans leur centre de recherche de Karlsruhe. Thanks to Charles for its friendship, help and advices during the 6-months visit I made in his laboratory in Karlsruhe. Thanks to him also for the numerous corrections of the articles and of my poor English. Thanks also to Melanie and Anna for their welcoming and help during this visit.

Je tiens également à remercier le Dr. P. Fickers du Centre pour l'Ingénierie des Protéines (ULg) pour son encadrement, ses conseils, l'expérience qu'il m'a permis d'acquérir en biologie moléculaire, son point de vue critique et les corrections, recorrections, rerecorrections… d'articles…

Plusieurs personnes ont contribué à cette thèse. Merci donc à Dora, Emilie et Ludovic qui ont, par l'intermédiaire de leur travail de fin d'étude, permis sa réalisation.

Ensuite, je ne peux que remercier les membres du Centre Wallon de Biologie Industrielle qui m'ont permis de réaliser cette thèse dans un environnement de travail agréable. Merci à Cécile et Marina pour leur calme olympien en toute situation, leur soutien, les commandes rapides, les rappels lors des oublis de demande de mission… Merci également à Manu, mon clown de collègue de bureau, futur maître du monde qui passe ses bracelets de pouvoir quand rien ne va plus (hum hum hum)… Merci encore à Brigitte et Serge, toujours prêts à faire des heures sup'

pour faire tourner un fermenteur. Merci encore à Robin, Fred, Christian, Marc, Valérie, Isabelle, Jean-Jacques, Thami, Sabri, Jérémy, Anna, Hakim, Privat, Michel et ceux que j'oublie sûrement pour leur bonne humeur durant toute cette thèse.

Pour terminer, j'aimerais remercier ma famille, un soutien indéfectible depuis toujours, mes amis… et Pierre, pour ses bras réconfortant…

Enfin, je dédicace ce travail à Fabien. J'espère que de là-haut, tu es fier de ta petite sœur…

Table of content

LISTE DES ABBRÉVIATIONS

ADN : acide désoxyribonucléique

ATP : adénosine triphosphate

AU ml^{-1} : unité d'activité par millilitre

bp : paire de base

cDNA : ADN complémentaire

cfu : Unité formant colonie

d : jours (days)

Da : dalton

$DiSC_3(5)$: 3,3'-dipropylthiadicarbocyanine iodide

$\Delta\Psi$: potentiel membranaire

Δ pH : gradient de pH

EDTA : ethylène-diamine-tetraacétique acide

g : gramme

GlcNAc : N-acetylglucosamine

h : heure

HGT: horizontal gene transfer

HPLC: high performance liquid chromatography

kb: kilobase

kDa : kilodalton

kV : kilovolt

l : litre

λ : longueur d'onde

MALDI-TOF : matrix assisted lazer desorption ionization – time of flight

mg : milligramme

ml : millilitre

mm : millimètre

mM : millimolaire

MurNAc : N-acetylmuramic acid

ng : nanogramme

nm : nanomètre

OD : densité optique

PCR : polymerase chain reaction

ppm : part par million

RNA : acide ribonucléique

RNase : ribonucléase

RT-PCR : reverse transcriptase polymerase chain reaction

s : seconde

SDS-PAGE : sodium dodecyl sulfate polyacrylamide gel electrophoresis

µl : microlitre

µm : micromètre

UV : ultraviolet

V : volt

Chapitre 1 : General Introduction

1.1 Introduction

Malgré l'utilisation de différentes techniques de préservation des aliments et de bonnes pratiques d'hygiène, la contamination des denrées alimentaires par des microorganismes pathogènes tels que *Listeria monocytogenes* reste très présente. En effet, suivant les données de l'Agence Fédérale pour la Sécurité de la Chaîne Alimentaire Belge (AFSCA), entre 1997 et 2005, 545 cas de listérioses ont été observés en Belgique. Le nombre de cas par année est stable. La contamination des produits alimentaires l'est également.

L. monocytogenes est une bactérie Gram-positive, catalase positive et non sporulée qui est responsable de la listériose. L'infection survient habituellement après l'ingestion de produits d'origine animale ou après contact direct avec un animal infecté. Les listérioses humaines sont rares mais contrairement aux autres zoonoses telles que celles causées par *Salmonella* ou *Campylobacter*, elles sont associées à un haut taux de mortalité, même si les traitements antimicrobiens adéquats sont appliqués. C'est une bactérie ubiquiste qui peut être retrouvée dans une large gamme d'aliments tels que les produits laitiers, les crustacés et poissons et les produits viandeux. Les produits sont infectés pendant leur transformation ou après transformation, avant l'emballage. Elle peut survivre et croître à de basses températures (2 – 4°C), à un pH acide et à une haute concentration en sel, et donc dans les produits alimentaires au cours de leur stockage (Gandhi *et al*., 2007 ; Lecuit, 2007).

La demande croissante des consommateurs de pouvoir bénéficier de produits frais prêts à l'emploi avec une innocuité et une durée de conservation maximale tout en ne contenant qu'une quantité minimale de conservateurs chimiques pousse les professionnels de l'agro-alimentaire à se tourner vers des moyens alternatifs de préservation des aliments. Des moyens biologiques de préservation sont actuellement envisagés tels que l'utilisation des bactéries lactiques produisant des bactériocines, des peptides antimicrobiens dont beaucoup ont une action contre *L. monocytogenes*. Comme nous allons le voir tout au long de l'introduction bibliographique, cette famille des bactériocines englobe une grande variété de peptides aux structures, modes d'action et potentialités d'application variées.

Notre travail se situe dans ce cadre et vise à contribuer à l'étude des bactériocines produites par les bactéries lactiques. Pour ce faire, différentes souches produisant des bactériocines ont été isolées de produits variés. Les bactériocines produites ont été identifiées. Sur base de ses potentialités d'application, une des souches sélectionnées a été plus

spécifiquement étudiée. Sa bactériocine a été caractérisée, ce qui a permis d'en déterminer le mode et le spectre d'action. D'autre part, l'impact de certains facteurs sur la production a été étudié. Enfin, les potentialités d'application de la souche dans les produits viandeux ont été évaluées.

1.2 Les bactéries lactiques et leurs propriétés antimicrobiennes

1.2.1 Définition des bactéries lactiques

Les bactéries lactiques sont un groupe hétérogène de microorganismes produisant de l'acide lactique comme produit principal du métabolisme. Elles sont non pigmentées, aérobie-anaérobie facultatives, Gram-positif, catalase négatives à l'exception de certains genres à pseudocatalase et tolérantes à des pH acides. Leur forme peut être coccoïde, coccobacillaire, ou bacillaire. Elles sont généralement mésophiles. Sur la base des caractéristiques de fermentation, les bactéries lactiques sont homofermentaires ou hétérofermentaires. Dans le premier cas, seul l'acide lactique est produit. Dans le second, en plus de l'acide lactique sont produits de l'acide acétique, de l'éthanol, du dioxyde de carbone et de l'acide formique. Actuellement, les bactéries lactiques regroupent douze genres bactériens différents : *Lactobacillus*, *Leuconostoc*, *Lactococcus*, *Enterococcus*, *Streptococcus*, *Pediococcus*, *Carnobacterium*, *Oenococcus*, *Weissella*, *Aerococcus*, *Tetragenococcus* et *Vagococcus*.

1.2.2 Les propriétés antimicrobiennes des bactéries lactiques

Les propriétés antimicrobiennes des bactéries lactiques peuvent être associées à de nombreux éléments. Elles résultent de l'effet combiné de différents facteurs biologiques provenant de leurs activités métaboliques.

1.2.2.1 Le pH et les acides organiques

Les produits principaux du métabolisme des bactéries lactiques sont les acides organiques. Les acides organiques sont produits soit par la voie homofermentaire, soit par la voie hétérofermentaire. Le métabolisme du pyruvate conduit à la formation uniquement d'acide lactique chez les homofermentaires tandis qu'il conduit à la formation d'acide lactique, acétique et formique, d'éthanol et de dioxyde de carbone chez les hétérofermentaires (Liu, 2003).

Grâce à cette production d'acides organiques, les bactéries lactiques diminuent le pH du milieu dans lequel elles se multiplient en inhibant une partie de la flore qui s'y développe.

Leur compétitivité est améliorée étant donné leur grande tolérance aux pH bas extra et intra cellulaires. Outre la diminution du pH du milieu, l'effet antagoniste des acides organiques résulte de l'action de leur forme non dissociée. En effet, la forme non dissociée de l'acide peut traverser passivement la membrane et acidifier le cytoplasme par libération du proton, ce qui affecte le métabolisme cellulaire en inhibant certaines fonctions (Klaenhammer, 1993; Brul *et al.*, 1999; Caplice *et al.*, 1999; Hsiao *et al.*, 1999; Cotter *et al.*, 2003; Janssen *et al.*, 2007).

Les acides organiques sont un des agents classiques de préservation des aliments (Brul *et al.*, 1999) et sont reconnus comme des additifs alimentaires. Les acides couramment utilisés sont les acides benzoïque, sorbique, acétique, fumarique, propionique et lactique. Ils sont utilisés pour prévenir ou retarder la croissance des bactéries dégradant la nourriture *(*Hsiao *et al*., 1999). Le principal problème consécutif à leur utilisation est la haute concentration nécessaire pour inhiber les bactéries pathogènes ou indésirables et qui est parfois inacceptable pour le consommateur (Kobilinsky *et al*., 2007).

La concentration inhibitrice minimale, qui est la plus petite quantité d'acide qui peut empêcher la croissance d'un microorganisme, de chacun de ces acides doit être déterminée dans des conditions précises de pH mais aussi d'activité d'eau, de température… (Hsiao *et al*., 1999). Elle varie avec chaque microorganisme à inhiber. Hsiao *et al*. (1999) ont montré qu'une concentration en acide acétique de 0,105 g l^{-1} inhibe la croissance de *Bacillus subtilis* à un pH de 5,3 alors qu'il faut une concentration de 27,5 g l^{-1} pour inhiber *Lactobacillus plantarum* et une concentration de 1,6 g l^{-1} pour inhiber *Escherichia coli* dans les mêmes conditions. *Listeria monocytogenes* est inhibé par de l'acide lactique à 9,0 g l^{-1} et un pH de 3,7 tandis qu'il l'est à un pH de 3,4 par l'acide chlorhydrique à la même concentration (Gravesen *et al.*, 2004).

Les bactéries pathogènes peuvent développer certains mécanismes de résistance appelés « acid tolerance response » vis à vis de l'exposition à des pH bas. Ceux-ci leur sont également utiles pour survivre au transit intestinal. *L. monocytogenes* par exemple peut survivre à une exposition de 60 minutes à un pH de 3 (Brul *et al*., 1999; Gahan *et al.*, 1999; Cotter *et al*., 2003).

1.2.2.2 Le peroxyde d'hydrogène

Les bactéries lactiques ne possèdent pas de catalase typique contenant un noyau hème pour dégrader le peroxyde d'hydrogène en oxygène et en eau. Il peut s'accumuler et être inhibiteur de différents micro-organismes par l'oxydation des lipides membranaires et la

destruction des structures des protéines cellulaires (Zalan *et al.*, 2005). Certaines bactéries lactiques peuvent néanmoins se protéger contre le peroxyde d'hydrogène qu'elles produisent par la synthèse de catalase hexamérique ou tétramérique contenant du manganèse et qui sont parfois décrites comme étant des pseudocatalases (Strus *et al.*, 2006). La concentration de peroxyde d'hydrogène produite par des *Lactobacilli* varie entre 0,001 et 8 mM, en fonction de l'espèce, de la souche et des conditions de cultures (Sakamoto *et al.*, 1998; Kullisaar *et al.*, 2002; Zalan *et al.*, 2005; Strus *et al*., 2006). Son action se manifestera aussi bien sur les germes indésirables que sur ceux qui sont indispensables au bon déroulement de la fermentation. Il est donc rarement utilisé pour son activité inhibitrice. D'autre part, son action oxydante peut avoir un effet néfaste sur la santé humaine (Zalan *et al.*, 2005).

1.2.2.3 Le dioxyde de carbone

Celui-ci est formé pendant la fermentation hétérolactique et crée un environnement anaérobie qui inhibe les microorganismes aérobies. L'accumulation de dioxyde de carbone dans la bicouche lipidique peut causer un dysfonctionnement de la perméabilité (Ammor *et al.*, 2006).

1.2.2.4 Le diacétyl

Il est synthétisé par différents genres de bactéries lactiques comme *Lactococcus* sp., *Leuconostoc* sp., *Lactobacillus* sp. et *Pediococcus* sp. Le diacétyl ($C_4H_6O_2$) est un des composants aromatiques essentiels du beurre. Il a des propriétés antimicrobiennes qui sont dirigées contre les levures, les bactéries Gram-négatif et les bactéries Gram-positif non lactiques, ces dernières y sont néanmoins moins sensibles (El Ziney *et al.*, 1998). Les concentrations nécessaires à l'obtention d'une inhibition sont de l'ordre de 100 ppm, et sont supérieures à celles présentes dans le beurre et susceptibles de provoquer son arôme (2 à 7 ppm) (Caplice *et al.*, 1999).

1.2.2.5 La reutérine

La reutérine (ou 3-hydroxypropionaldehyde) est une substance antimicrobienne qui est produite comme métabolite intermédiaire pendant la fermentation anaérobique du glycérol par certaines espèces de *Lactobacillus* ainsi que par d'autres genres bactériens non lactiques tels que *Bacillus*, *Klebsiella*, *Citrobacter*, *Enterobacter* et *Clostridium* (El-Ziney *et al.*, 1998). La fermentation du glycérol se déroule en deux étapes. Le glycérol sera tout d'abord déshydraté

par une « glycerol deshydratase » pour former de la reutérine qui sera ensuite réduite en 1,3-propanediol par une oxydoréductase. Cette deuxième étape est inhibée en l'absence de glucose. La reutérine s'accumule alors dans le microorganisme producteur. A haute concentration, elle est excrétée dans le milieu. Sa toxicité contre la cellule productrice limite sa production, certaines espèces comme *Lactobacillus reuteri* y sont plus résistantes (Vollenweider, 2004).

La reutérine a un large spectre d'activité. Elle a une action contre les procaryotes (Gram-positif ou Gram-négatif), les eucaryotes, les virus, les champignons et les protozoaires. Elle interfère avec la réplication de l'ADN. Elle a des applications aussi bien dans le domaine médical que dans le domaine alimentaire (Vollenweider, 2004).

1.2.2.6 Les bactériocines

Les bactériocines sont des protéines, ou complexes de protéines, avec une activité bactéricide contre les espèces proches de la souche productrice. Les bactériocines représentent une large classe de substances antagonistes qui varient considérablement du point de vue de leur poids moléculaire, de leurs propriétés biochimiques, de leur spectre d'action et de leur mode d'action (Klaenhammer, 1988).

1.3 Les bactériocines produites par les bactéries lactiques : caractéristiques et intérêt pour la bioconservation des produits alimentaires

1.3.1 Définition des bactériocines

Le premier prototype des bactériocines produites par les bactéries lactiques fût découvert en 1928 quand l'inhibition de la croissance de différentes bactéries lactiques par un métabolite produit par *Streptococcus lactis* fut observée (maintenant classifié comme *Lactococcus lactis*) (McAuliffe *et al.*, 2001). En 1953, Jacob *et al.* proposèrent le terme général « bactériocine ». Elles furent définies comme des antibiotiques protéiques, caractérisés par une activité bactéricide intra-espèce et une absorption par des récepteurs spécifiques sur la surface des cellules cibles (Jack *et al.*, 1995). En 1976, Tagg *et al.* les définirent comme des composés protéiques ayant une action bactéricide contre les espèces apparentées à la souche productrice. Cette définition est correcte pour la majorité des bactériocines mais est trop restrictive étant donné que certaines peuvent avoir une activité bactéricide contre des espèces plus distantes de la souche productrice.

En 1988, Klaenhammer proposa la définition qui reste la plus largement acceptée. Il définit les bactériocines comme des protéines, ou complexes de protéines, avec une activité bactéricide contre les espèces proches de la souche productrice. Les bactériocines représentent une large classe de substances antagonistes qui varient considérablement du point de vue de leur poids moléculaire, de leurs propriétés biochimiques, de leur spectre d'action et de leur mode d'action (Klaenhammer, 1988). Toutes les bactériocines produites par des bactéries lactiques décrites jusqu'à présent ont une activité dirigée contre les bactéries Gram-positive. Aucune bactériocine produite par des bactéries lactiques avec une activité contre des bactéries Gram-négatif n'a été décrite. En effet, la membrane externe des bactéries Gram-négatif ne permet pas aux bactériocines d'atteindre la membrane interne, siège de leur activité.

1.3.2 Classification des bactériocines

Les bactériocines produites par les bactéries lactiques sont réparties en quatre classes, comme proposé par Klaenhammer (1993).

1.3.2.1 Classe I : Les lantibiotiques

Ce sont des peptides de taille inférieure à 5kDa qui contiennent des acides aminés inhabituels soufrés formés post-traductionnellement c'est à dire la lanthionine, la β-méthyl lanthionine, la déhydrobutyrine et la déhydroalanine. Ils sont stables à la chaleur. Ils peuvent être divisés en deux types : la classe Ia qui contient des peptides cationiques hydrophobes allongés contenant jusqu'à 34 acides aminés et la classe Ib qui contient les peptides globulaires chargés négativement ou sans charge nette et contenant jusqu'à 19 acides aminés. (Mc Auliffe *et al.,* 2001 ; Twomey *et al.,* 2002). Les séquences et structure de quelques lantibiotiques se trouvent à la figure 1.

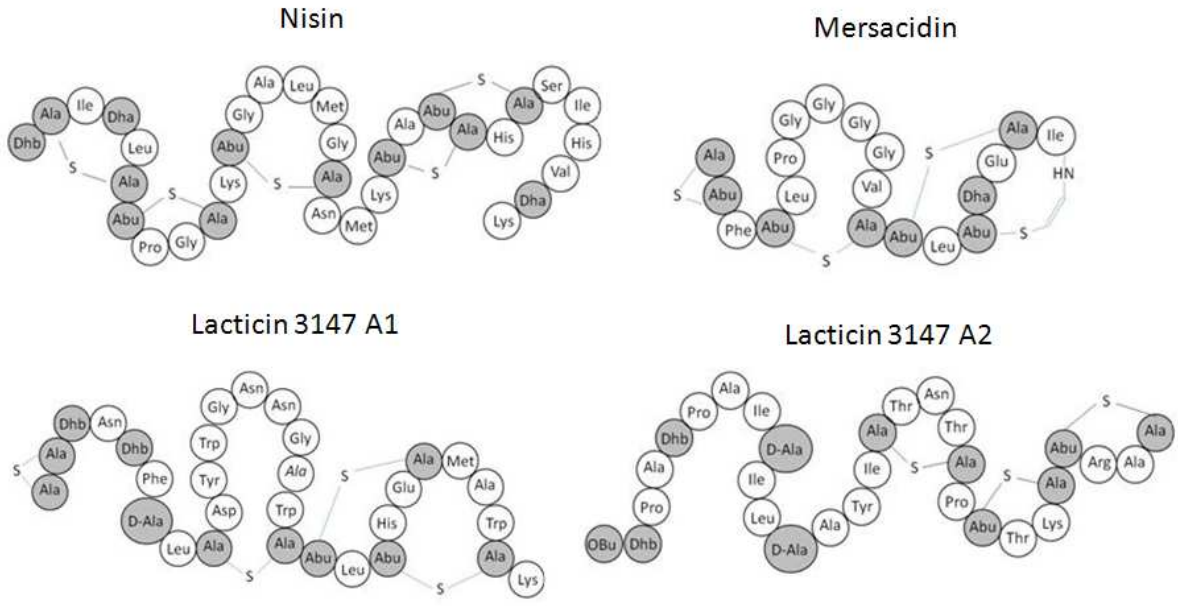

Figure 1 : Séquence et structure de lantibiotiques de type A (nisin) et B (mersacidin) et de « two-peptides lantibiotic » (lacticin 3147 A1 et A2)

1.3.2.2 Classe II

Peptides de taille inférieure à 10kDa, stables à la chaleur, ne contenant pas d'acides aminés modifiés et chargés positivement à un pH neutre. Les séquences de quelques bactériocines appartenant à cette classe se trouvent au tableau 1. Cette classe est divisée en trois sous classes.

Tableau 1 : Liste non exhaustive et séquences de bactériocines appartenant à la classe II

Classe IIa : "Pediocin-like bacteriocin"		
Mesentericin Y105		MTNMKSVEAYQQLDNQNLKKVV**GG**K**YYGNGV**H**C**TKSG**C**SVNWGEAASAGIHRLANGGNGFW
Leucocin A		MMNMKPTESYEQLDNSALEQVV**GG**K**YYGNGV**H**C**TKSG**C**SVNWGEAFSAGVHRLANGGNGFW
Sakacin P		------MEKFIELSLKEVTAIT**GG**K**YYGNGV**H**C**GKHS**C**TVDWGTAIGNIGNNAAANWATGWNAGG
Curvacin A		-----MNNVKELSMTELQTIT**GG**ARS**YGNGV**Y**C**NNKK**C**WVNRGEATQSIIGGMISGWASGLAGM
Piscicolin 126		------MKTVKELSVKEMQLTT**GG**K**YYGNGV**S**C**NKNG**C**TVDWSKAIGIIGNNAAANLTTGGAAGWNKG
Carnobacteriocin Bm1		-----MKSVKELNKKEMQQIN**GG**AIS**YGNGV**Y**C**NKEK**C**WVNKAENKQAITGIVIGGWASSLAGMGH
Carnobacteriocin B2		------MNSVKELNVKEMKQLH**GG**VN**YGNGV**S**C**SKTK**C**SVNWGQAFQERYTAGINSFVSGVASGAGSIGRRP
Pediocin PA-1		------MKKIEKLTEKEMANII**GG**K**YYGNGV**T**C**GKHS**C**SVDWGKATT**C**IINNGAMAWATGGHQGNHK**C**
Enterocin A		-MKHLKILSIKETQLIY**GG**TTHSGK**YYGNGV**Y**C**TKNK**C**TVDWAKATT**C**IAGMSIGGFLGGAIPGK**C**
Sakacin G		------MKNTRSLTIQEIKSIT**GG**K**YYGNGV**S**C**NSHG**C**SVNWGQAWT**C**GVNHLANGGHGGV**C**
Classe IIb : "Two-peptide bacteriocin"		
ABP-118	α	KRGPNCVGNFLGGLFAGAAAGVPLGPAGIVGGANLGMVGGALTCL
	β	KNGYGGSGNRWVHCGAGIVGGALIGAIGGPWSAVAGGISGGFTSCR
Lactacin F	X	NRWGDTVLSAASGAGTGIKACKSFGPWGMAICGVGGAAIGGYFGYTHN
	A	RNNWQTNVGGAVGSAMIGATVGGTICGPACAVAGAHYLPILWTGVTAATGGFGKIRK
Lactocin 705	α	MDNLNKFKKLSDNKLQATIGG
	β	MESNKLEKFANISNKDLNKITGG
Lactococcin G	α	GTWDDIGQGIGRVAYWVGKAMGNMSDVNQASRINRKKKH
	β	KKWGWLAWVDPAYEFIKGNIGKGAIKEGNKDKWKN
Lactococcin MN	M	IRGTGKGLAAAMVSGAAMGGAIGAFGGPVGAIMGAWGGAVGGAMKYSI
	N	GSIWGAIAGGAVKGAIAASWTGNPVGIGMSALGGAVLGGVTYARPVH
Plantaricin EF	E	FNRGGYNFGKSVRHVVDAIGSVAGIRGILKSIR
	F	VFHAYSARGVRNNYKSAVGPADWVISAVRGFIHG
Plantaricin JK	J	GAWKNFWSSLRKGFYDGEAGRAIRR
	K	RRSRKNGIGYAIGYAFGAVERAVLGGSRDYNK
Plantaricin S	α	RNKLAYNMGHYAGKATIFGLAAALLA
	β	KKKKQSWYAAAGDAIVSFGEGFLNAW
Thermophilin 13	A	YSGKDCLKDMGGYALAGAGSGALWGAPAGGVGALPGAFVGAHVGAIAGGFACMGGMIGNKFN
	B	QINWGSVVGHCIGGAIIGGAFSGGAAAGVGCLVGSGKAIINGL
CLASSE IIc		
Plantaricin A		MKIQIKGMKQLSNKEMQKIVGGKSSAYSLQMGATAIKQVKKLFKKWGW
Lactococcin A		MKNQLNFNIVSDEELSEANGGKLTFIQSTAAGDLYYNTNTHKYVYQQTQNAFGAAANTIVNGWMGGAAGGFGLHH
Lactococcin 972		MKTKSLVLALSAVTLFSAGGIVAQAEGTWQHGYGVSSAYSNYHHGSKTHSATVVNNNTGRQGKDTQRAGVWAKAT VGRNLTEKASFYYNFW

Les bactériocines de la sous classe IIa contiennent entre 27 et 48 acides aminés qui ont toutes une partie N-terminale hydrophobe contenant la séquence consensus YGNGV ainsi qu'un pont disulfure et une partie C-terminale moins conservée, hydrophobe ou amphiphile qui détermine la spécificité d'action (Fimland *et al.,* 2000 ; Richard *et al*., 2006). Leur point isolélectrique varie entre 8 et 10. Elles ont toutes une activité contre *L. monocytogenes*. Certaines bactériocines de cette sous-classe contiennent également un deuxième pont disulfure dans leur domaine C-terminale qui semble être important dans la stabilisation de la structure tertiaire. Ces bactériocines semblent par ailleurs avoir une meilleure activité antimicrobienne, une meilleure résistance à l'exposition à des hautes températures et un spectre d'action plus large (Eijsink *et al.,* 1998 ; Fimland *et al.,* 2000 ; Drider *et al.,* 2006 ; Richard *et al*., 2006). Cependant, d'autres études ont suggérés que la séquence en acides aminés de ce domaine C-terminal était le facteur prédominant (Fimland *et al.,* 2002 ; Johnsen *et al.,* 2005).

La sous classe IIb contient les bactériocines ayant besoin de deux peptides pour avoir une activité. Deux types de bactériocines de classe IIb peuvent être distingués : le type E (Enhancing) où la fonction d'un des deux peptides est d'augmenter l'activité de l'autre et le type S (Synergy) où les deux peptides sont complémentaires, ils doivent être présents pour avoir une activité.

La sous classe IIc contient les bactériocines ne pouvant pas être classées dans les autres sous classes.

1.3.2.3 Classe III

Elle contient les protéines de taille supérieure à 30kDa et sensibles à la chaleur. La structure et le mode d'action de ces bactériocines diffèrent complètement des autres bactériocines produites par les bactéries lactiques. Cette classe ne contient que quatre bactériocines : l'helveticin J produite par *Lactobacillus helveticus* A, l'enterolysin A produite par *Enterococcus faecium*, la zoocin A produite par *Spreptococcus zooepidemicus* et la millericin B produite par *Streptococcus milleri* (Nilsen *et al*., 2003 ; Papagianni, 2003 ; Nigutova *et al.,* 2007).

1.3.2.4 Classe IV

Elle contient les peptides requérant une partie carbohydratée ou lipidique pour avoir une activité. Aucune bactériocine de cette classe n'a été décrite.

1.3.3 Le mécanisme de production des bactériocines et sa régulation

Différentes protéines sont impliquées dans la production des bactériocines et sa régulation. Les bactériocines sont produites sous forme d'un prépeptide non-biologiquement actif qui subira des modifications post-traductionnelles pour aboutir au peptide actif. Cette production est souvent régulée par un système de Quorum Sensing, un mécanisme permettant à certains gènes d'être exprimés en fonction de la densité de la population bactérienne.

1.3.3.1 Les lantibiotiques

L'organisation génétique et de la production d'un lantibiotique, la nisine, est montrée à la figure 2. Les gènes de biosynthèse des lantibiotiques ont été désignés par le symbole commun *Lan*, avec un nom plus spécifique pour chaque lantibiotique (*nis* pour la nisine par exemple). Le gène de structure, *LanA*, code pour un peptide précurseur du lantibiotique contenant une séquence N-terminale de 23 à 30 acides aminés qui sera clivée lors du transport à l'extérieur de la cellule. Ce prépeptide subira différentes modifications post-traductionnelles afin d'acquérir les acides aminés inhabituels que sont la lanthionine et la β-méthyllanthionine. La première étape de ces modifications consiste en la déshydratation de la sérine et de la thréonine pour former la déhydroalanine et la déhydrobutyrine. La deuxième étape consiste en la formation d'un lien thioéther entre ces résidus déshydratés et les cystéines environnantes, donnant aux lantibiotiques une structure cyclique. Les enzymes impliquées sont une déshydratase et une cyclase codée soit par les gènes *LanB* et *LanC* ou le gène *LanM*. Après ces modifications, le prépeptide sera clivé lors de l'excrétion hors de la cellule par la protéase codée par *LanP* ou le domaine protéasique de l'ABC transporteur codé par le gène *LanT*. Cette dernière modification permettra d'avoir le peptide biologiquement actif *(*McAuliffe *et al*., 2001; Kleerebezem, 2004; Xie *et al*., 2004; Patton *et al*., 2005).

La production des lantibiotiques est sous le contrôle d'un mécanisme de régulation à deux composantes basé sur le Quorum Sensing. Ces deux composantes sont une histidine kinase, codée par le gène *LanK*, qui réagira à un stimulus extérieur et induira la phosphorylation d'un régulateur de réponse, le deuxième composant, codé par le gène *LanR*. Une fois phosphorylé, il va permettre l'activation de l'expression de l'opéron. Le stimuli extérieur est la bactériocine elle-même qui est présente dans la culture à basse concentration en début de croissance. Elle s'accumule et, quand un certain seuil est atteint, elle interagit

avec le système de régulation pour activer la transcription du gène de structure mais également de ceux d'immunité et de transport. On parlera donc d'autorégulation (McAuliffe *et al.*, 2001; Twomey *et al.*, 2002; Kleerebezem, 2004; Patton *et al.*, 2005). Les gènes d'immunités sont *LanI* qui code pour une protéine d'immunité et *LanF*, *LanE* et *LanG* qui code pour un ABC transporteur. Ces protéines permettent de protéger la bactérie productrice de l'action de sa propre bactériocine.

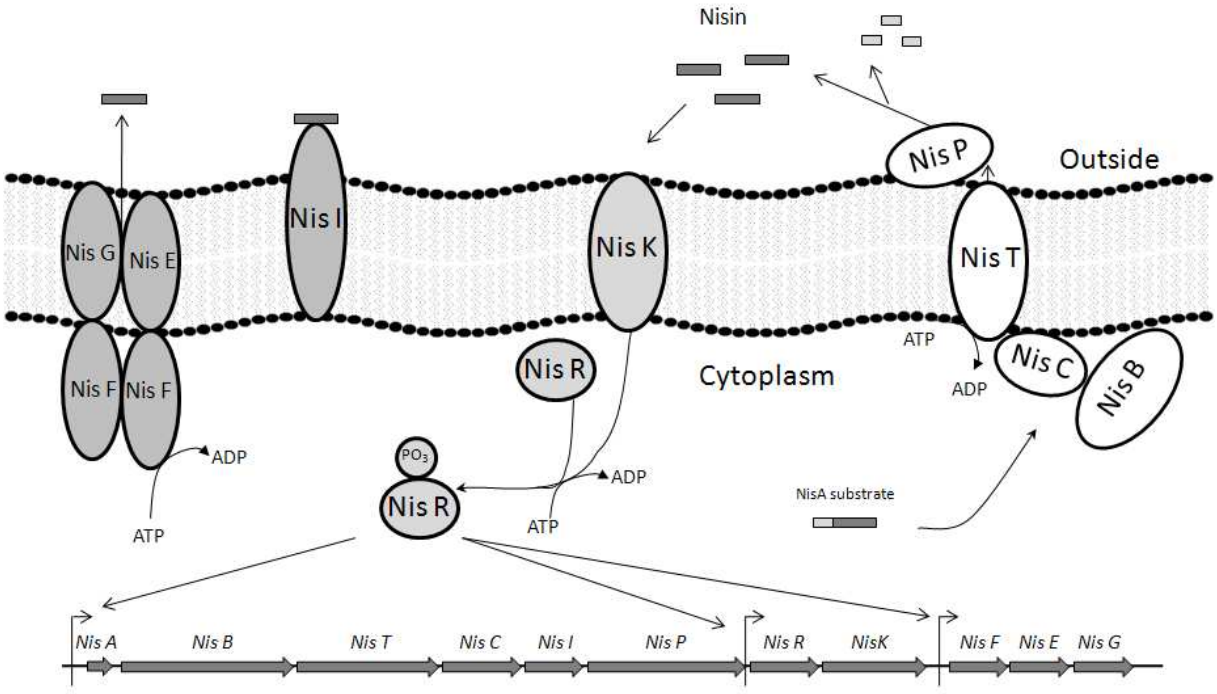

Figure 2 : Régulation de la production, modifications post-traductionnelles et auto-immunité de la nisine. « NisA substrate » est le prépeptide non biologiquement actif qui sera déshydraté par NisB et cyclisé par NisC avant sa translocation par l'ABC transporteur NisT et le clivage de la séquence signal par la protéase NisP. Ces modifications conduiront au peptide biologiquement actif. La nisine interagira avec l'histidine kinase NisK, ce qui induira la phosphorylation du régulateur de réponse NisR et l'activation de la transcription des gènes nécessaires à la production de la nisine. La protection de la cellule vis à vis de la nisine est réalisée par deux mécanismes : la lipoprotéine d'immunité NisI et l'ABC transporteur formé par NisG, NisE et NisF (inspiré de Patton *et al.*, 2005).

1.3.3.2 Les bactériocines de classe II

La figure 3 montre l'organisation génétique de deux bactériocines de classe IIa, la sakacin P et la piscicolin 126, ainsi que d'une bactériocine de classe IIb, l'ABP118. Les bactériocines de classe IIa sont également produites sous forme d'un pré-peptide non biologiquement actif dont la séquence N-terminal, appelée séquence signal et contenant une vingtaine d'acides aminés avec des séquences très conservées, sera clivée du côté C-terminal d'un motif GG par le domaine protéasique de l'ABC transporteur lors de la translocation membranaire pour donner le peptide biologiquement actif (Ennahar *et al*., 2000). Avec la formation d'un ou deux ponts disulfures cruciaux pour l'activité, ce sera la seule transformation post-traductionnelle (Drider *et al*., 2006). Certaines bactériocines de classe IIa sont excrétées par le « sec-dependent pathways ». Les peptides signaux de ces bactériocines ne contiennent donc pas de doublet glycine mais bien la séquence signal typique des protéines secrétées par ce système qui sera clivé par une peptidase durant la translocation (van Wely *et al*., 2001 ; De Kwaadsteniet *et al*., 2006 ; Sanchez *et al*., 2007).

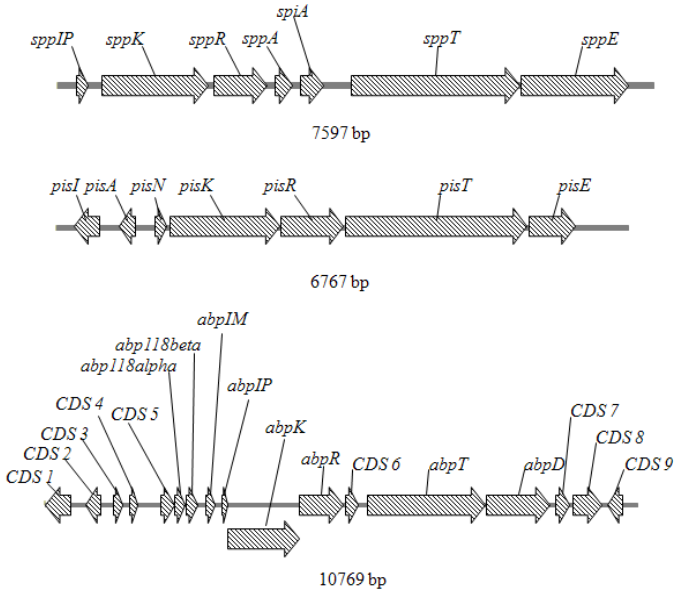

Figure 3 : Organisation génétique de bactériocines de classe II. Spp : Sakacin P, pis : piscicolin 126, abp118 : Bactériocine abp118. *sppA, pisA, abp118alpha* et *abp118 beta* : gène de structure, *spiA, pisI, AbpIM* : gène d'immunité, *sppK, pisK, abpK* : gène de l'histidine kinase, *sppR, pisR, abpR* : gène du régulateur de réponse, *sppIP, pisN, abpIP* : peptide d'induction, *sppT, pisT et abpT* : gène de l'ABC transporteur, *sppE, pisE* et *abpD* : protéine accessoire, *CDS1* à *9* : protéines hypothétiques dont la fonction n'est pas connue.

La régulation de la production des bactériocines de classe IIa est sous le contrôle d'un système de Quorum Sensing à trois composantes qui sont : un peptide d'induction, une histidine kinase et un régulateur de réponse. Les gènes codant pour ces trois protéines sont co-transcrits (Eijsink *et al*., 2002). Le peptide d'induction est produit à basse concentration comme un prépeptide de bas poids moléculaire, sans ou avec une très faible activité inhibitrice, stable à la chaleur, cationique et hydrophobe et dont la partie N-terminal est clivée au niveau d'un doublet glycine lors de l'excrétion en dehors de la cellule par l'ABC transporteur également impliqué dans l'excrétion de la bactériocine (Ennahar *et al.,* 2000; Eijsink *et al.,* 2002). A une certaine concentration externe du peptide d'induction, l'histidine kinase transmembranaire est activée, ce qui mène à sa phosphorylation. Le groupement phosphate est transféré au régulateur de réponse qui va activer l'expression des gènes de structure, immunité et transport de la bactériocine mais également du système de régulation à trois composantes. Le système est donc auto-induit (Eijsink *et al.,* 2002; Drider *et al.,* 2006). Cependant, il a été récemment suggéré que le gène codant pour le régulateur de réponse de la sakacin P (*sppR*) pouvait produire deux protéines : une protéine complète et cette même protéine dont l'extrémité N-terminal est tronquée. Cette deuxième protéine tronquée peut alors réprimer l'expression des gènes codant pour la sakacin P, probablement en interférant avec l'action de la molécule complète (Straume *et al.*, 2007).

Les gènes codant pour la production des bactériocines de classe IIa sont, la plupart du temps, organisés en trois opérons, le premier contenant les gènes de structure et d'immunité, le deuxième les gènes nécessaires à la sécrétion de la bactériocine (l'ABC transporteur et une protéine accessoire) et le troisième les gènes du système de régulation à trois composantes. Le gène d'immunité code pour une protéine permettant à la bactérie d'être résistante à la bactériocine qu'elle produit.

Peu d'informations sont disponibles concernant la régulation de la production des bactériocines de classe IIb. Néanmoins, il a été montré qu'un système de régulation à trois composantes identique à celui retrouvé pour les bactériocines de classe IIa est impliqué dans la production de l'ABP-118 et des plantiricin E/F et J/K (Flynn *et al.,* 2002 ; Oppegard *et al.,* 2007).

1.3.4 Les mécanismes d'action des bactériocines

Le siège d'activité des bactériocines est la membrane cellulaire, raison pour laquelle les bactériocines n'ont pas d'activité contre les bactéries Gram-négatif. Cependant, les modes d'action des bactériocines sur la membrane sont variés.

1.3.4.1 Les lantibiotiques

Les lantibiotiques interagissent avec la membrane cellulaire par des interactions électrostatiques ou par liaison à des récepteurs spécifiques tels que le lipide II, un précurseur de peptidoglycanes dont la structure se trouve à la figure 4. Suite à cette liaison, les lantibiotiques peuvent former des pores larges et non spécifiques dans la membrane cytoplasmique, ce qui va causer l'efflux rapide de petits composés cytoplasmiques tels que les ions, les acides aminés, l'ATP… Cette augmentation de la perméabilité membranaire va conduire à la dissipation des deux composantes de la force proton motrice, c'est à dire le potentiel transmembranaire ($\Delta\Psi$) et le gradient de pH (ΔpH), à la cessation rapide des activités cellulaires et à la mort de la cellule. L'interaction avec le lipide II permet d'augmenter la stabilité des pores formés et de réduire la concentration du lantibiotique nécessaire pour former un pore (Patton *et al.,* 2005). La liaison des lantibiotiques avec le lipide II peut également conduire à l'inhibition de la synthèse de la paroi cellulaire. D'autre part, certains lantibiotiques peuvent inhiber la sporulation (McAuliffe *et al*., 2001; Twomey *et al*., 2002; Bauer *et al.*, 2005; Patton *et al.*, 2005).

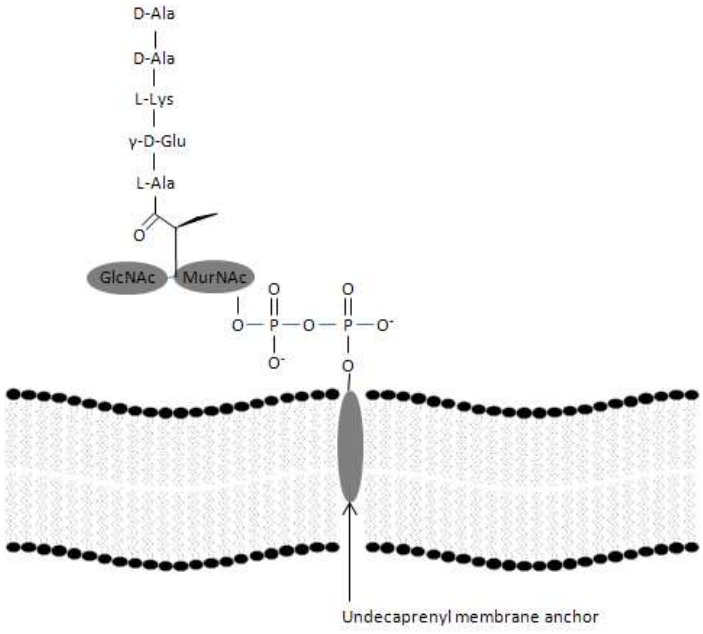

Figure 4 : Structure du lipide II. La nisine interagit au niveau du MurNAc tandis que la mersacidine interagit au niveau du GlcNAc.

Les lantibiotiques de type A dissipent la force proton-motrice par formation de pores et interfèrent avec la synthèse des peptidoglycanes alors que la plupart des lantibiotiques de type B agissent par inhibition de la synthèse des peptidoglycanes. Néanmoins, certains forment également des pores dans la membrane des cellules cibles (Bauer *et al.*, 2005; Patton *et al.*, 2005). La nisine, un lantibiotique de type A, interagit avec le lipide II au niveau du MurNAc tandis que la mersacidine, un lantibiotique de type B, interagit avec le GlcNAc du lipide II (Willey *et al.*, 2007).

Certains lantibiotiques ont besoin de deux peptides qui agissent ensemble pour avoir une activité. Ces lantibiotiques sont aussi produits comme des prépeptides qui seront ensuite modifiés comme les lantibiotiques simples afin d'avoir tous les deux des acides aminés modifiés. La lacticin 3147, un « two-peptide lantibiotic » dont la séquence se trouve à la figure 2, agit également par formation de pores dans la membrane des cellules cibles (McAuliffe *et al.*, 2001). Elle a un spectre d'action large. Un des deux peptides, la lacticin A1, a une activité qui est plus élevée en présence du deuxième peptide, la lacticin A2. Il a été récemment proposé que la lacticin A1 agit en se liant au lipide II, inhibant la synthèse des peptidoglycanes et permettant à la lacticin A2 de former un pore dans la membrane de la cellule cible (Morgan *et al.*, 2005; Wiedemann *et al.*, 2006).

1.3.4.2 Les bactériocines de classe II

Le mécanisme d'action supposé des bactériocines de classe IIa est l'interaction de la bactériocine avec la membrane ou un récepteur, la mannose perméase, pour ensuite former un pore dans la membrane de la cellule ce qui induit la perméabilisation de la membrane et la mort de la cellule (Dalet *et al*., 2000; Héchard *et al*., 2001; Gravesen *et al.*, 2002a; Arous *et al.*, 2004; Vadyvaloo *et al*., 2004a). Le mécanisme de formation des pores n'est pas connu même si l'hypothèse la plus courante est l'assemblage de différentes molécules de la bactériocine (Ennahar *et al*., 2000; Fimland *et al.*, 2000; Diep *et al*., 2007). Les pores formés par les bactériocines de classe IIa causent la perte d'ion potassium ainsi que d'acides aminés et d'autres molécules de faible poids moléculaire ce qui dissipe les deux composantes de la force proton motrice (Bauer *et al.*, 2005).

Les bactériocines de classe IIb ont en général un spectre d'action inhibant une large gamme de bactéries Gram-positif. Elles rendent la membrane perméable à différentes petites molécules, des cations monovalents ou des anions, ce qui dissipe une ou les deux composantes de la force proton motrice. Les ions transportés sont spécifiques de la bactériocine (Oppegard *et al.*, 2007). Le ratio optimal d'activité de la plupart de ces bactériocines est 1:1 (Oppegard *et al.*, 2007). Pour la lactocin 705, il est de 4:1 (Castellano *et al.*, 2007). Néanmoins, les mécanismes d'interaction des deux bactériocines entre elles et avec la membrane cellulaire ne sont que très peu connus. Il a été montré qu'il n'y avait pas de liaison au même récepteur que pour les bactériocines de classe IIa (la mannose transférase) (Diep *et al*., 2007). Castellano *et al.* (2007) ont récemment montré que les deux peptides composant la lactocin 705 ont des activités bien spécifiques. La lactocin 705α interagit avec la surface de la membrane cellulaire et la déshydrate. La lactocin 705β interagit avec cette partie déshydratée pour former des pores.

1.3.4.3 Les bactériocines de classe III

Le mode d'action de ces bactériocines diffère complètement des bactériocines des autres classes. En effet, l'enterolysin A, la zoocin A et la millericin B agissent par l'hydrolyse des liens peptidiques des peptidoglycanes des cellules sensibles. La zoocin A a un spectre d'action étroit alors que l'enterolysin A et la millericin B ont un spectre d'action large. L'helveticin J a un mode d'action bactéricide (Nilsen *et al*., 2003).

1.3.5 L'auto-immunité et la résistance aux bactériocines

1.3.5.1 L'auto-immunité

L'auto-immunité consiste en la protection de la cellule productrice de bactériocines contre la bactériocine qu'elle produit.

1.3.5.1.1 L'auto-immunité des lantibiotiques

Deux mécanismes peuvent être responsables de l'auto-immunité :

- La production d'une lipoprotéine d'immunité codée par le gène *LanI* : Cette protéine s'attache à la surface externe de la membrane et interagit avec le lantibiotique afin de l'empêcher de former des pores dans la membrane de la cellule productrice. La structure de ces protéines est très variable, ce qui permet de supposer une certaine spécificité d'interaction avec le lantibiotique (McAuliffe *et al.*, 2001; Twomey *et al.*, 2002). Pour la nisine, il semblerait que l'extrémité C-terminal de cette lipoprotéine d'immunité soit impliquée dans l'interaction spécifique avec la bactériocine (Takala *et al.*, 2006).
- L'ABC transporteur codé par les gènes *LanE*, *LanF* et *LanG* : Ce système permettrait d'exporter le lantibiotique à l'extérieur de la membrane cellulaire, permettant de garder la concentration intracellulaire en dessous du seuil critique. Le mode d'action est toujours sous étude. Néanmoins, même s'il est évident que ce système permet d'augmenter l'auto-immunité, il semblerait qu'il ne soit pas suffisant pour conférer une immunité totale et doive être complété par le premier (McAuliffe *et al.*, 2001; Twomey *et al.*, 2002; Stein *et al*., 2003; Li *et al*., 2006).

1.3.5.1.2 L'auto-immunité des bactériocines de classe II

1.3.5.1.2.1 La classe IIa

Pour les bactériocines de classe IIa, l'auto-immunité semblerait provenir de la production d'une protéine d'immunité intracellulaire contenant entre 88 et 115 acides aminés (Ennahar *et al*., 2000). Ces protéines globulaires sont cationiques et hydrophobes (Ennahar *et al.*, 2000). Le gène les codant est la plupart du temps cotranscrit avec le gène de structure de la bactériocine, les deux gènes constituant un opéron. Cependant, des gènes d'immunité indépendants ont déjà été décrits comme *orfY* qui donne une protection contre la leucocin A et

l'enterocin A (Fimland *et al.*, 2002). Les protéines d'immunités des bactériocines de classe IIa sont classées en trois sous-groupes sur la base de leur alignement de séquence.

Figure 5 : Sous-groupe et alignement de quelques protéines d'immunité des bactériocines de classe IIa (Drider *et al*., 2006)

Comme montré à la figure 5, il y a une grande variation entre les séquences des différentes protéines d'immunité, malgré le haut degré d'homologie des bactériocines correspondantes (Fimland *et al*., 2002). Les protéines d'immunité ont un haut degré de spécificité. Néanmoins, elles peuvent donner une immunité aux bactériocines contenant des séquences C-terminales homologues. Une classification des bactériocines sur la base de ces séquences a donc été réalisée. Brièvement, le sous-groupe1 contient l'enterocin A, la pediocin PA-1, la sakacin P et la piscicolin 126 ; le sous-groupe 2 contient la leucocin A et la mesentericin Y105 tandis que le sous-groupe 3 contient la curvacin A, la carnobacteriocin Bm1 et l'enterocin P (Fimland *et al.*, 2002). De plus, il semble que la séquence C-terminale des protéines d'immunité permette également de déterminer la spécificité d'action (Johnsen *et al.*, 2004). La fonctionnalité de la protéine d'immunité est par ailleurs dépendante de la souche (Drider *et al.*, 2006).

Les protéines d'immunité ont une action intracellulaire. Différentes études ont pu montrer qu'elles se trouvaient principalement dans le cytoplasme et qu'elles interagissent peu avec la membrane cellulaire (Johnsen *et al.*, 2004). Cependant, leur mode d'action reste peu compris (Ennahar *et al.*, 2000; Fimland *et al.*, 2002; Drider *et al*., 2006). Diep *et al.* (2007) ont montré récemment que les protéines d'immunité de la lactococcin A mais aussi de bactériocines de classe IIa (l'enterocin P, la sakacin A et la pediocin PA-1) agissaient en se liant à un complexe formé par les sous unités IIC et IID de la mannose transférase et la bactériocine en inhibant ainsi son action, probablement en l'empêchant de former les pores

qui mènent à la mort de la cellule. En l'absence de la bactériocine dans le milieu de culture, il n'y a pas ou très peu d'interaction entre la protéine d'immunité et les sous-unités IIC et IID de la « mannose transférase ». Ce modèle de mécanisme d'action est montré à la figure 6.

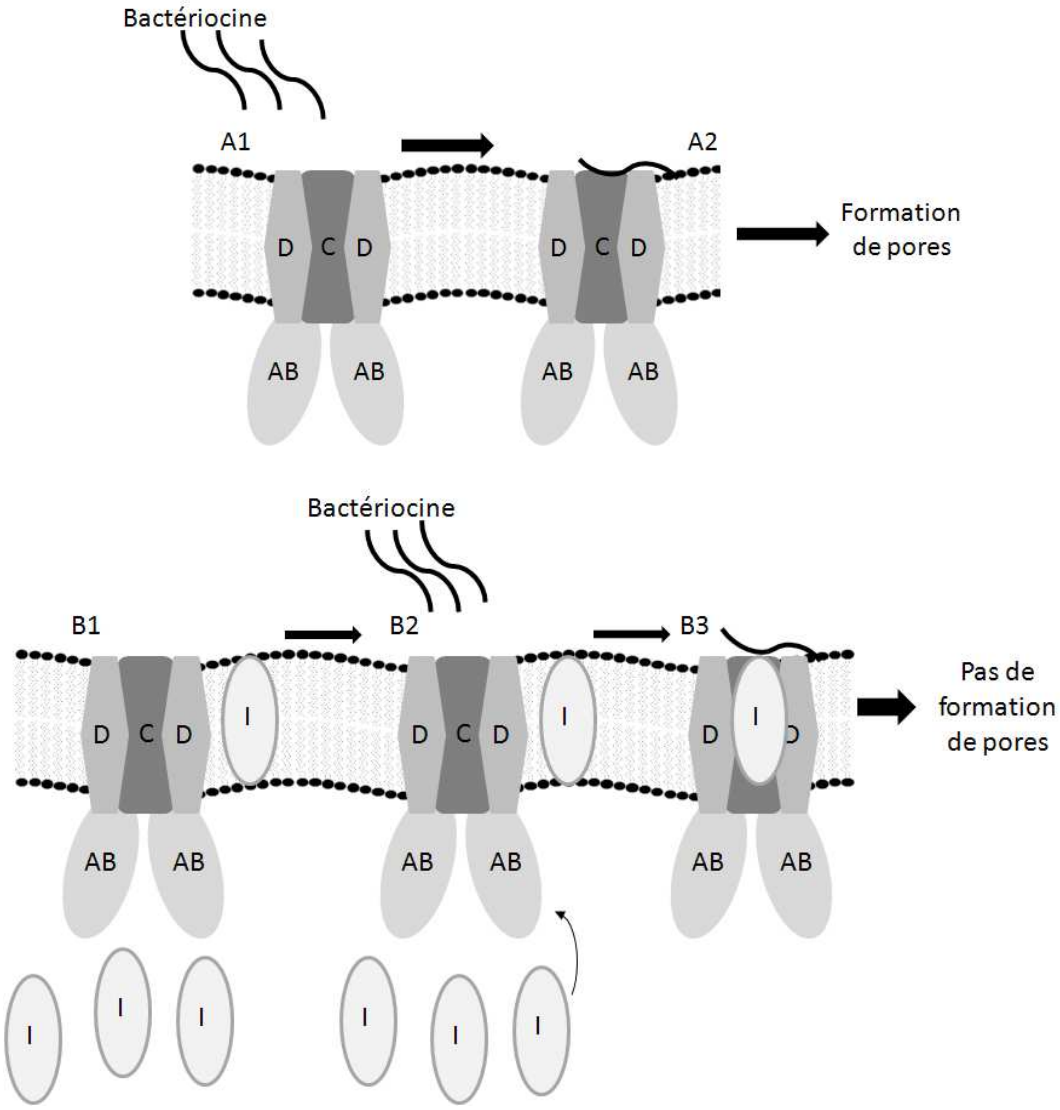

Figure 6 : Modèle d'action de la lactococcin A et de bactériocines de classe IIa (A) ainsi que de leurs protéines d'immunité (B). En absence de protéine d'immunité (A1 et A2), la bactériocine interagit avec la « mannose permease » (AB, C et D) pour ensuite former des pores. Si les protéines d'immunité (I) sont présentes, elles se trouvent sous forme libre dans le cytoplasme et n'interagissent que faiblement avec la membrane (B1). En présence de la bactériocine, elles interagissent avec le complexe formé entre la bactériocine et la « mannose perméase » et empêche la bactériocine de former des pores (B2 et B3) (inspiré de Diep *et al.*, 2007).

1.3.5.1.2.2 La classe IIb

Comme pour la classe IIa, la résistance de la cellule à la bactériocine qu'elle produit se fait par l'intermédiaire de la production d'une protéine d'immunité. Il n'y a qu'un seul gène d'immunité. L'information génétique est localisée sur le même opéron que celui des deux gènes de structure (Garneau *et al.*, 2002 ; Oppegard *et al*., 2007).

1.3.5.2 Le mécanisme de résistance des cellules cibles aux bactériocines

Etant donné qu'une des utilisations principales des bactériocines est leur application dans l'industrie alimentaire pour prévenir des contaminations par des bactéries pathogènes telles que *L. monocytogenes*, le développement de souches résistantes peut être un problème.

1.3.5.2.1 La résistance aux lantibiotiques

Le mécanisme de résistance des lantibiotiques le plus étudié est celui de la résistance à la nisine. Les résistances vis à vis de la nisine dans des souches non résistantes apparaissent à la fréquence de 10^{-9} à 10^{-2} pour *L. monocytogenes* et 10^{-8} à 10^{-2} pour les autres microorganismes après exposition à des concentrations croissantes. Leur stabilité est variable (Gravesen *et al*., 2002b; Guinane *et al.*, 2006; Kramer *et al.*, 2006; Guinane *et al*., 2007). La fréquence d'apparition des résistances varie en fonction de la souche cible et des conditions environnementales (pH, concentration en chlorure de sodium et température). L'apparition d'une résistance est souvent associée à une diminution du taux de croissance maximale de la cellule (Gravesen *et al*., 2002a).

Il a été montré que les mutants résistants n'avaient pas de modification au niveau du lipide II (Kramer *et al.*, 2004). La résistance à la nisine a été attribuée à une différence de la composition en acide gras dans la membrane et à des modifications au niveau de la paroi cellulaire (Kramer *et al.*, 2006; Naghmouchi *et al*., 2007). Récemment, Kramer *et al.* (2006) ont montré que des souches de *Lactococcus lactis* résistantes à la nisine exprimaient différemment 93 gènes par rapport à la souche sauvage non résistante, 62 étant plus exprimés et 31 étant sous exprimés. Cette étude a permis de montrer que le mécanisme principal de résistance semblait être d'empêcher la bactériocine d'atteindre le lipide II par différents modes (Kramer *et al.*, 2006) :

- Changer la paroi cellulaire en l'épaississant, la faisant plus dense ou en changeant sa charge
- Elever le pH local à l'extérieur de la membrane cytoplasmique
- Changer l'expression de l'opéron fab qui est impliqué dans l'élongation et la saturation des phospholipides, ce qui augmente la fluidité de la membrane et empêche la nisine de former des pores
- Changer les ABC transporteurs présents. Ils peuvent être impliqués dans l'excrétion de la nisine hors de la membrane cytoplasmique, l'empêchant d'atteindre le lipide II.

D'autre part, il a été montré que l'adsorption de la lacticin 3147 sur la membrane des cellules cibles était diminuée chez les mutants résistants (Guinane *et al.*, 2006).

L'apparition d'une résistance contre un lantibiotique peut induire une résistance contre un autre. Par exemple, l'apparition d'une résistance contre la lacticin 3147 induit une résistance à la nisine (Guinane *et al*., 2007).

1.3.5.2.2 La résistance aux bactériocines de classe IIa

Les résistances aux bactériocines de classe IIa sont souvent très élevées avec une augmentation de la MIC (concentration inhibitrice minimale) d'un facteur 1000. 1 à 8 % des souches sauvages de *L. monocytogenes* isolées sont résistantes (Gravesen *et al*., 2002a). Les mutations spontanées apparaissent après exposition à la bactériocine. *L. monocytogenes* développe des résistances aux bactériocines de classe IIa à la fréquence de 10^{-3} à 10^{-6} (Gravesen *et al.*, 2004). Cette fréquence d'apparition des résistances varie en fonction de la souche, de la bactériocine et de facteurs environnementaux tels que le pH, la concentration en chlorure de sodium et la température (Gravesen *et al.*, 2002a). La stabilité de ces mutants est variable. Par exemple, des mutants spontanés de *L. monocytogenes* 412 résistants à la pediocin PA-1 gardent cette résistance après 100 générations (Gravesen *et al.*, 2002a). Différents mécanismes sont à l'origine de cette résistance :

- L'augmentation du pourcentage d'acides gras insaturés dans la membrane et du nombre de chaînes courtes. Ces modifications augmentent la fluidité de la membrane en diminuant les potentialités d'insertion de la bactériocine et la formation des pores (Vadyvaloo *et al*.. 2002).

- L'absence de la mannose phosphoenolpyruvate-dependent phosphotransférase (PSTS), la molécule transmembranaire réceptrice de la bactériocine (Vadyvaloo *et al.*, 2004b). Il a été montré que, chez *L. monocytogenes*, l'inactivation du gène *rpo*N et de l'opéron *mpt* codant pour la mannose perméase induit la résistance de *L. monocytogenes*. La mannose perméase est une protéine transmembranaire qui assure le transport du sucre (mannose, glucose et 2-deoxyglucose chez *L. monocytogenes*) et sa phosphorylation de part et d'autre de la membrane cellulaire. Chez *L. monocytogenes*, cette molécule contient trois sous-unités. Le gène *rpo*N code pour la sous-unité σ^{54} de la RNA polymérase bactérienne qui dirige l'activation de la transcription de l'opéron *mpt*. Dans les mutants résistants, une diminution de l'expression du gène codant pour la mannose perméase et une augmentation de l'expression du gène codant pour la phosphotransférase spécifique des β-glucosides ont été remarquées (Gravesen *et al.*, 2002a; Gravesen *et al.*, 2002b; Gravesen *et al.*, 2004; Vadyvaloo *et al.*, 2004). Par ailleurs, il a été montré que des mutants résistants avaient un taux de croissance maximal réduit (Gravesen *et al.*, 2002a). Cette réduction peut être attribuée à la diminution de la capacité à transporter des nutriments, comme le glucose, de part et d'autres de la membrane cellulaire (Naghmouchi *et al.*, 2007).

1.3.5.2.3 Les cross-résistances

Les mécanismes de résistances aux lantibiotiques et aux bactériocines de la classe IIa ne sont pas les mêmes. Cependant, la résistance aux lantibiotiques peut parfois entraîner une résistance aux bactériocines de classe IIa ou inversément. Par exemple, Naghmouchi *et al.* (2007), ont montré que la résistance de *L. monocytogenes* acquise à la nisine A ou Z augmente la résistance à la pediocin PA-1 et à la divergicin M35, deux bactériocines de classe IIa. D'autre part, les mutants résistant à la divergicin M35 montrent une résistance accrue à la nisine Z mais réduite à la nisine A. Les mutants résistant à la pediocin PA-1, quand à eux, ont une résistance plus élevée à la nisine Z et la divergicine mais diminuée à la nisine A.

1.3.6 La production et le conditionnement des bactériocines

1.3.6.1 La production des bactériocines

Les bactériocines sont généralement produites à la fin de la phase exponentielle et au début de la phase stationnaire de croissance. Elles peuvent ensuite être dégradées par les protéases produites par la bactérie lactique productrice (Savijoki *et al.,* 2006) ou être adsorbées à sa surface ce qui mène à la baisse de la concentration de bactériocines dans la culture. Les facteurs influençant la production de bactériocines sont principalement la souche productrice, la température, le pH, la composition du milieu et la technologie de fermentation employée.

Comme l'ont montré Moretro *et al.* (2000) pour la production de sakacin P par *Lb. sakei*, une même bactériocine peut être produite par des souches ou espèces différentes dont la capacité de production peut être variable. Lors d'une optimisation de production, si différentes souches sont disponibles, le choix de celle-ci pourra être déterminant.

Les conditions de culture influencent fortement la production de bactériocines. En effet, l'optimalisation de la croissance ne résulte pas nécessairement en l'optimalisation de la production de bactériocines (Parente *et al.,* 1999). Il a même été suggéré que des conditions de croissance défavorables permettent de stimuler leur production (Verluyten *et al.,* 2004). Les températures et pH optimaux de production sont souvent inférieurs à ceux optimaux pour la croissance. C'est par exemple le cas pour la production de bactériocine par *Lb. curvatus* LTH1174 (Messens *et al.,* 2003), *Leuconostoc mesenteroides* L124, *Lb. curvatus* L442 (Mataragas *et al.,* 2003), de sakacin P par *Lb. sakei* CCUG42687 (Moretro *et al.,* 2000), d'amylovorin L471 par *Lb. amylovorus* DCE471 (De Vuyst *et al.,* 1996) et de pediocin PA-1 par *Pediococcus damnosus* (Nel *et al.,* 2001).

La composition du milieu, tout particulièrement les sources et concentrations de carbone et azote, affectent fortement la production de bactériocines. Les bactéries lactiques productrices requièrent de nombreux nutriments pour leur croissance et des milieux riches, contenant de l'extrait de viande, de levure et des hydrolysats de protéines, sont nécessaires. Il a déjà été montré que l'augmentation des concentrations en extrait de levure, extrait de viande ou peptone peut permettre une augmentation de la production de bactériocines (Aasen *et al.,*

2000 ; Nel *et al.,* 2001 ; Mataragas *et al.,* 2004 ; Todorov *et al.,* 2004 ; Verluyten *et al.,* 2004). D'autre part, quelques études ont montré que la source de carbone utilisée et sa concentration est un facteur important dans l'optimisation de la production de bactériocines (Leal-Sanchez *et al.,* 2002 ; Leroy *et al.,* 2006 ; Chen *et al.,* 2007 ; Anastasiadou *et al.,* 2008). L'ajout de ces nutriments lors d'une culture fed-batch permet souvent d'augmenter la production comparativement à une culture en batch (Callewaert *et al.,* 2000 ; Guerra *et al.,* 2005 ; Lv *et al.,* 2005).

L'utilisation de la technique des cellules immobilisées peut permettre d'augmenter la durée et la stabilité de la production de bactériocines. Les cellules peuvent être immobilisées dans des biofilms ou des billes d'alginates de calcium. Cette technique a déjà été utilisée avec succès pour la lacticin 3147 et la nisine (Scannell *et al.,* 2000 ; Pongtharangkul *et al*., 2006b).

1.3.6.2 Le conditionnement des bactériocines

Il est très difficile de conditionner les bactériocines sous une forme purifiée. La purification des bactériocines est une procédure longue et coûteuse qui nécessite la mise en œuvre de nombreuses techniques à savoir une précipitation des protéines au sulfate d'ammonium, différentes combinaisons de chromatographies sur colonne telles que des échanges d'ions ou des interactions hydrophobes et une étape finale de chromatographie liquide à haute performance en phase inverse. Ces traitements ne sont pas applicables à l'échelle industrielle. La stratégie souvent mise en œuvre consiste dès lors en l'adsorption de la bactériocine sur la cellule productrice suivie d'une centrifugation ou d'une ultrafiltration de la culture et de la désorption de la bactériocine par abaissement du pH à 2 et augmentation de la concentration en chlorure de sodium. Les bactériocines semi-purifiées peuvent alors être conditionnées sous forme sèche par atomisation ou lyophilisation par exemple (Parente *et al*., 1999). La nisine, la seule bactériocine légalement approuvée comme additif alimentaire, est commercialisée sous une forme semi-purifiée.

1.3.7 Les applications des bactériocines dans l'industrie alimentaire

1.3.7.1 Les propriétés avantageuses des bactériocines pour une application alimentaire

Les bactériocines sont habituellement reconnues comme sûres, sont sensibles aux protéases digestives et ne sont pas toxiques pour les cellules eucaryotes (Wijaya *et al.*, 2006). Elles ont une grande tolérance aux variations de pH et aux traitements thermiques. Leur spectre antimicrobien peut être large ou étroit, elles peuvent donc cibler sélectivement des bactéries pathogènes ou détériorantes sans inhiber les bactéries indispensables et ont un mode d'action bactéricide (Galvez *et al.*, 2007). Les bactériocines doivent cependant être considérées comme un moyen de préservation complémentaire à ceux déjà existant (Deegan *et al.*, 2006).

1.3.7.2 L'application des bactériocines dans le secteur alimentaire

Les bactériocines peuvent être appliquées sous un forme purifiée, semi-purifiée ou sous la forme d'une concentré obtenu après fermentation d'un substrat alimentaire. Les bactéries productrices peuvent également être appliquées dans les produits alimentaires, la bactériocine sera alors produite *in situ*.

1.3.7.2.1 Application de la bactériocine purifiée ou semi-purifiée

Les bactériocines purifiées ou semi-purifiées sont appliquées après production en fermenteur, purification ou semi-purification et conditionnement par les techniques adéquates, qui peuvent être relativement coûteuses. D'un point de vue législatif, une telle préparation est considérée comme un additif alimentaire. Jusqu'à présent, seule la nisine, un lantibiotique, est acceptée comme additif alimentaire (E234) (Guinane *et al.*, 2005).

Les bactériocines peuvent également être appliquées sous la forme d'un concentré obtenu après fermentation par la souche productrice et atomisation d'un substrat alimentaire tel que le lait par exemple. Cette préparation sera considérée comme un ingrédient fermenté. Elle contiendra la bactériocine mais également d'autres métabolites antimicrobiens tels que l'acide lactique. La pediocin, une bactériocine de classe IIa, est commercialisée sous cette forme sous le nom ALTA 2341. Des essais ont été récemment fait avec la lacticin 3147, un lantibiotic (Deegan *et al.*, 2006 ; Galvez *et al.*, 2007). Au niveau législatif, cette forme ne

nécessite pas d'approbation. Cependant, si la culture n'est pas traditionnellement consommée, il faudra se référer à la législation sur les « novel food » (EC258/97).

Un autre mode d'application des bactériocines consiste en leur immobilisation sur les cellules productrices, dans des gels ou des films tels que l'alginate de calcium, la gélatine, la cellulose, les protéines de soja, des films de polysaccharides,… La bactériocine sera alors libérée dans le produit au cours de sa conservation. Depuis peu, des emballages en polyéthylène ou d'autres films plastiques contenant des bactériocines ont été développés. Ces emballages permettent de réduire la croissance des microorganismes pathogènes ou indésirables pouvant se développer en surface durant la conservation du produit (Luchansky *et al*., 2004, Deegan *et al*., 2006 ; Ghalfi *et al*., 2006a ; Galvez *et al*., 2007).

1.3.7.2.2 Application de la bactérie productrice de bactériocines

L'utilisation des bactéries productrices de bactériocines peut être intéressante tant au niveau législatif qu'économique. Les bactéries productrices de bactériocines peuvent être ajoutées comme starter dans des produits fermentés ou comme culture protectrice. Elles doivent être capables de croître et de produire des bactériocines dans l'aliment à conserver. La composition du produit (nutriments accessibles, pH, additifs alimentaires…) et les conditions de stockage (température, atmosphère, activité d'eau…) doivent donc permettre la croissance et la production de bactériocines. Cette production étant souvent sous le contrôle d'un système de quorum sensing, la concentration en molécule inductrice doit être suffisante pour l'induire. Son interaction avec la matrice alimentaire peut donc être un facteur limitant.

Si les bactéries sont ajoutées en tant que starter dans des produits fermentés, elles doivent pouvoir conférer au produit les propriétés organoleptiques désirables tout en produisant des bactériocines. Les bactéries productrices de bactériocines peuvent être également ajoutées en combinaison avec un autre starter qui produira les propriétés organoleptiques désirables. Dans ce cas, la bactérie productrice de bactériocines ne doit pas détériorer les qualités organoleptiques de l'aliment fermenté et la bactériocine produite ne doit pas avoir d'activité contre le starter (Deegan *et al.,* 2006; Galvez *et al.,* 2007).

Si la bactérie est appliquée en tant que culture protectrice, elle doit être capable de produire sa bactériocine sans modifier les propriétés organoleptiques (Rodgers, 2001). La concentration cellulaire maximale atteinte dans le produit doit par ailleurs être inférieure à la limite de 10^6 cfu g^{-1} généralement admise pour les produits non fermentés.

1.3.7.3 Les facteurs influençant l'activité des bactériocines dans les produits alimentaires

Lors d'une application alimentaire, la composition du produit est un des premiers facteurs pouvant réduire ou totalement dissiper l'activité des bactériocines de part son adsorption sur des composantes du produit, la limitation de sa solubilité et de sa diffusion dans le produit, sa dégradation par les protéases, l'interaction avec des additifs alimentaires ou des ingrédients et/ou un pH inapproprié. Les traitements appliqués aux produits constituent un deuxième facteur pouvant limiter l'activité inhibitrice des bactériocines dans un produit alimentaire. En effet, des traitements thermiques trop élevés peuvent dégrader les bactériocines présentes. La température de stockage pourra également réduire l'activité des bactériocines, qui varie en fonction de la température (Galvez *et al.,* 2007).

Un autre facteur limitant l'activité des bactériocines est la flore autochtone, principalement sa concentration, la présence de bactéries résistantes, la présence de microorganismes produisant des protéases dégradant la bactériocine et l'état physiologique de cette flore. Un état physiologique stationnaire ou stressé ainsi que la formation de spores peut conduire à une résistance accrue. En outre, dans les produits solides, les bactéries forment des microcolonies ou des biofilms dont la résistance aux bactériocines peut être plus élevée (Schöbitz *et al.,* 2003).

Ces phénomènes peuvent conduire à (i) une absence totale d'activité antimicrobienne, (ii) une inhibition partielle des bactéries cibles, (iii) une diminution initial de la concentration des bactéries cibles sous la limite de détectabilité suivie d'une reprise de croissance au cours du stockage, un phénomène appelé « rebond » (Bouttefroy *et al.,* 2000 ; Vignolo *et al.,* 2000 ; Schöbitz *et al.,* 2003).

1.3.7.4 La combinaison de différentes bactériocines pour augmenter la durée de vie du produit

La combinaison de différentes bactériocines permet d'augmenter l'activité et le spectre d'action, tout particulièrement en combinant des bactériocines appartenant à des classes différentes (Vignolo *et al.,* 2000). Cependant, une attention toute particulière devra être portée au développement de résistances chez les bactéries cibles. Le mécanisme de résistance aux bactériocines de classe IIa, par exemple, semble être identique pour toutes les bactériocines de cette sous-classe. Une bactérie résistante à une bactériocine de classe IIa sera donc résistante à d'autres bactériocines de classe IIa. D'autre part, l'apparition de résistance à des bactériocines

de classes différentes chez des bactéries cibles peut également être observé, un phénomène appelé « cross-résistance » (Naghmouchi *et al.*, 2007; Deegan *et al*., 2006).

1.3.7.5 La combinaison des bactériocines avec d'autres agents

La combinaison des bactériocines avec d'autres traitements de conservation chimique ou physique donne des résultats prometteurs pour la conservation des aliments. Les molécules chimiques peuvent être des acides organiques, le nitrite, le chlorure de sodium, l'éthanol, des huiles essentielles (l'impact sur les propriétés organoleptiques doit être soigneusement évalué) ou des agents chélatants tel que l'EDTA, le phosphate trisodique, le citrate. Ces agents chélatants permettent de séquestrer les ions magnésium des lipopolysaccharides de la membrane externe des bactéries Gram-négatif permettant aux bactériocines d'atteindre la membrane interne, siège de leur activité. Les traitements physiques peuvent être des traitements thermiques, le stockage sous atmosphère contrôlé, l'application de champs électriques ou l'application de hautes pressions (Rodgers, 2004 ; Deegan *et al*., 2006; Galvez *et al.,* 2007). D'autre part, l'utilisation d'inhibiteurs de protéases ou de protéines de soja a été suggérée afin de prévenir la dégradation des bactériocines par les protéases présentes dans le produit à conserver.

1.4 Conclusion

Les études réalisées précédemment ont mis en évidence une grande variabilité de séquences, structures, mode et spectre d'action entre les différentes bactériocines produites par les bactéries lactiques. Cette variabilité implique également des spécificités d'application alimentaire. Il apparaît donc important de profiter au maximum de la diversité microbienne afin de disposer de collections élargies de bactéries lactiques produisant des bactériocines variées avec des potentialités d'applications diverses.

D'autre part, même s'il existe de nombreuses données concernant les bactériocines, de nombreux facteurs restent à être compris afin d'optimiser leurs applications industrielles potentielles. En voici une liste non exhaustive :

- Comprendre l'impact de la séquence et de la structure sur le mode et le spectre d'action
- Comprendre l'impact des facteurs nutritionnels et environnementaux sur la production de bactériocines
- Comprendre l'impact des facteurs environnementaux sur l'activité des bactériocines
- Comprendre la façon dont les gènes codant pour les bactériocines se transmettent phylogénétiquement.

Notre travail a eu pour objectif de contribuer à la compréhension de ces facteurs. Il a consisté tout d'abord à profiter de la biodiversité de produits alimentaires divers sénégalais et belges pour sélectionner des bactéries lactiques produisant des bactériocines avec une activité contre *L. monocytogenes*, de les identifier, de décrire les propriétés et structures des bactériocines produites et d'évaluer leurs potentialités d'applications dans de la viande. Ensuite, la souche produisant la bactériocine la plus originale et présentant le plus d'applications potentielles a été étudiée plus spécifiquement. Son spectre d'action, les facteurs régulant la croissance et la production ainsi que la caractérisation partielle des gènes nécessaires à la production de la bactériocine ont été étudiés. Ses potentialités d'applications dans de la viande ont ensuite été évaluées.

Chapitre 2 : Objective

The aim of this work is to contribute to the comprehension of the phenomenons involved in the production and the food applications of bacteriocins and the producer lactic acid bacteria to decrease the contamination of food products by *L. monocytogenes*. The objectives of this thesis are:

- The evaluation of the antimicrobial properties of lactic acid bacteria isolated from food product and the identification of the compounds involved
- The selection of bacteriocin producing lactic acid bacteria with activity against *L. monocytogenes*, the study of the properties of these bacteriocins and their genetic identifications
- The selection of the lactic acid bacteria that have the wider applications potential
- The characterisation of the produced bacteriocin, at a biochemical and genetic level
- The understanding of some factors controlling the growth and the bacteriocin production
- The evaluation of the impact of the bacteriocin production during the raw meat applications of the producing strains to inhibit *L. monocytogenes*.

Chapitre 3 Material and methods

3.1 Microorganisms

3.1.1 Lactic acid bacteria

Lactic acid bacteria used in this study are:

- *Lactobacillus sakei* LMG17302, a non bacteriocin-producer isolated from raw sausage.
- *Lactobacillus curvatus* CWBI-B28 isolated from raw meat and producing an unidentified bacteriocin (Ghalfi *et al*., 2006a; Ghalfi *et al*., 2006b; Ghalfi *et al.*, 2007a; Ghalfi *et al.*, 2007b).
- *Lactobacillus sakei* CWBI-B1365 isolated from raw poultry during this study.
- *Carnobacterium maltaromaticum* CWBI-B1369 isolated from raw poultry during this study.
- *Carnobacterium maltaromaticum* CWBI-B1436 isolated from raw poultry during this study.
- *Lactococcus lactis lactis* CWBI-B1437 isolated from fermented fish during this study
- *Carnobacterium piscicola* (now *Carnobacterium maltaromaticum*) 8A, which produces carnobacteriocins BM1 and A.
- *Lactobacillus sakei* Lb 706, which produces sakacin A (Diep *et al.*, 2000).
- *Lactobacillus sakei* 2521, which produces sakacin G (Simon *et al.*, 2002).
- *Lactobacillus curvatus* DSM 20019 used as type strain for DNA-DNA hybridisation experiments.
- *Lactobacillus sakei* DSM20017 used as type strain for DNA-DNA hybridisation experiments.

Culture conditions are indicated below.

3.1.2 Indicator strains

Indicator strains used in this study were:

- *Listeria monocytogenes* CWBI-B715, BFE 181, BFE 104, BFE 250, BFE 172, BFE 188, BFE 411, BFE 286, BFE 171, BFE 384, BFE 236, BFE 422, BFE 227, BFE 60, LMG21263 and LMG23905 originating from various fish and meat products
- *Salmonella* Typhimurium isolated from poultry

3.2 Media

- MRS (de Man, Rogosa and Sharp): casein peptone (10 g l^{-1}) (Organotechnie, La Courneuve, France), yeast extract (5 g l^{-1}) (Organotechnie, La Courneuve, France), meat extract (5 g l^{-1}) (Organotechnie, La Courneuve, France), ammonium sulphate (2 g l^{-1}), dipotassium hydrogen phosphate (2 g l^{-1}), Tween 80 (1 ml l^{-1}), sodium acetate (5g l^{-1}), magnesium sulphate (0.1 g l^{-1}), manganese sulphate (0.05 g l^{-1}), glucose (20 g l^{-1}). For solid medium: agar(15 g l^{-1}).
- MMRS: meat extract (5g l^{-1}) (Organotechnie, La Courneuve, France), ammonium sulphate (2 g l^{-1}), dipotassium hydrogen phosphate (2g l^{-1}), Tween 80 (1 ml l^{-1}), sodium acetate (5 g l^{-1}), magnesium sulphate (0.1 g l^{-1}), manganese sulphate (0.05 g l^{-1}), glucose (20 g l^{-1}).
- MRSS the same as MRS except that glucose is replaced by 20 g of sucrose;
- MRST, the same as MRS except that glucose is replaced by 20 g of trehalose;
- MRSM, the same as MRS with 20 g of meat extract;
- MRSSM, the same as MRSS with 20 g of meat extract
- PALCAM: agar (10 g l^{-1}), ammonium ferric citrate (0.5 g l^{-1}), esculin (0.8 g l^{-1}), glucose (0.5 g l^{-1}), lithium chloride (15 g l^{-1}), D-mannitol (10 g l^{-1}), peptone (23 g l^{-1}), phenol red (0.08 g l^{-1}), sodium chloride (5 g l^{-1}), starch (1 g l^{-1}), Polymyxin-B-sulfate (5 g l^{-1}), Ceftazidim (0.01 g l^{-1}), acriflavine (0.0025 g l^{-1}), final pH 7.
- Peptone water: NaCl (5 g l^{-1}), casein peptone (1 g l^{-1}), and Tween 80 (1 ml l^{-1})
- M17 broth: soy peptone (5 g l^{-1}), meat peptone (2.5 g l^{-1}), casein peptone (2.5 g l^{-1}), yeast extract (2.5 g l^{-1}), meat extract (5 g l^{-1}), glucose (5 g l^{-1}), ascorbic acid (0.5 g l^{-1}), magnesium sulphate (0.25 g l^{-1}), agar (15 g l^{-1}), dipotassium hydrogenophosphate (13.3 g l^{-1}), potassium dihydrogenophosphate (5.7 g l^{-1}).
- Standard-I broth: peptone (15 g l^{-1}), yeast extract (3 g l^{-1}), sodium chloride (6 g l^{-1}), glucose (1 g l^{-1}), agar (12 g l^{-1}) (not present in the broth).
- Plate count agar: casein peptone (0.5 g l^{-1}), yeast extract (2.5 g l^{-1}), glucose (1 g l^{-1}), agar (15 g l^{-1}).

Culture conditions are indicated below.

3.3 PCR primers

Table 2 shows the sequence of the primers used during this study.

Table 2: Primers used during this study

Name	Sequence (5'-3')
16Sp0	GAAGAGTTTGATCCTGGCTCAG
16Sp6	CTACGGCTACCTTGTTACGA
Cbfw	AAAGAAATG(C/A)AACAA(T/A)TT(C/A)(C/A)(T/C)GG
CbBm1rev	CCTCTTTAATGTCCCATTCC
CbB2rev	CACCACCTTGCTCTATATTG
CbArev	CCAACTCTTTGGTCTACAGTC
Piscfw	GATGTGATACAGTCAGCATG
Piscrev	GACTTTAATTATCCTTTGTTC
Nisfw	AGATTTTAACTTGGATTTGG
Nisrev	TTATTTGCTTACGTGAATAC
Sakfw	GAA(T/A)T(A/G)(C/A)(A/C)ANCAATTA(C/T)(A/C)GGTGG
SakArev	GGCCCAGTTTGCAGCTGCAT
SakPrev	GGCCCAGTTTGCAGCTGCAT
SakQ rev	TACCACCAGCAGCCATTCCC
PheSfw	CAYCCNGCHCGYCAYATGC
PheSrev	GGRTGRACCATVCCNGCHCC
sakimm5fw	AGCTTCGGGATTCTTAGCTATATCAATTTT
sakstruc4rev	ACGTAGCTTAACGATCCAAG
saktrans2fw	CTGAACAACCATGAGAGTTACAGCTAACACCAT
saktrans1rev	GCTTGCTGTCCTTGGTCAGCTATCG
sakimm6rev	CAACATCGAAGCTCTCTTGCACTTACTAGAA
saktrans3fw	GCAGCATTATTGAATTCTGCAATAATCGAAAACG
sakG2fw	GTAAAAATTATTTAACAGGAGG
sakG2rev	TTAGTGCTTTTTTATCTGGTA
sagCrev	CAATCTACCACACAAAAAACTCATAAC
sakgCfw	CGGATAATAACTCAGTTAAAGC
sakimmfw	GTGTTTTGGTATGTGCTCTGAG
16sp0	GAAGAGTTTGATCCTGGCTCAG
R3seq18	CCAACATCTCACGACACG
ImmQrev	GTTGACGCCTTAAAAAGAATATAACG
ImmQfw	GTGTCTCTAAACAAGCGTGTTGC
ImmArev	CAATACTAACTTACACATCTACTGC
ImmAfw	CAGACCACGCCTTAGGTGTTTC
OrfYr	TAAACGCGAGCGTATTTACGG
OrfYf	AACGCGCTTCTCGACATTACC
SppiAf	ATGAAAATATTGAAGTGGTATTCAGG

SppiAr	ATAGCCATATCTTATATTAGACAACG

3.4 General methods

3.4.1 Analytical procedure

3.4.1.1 Antimicrobial activity assay

Depending on the experiment, the antimicrobial activity was tested using cell-free supernatants, the semi-purified bacteriocin or on organic acid and hydrogen peroxide solutions. The preparation of the cell-free supernatants was made by centrifugation of a culture samples and filtration of the supernatants through a 0.2 µm cellulose acetate filters (Acrodisc, Sartorius).

Antimicrobial activity against indicator strains was tested by a modified agar well-diffusion method. Briefly, overnight cultures at 37°C of the indicator strains in 10 ml of the suitable medium (M17 for *L. monocytogenes* and nutrient broth for *S.* Typhimurium) were made. The overnight culture was used to inoculate 1 litre of the same growth medium at approx. 50°C. Following inoculation, the agar was poured immediately into Petri dishes and allowed to solidify. Wells of 7 mm diameter were made into the agar plates. 80 µl of the supernatants were placed into the wells. The plates were then incubated during 24 hours at 30°C.

For quantitative determination, the antilisterial activity was evaluated by the critical dilution method (Benkerroum *et al.*, 2002). Briefly, the sample was diluted using a two-fold dilution series in distilled water. One arbitrary unit per millilitre (AU ml^{-1}) was defined as the reciprocal of the highest two-fold dilution showing a definite zone of growth inhibition of the indicator strain. The specific production was defined as the bacteriocin production per unit of $OD_{600\ nm}$ (AU ml^{-1} OD^{-1}).

Quantitative determination was also performed by spotting ten µl of the neutralised cell-free supernatants on the solidified surface of Standard-I agar plates inoculated with the indicator strains. Plates were then incubated at 37°C for 16 hours. The inhibitory activity was evaluated by measuring the diameter of the inhibition zone.

3.4.1.2 Determination of sugar and organic acid concentration and pH in LAB culture supernatants

Cultures were centrifuged. The pellet was discarded. The pH of the supernatants was measured using a pH meter (Consort, Turnhout, Belgium). Lactic acid, acetic acid, glucose, sucrose and fructose concentration in the culture broth were determined by HPLC (Agilent 1100 series, Agilent Technologies, Massy France) using a C-610H ion exchange column (300 mm x 7.8 mm, 9 µM, Supelco, Bellefonte, PA) equipped with a refractometer detector (RID). The mobile phase was phosphoric acid 0.1 % in MilliQ water at a flow rate 0.5 ml min^{-1}. The temperature of the column was fixed at 30°C. A standard curve was generated in the range 0.125 to 4 g l^{-1}. A ten-fold dilution of the sample was prepared to be in this range.

3.4.2 General genetic techniques

Standard molecular genetic techniques were used (Sambrook *et al.*, 2001). Restriction enzymes and T4 DNA ligase were obtained from Fermentas GMBH (St. Leon-Rot, Germany) or New England Biolabs (Beverly, MA, USA). Total DNA was extracted using the Wizard Genomic DNA purification kit (Promega, Madison, USA) or using the guanidium extraction method of Pitcher *et al.* (1989) as modified by Björkroth and Korkeala (1996). Plasmid DNA was extract using the QIAGEN plasmid midi kit (Qiagen, Venlo, Netherland). RNA were isolated using RNA protect reagent and RNeasy Mini Kit (Qiagen, Hilden, Germany). For cDNA synthesis, the RevertAid H Minus First Strand cDNA synthesis kit (Fermentas, St Leon-Rot, Germany) or the RT-PCR beads (Amersham, Pharmacia, Freiburg, Germany) were used with random hexamer as primer. DNA and RNA concentration were spectrophotometrically determined by measuring absorbance at 260 nm. PCR amplification were performed with Taq polymerase (Amersham, Pharmacia, Freiburg, Germany), Taq DNA polymerase (Promega) and/or FideliTaq DNA polymerase (USB corporation, Staufen, Germany). PCR fragment were purified using Illustra GFX DNA and gel band purification kit (GE Healthcare) or using the Quantum Prep PCR Kleen Spin Columns (Biorad). DNA sequencing was performed at the GIGA Genomics facilities (University of Liège, Liège, Belgium) or at GATC Biotech (Konstanz, Germany). Similarity search were performed using Blast software (http://www.ncbi.nlm.nih.gov/blast/) (Altschul *et al.*, 1990).

3.5 Selection of bacteriocin producing lactic acid bacteria for applications in meat and genetic characterization of the bacteriocin

3.5.1 Collection of samples and isolation of lactic acid bacteria

Various food samples or samples associated with food production were collected in Senegal, Tunisia and Belgium. The samples collected in Senegal consisted of salted fish (17 samples) which were collected from artisanal processing operations for fish along the Atlantic coast. The samples from Tunisia consisted of minced beef (2 samples) and poultry intestine samples (2 samples). The samples collected in Belgium included poultry meat (2 samples) and poultry faeces (3 samples). All the samples were stored at 4 °C until analysis in the laboratory, which was done within 24 h, except for the Tunisian samples, which were frozen before analysis.

Ten grams of samples were diluted in 90 ml of peptone water and homogenised in a stomacher for 180 s. The samples were serially diluted using a 10-fold dilution series and plated onto MRS agar containing $CaCO_3$ (5 g l^{-1}) and novobiocin (0.05 g l^{-1}) to prevent growth of moulds. All plates were incubated at 30°C for 48 h. Predominant colonies were randomly collected and pure cultures were obtained by repeated streaking out onto MRS agar containing $CaCO_3$ (5 g l^{-1}) to visualize acidification properties. The isolates were characterised by Gram and catalase reaction. Only the Gram-positive and catalase-negative bacteria, i.e. presumptive lactic acid bacteria, were selected. They were stored at –80°C in 50% glycerol.

3.5.2 Selection of bacteriocin producing lactic acid bacteria

The LAB strains were grown for 16 h in 10 ml MRS-broth at 30°C, after which the activity of the cell-free supernatants was determined. Isolates showing antimicrobial activity were selected.

The antimicrobial activity of organic acids produced by the bacteria was suppressed by adjustment of the pH of the cell-free supernatants to pH 6.0 with 5 mol l^{-1} NaOH. In addition,

possible hydrogen peroxide activity was suppressed by treatment of the cell-free supernatants with catalase from bovine liver (Sigma, Steinheim, Germany). Briefly, the pH of the cell-free supernatant was adjusted to pH 6 with 5 mol l^{-1} NaOH. 300 µl of this neutralised cell-free supernatant were treated during 1 hours at 30°C with catalase at a final concentration of 1 mg ml^{-1}. The reference was the supernatants without added catalase. Antimicrobial activity against the indicator strains following the catalase treatment were determined as described before.

The proteinaceous nature of the inhibitory compound was confirmed by treatment of the neutralised cell-free supernatant with different proteases. 300 µl of the supernatant were treated with proteases (papaïne from *Carica papaya*, pronase from *Streptomyces griseus*, pepsin from hog stomack, α-chymotrypsine from bovine pancreas and trypsin from bovine pancreas, all from Fluka) at a final concentration of 1mg ml^{-1} and incubated at 37°C for 2 h. After this, the protease was denatured at 105°C for 5 min. 300 µl of the supernatants without proteases treatment serve as reference. Antimicrobial activities against the indicator strains following the protease treatment were determined as described before.

3.5.3 Determination of the minimal inhibitory concentration (MIC) value of organic acid and hydrogen peroxide

For the estimation of the minimal inhibitory concentration (MIC) of the lactic, acetic and propionic acids, solutions of each acid were made at pH 3.5, 4.0, 4.5 and 5.0. For lactic acid, 5.0, 10.0 and 15.0 g l^{-1} solutions were used, while for acetic and propionic acids 1.0 and 3.0 g l^{-1} solutions were used. In order to estimate the MIC of hydrogen peroxide, a 4 mol l^{-1} solution was diluted down to 0.12 mmol l^{-1} in saline peptone water at pH 6.0. For determination of the inhibitory activity, the well-diffusion method was used as described above.

3.5.4 Identification of bacteriocin-producing strains

The bacteriocin-producing strains were identified using biochemical identification techniques, as well as genotypic methods. The range of sugars fermented was assessed using the API 50CHL test kit (Biomérieux S.A., Marcy l'Etoile, France) and identification to the

species-level was done by 16SrRNA gene sequencing. PCR amplification of the 16S rDNA was done using the primers 16SP0 and 16SP6.

For DNA-DNA hybridisation, DNA isolation and spectrophotometric determination of DNA-reassociation was done by the method of Huss *et al*. (1983). The type strains used in DNA-DNA hybridisation studies included *Lactobacillus curvatus* DSM20019^T and *Lactobacillus sakei* DSM20017^T.

3.5.5 Effect of pH and temperature on the bacteriocin activity

The bacteriocin-producing isolates were grown in MRS broth at 30°C for 16 h and the cells were harvested by centrifugation at 8000x*g* for 10 min at 4°C. The effect of pH and temperature was studied by adjusting the pH of the supernatants to 4.0, 5.0, 6.0, 7.0, 8.0 and 10.0 with either 5 mol l^{-1} HCl or 5 mol l^{-1} NaOH (three samples for each pH). The aliquots were filtrated through a 0.2 µm filter (Acrodisc, Sartorius). In addition, supernatant was heated at 72°C for 15 min and at 105°C for 20 min, while cell free supernatant kept at 4°C was used as a control. The antimicrobial activity of the supernatants after treatment was tested against *L. monocytogenes* CWBI-B715 as described before.

3.5.6 Genetic characterization of the bacteriocin

3.5.6.1 Characterization of the bacteriocin structural gene

Numerous structurally different bacteriocins have been described to date. To determine whether the selected and identified bacteriocin-producing strains (*Lb. sakei* CWBI-B1365, *Lc. lactis* subsp. *lactis* CWBI-B1437 and *C. maltaromaticum* CWBI-B1369 and CWBI-B1436) in this study produced bacteriocins identical to those described previously, we performed PCR amplification experiments for detection of specific bacteriocin genes using specific primers. The specific primers for PCR amplification were custom designed on the basis of bacteriocin gene sequences deposited in the GenBank database. The bacteriocins under investigation, the specific primers pairs used and the expected amplicon size are shown in Table 3.

Table 3 : Identified bacteriocin produced by the species of the strains of the collection and primers pairs for specific PCR-amplification of the structural gene.

Bacteriocin structural gene targetted	Primer pair	Expected amplicon size (bp)	Bacteriocin reference
Carnobacteriocin Bm1	Cbfw-CbBm1rev	164	Quadri *et al.*, 1994
Carnobacteriocin B2	Cbfw-CbB2rev	205	Quadri *et al*., 1994
Carnobacteriocin A	Cbfw-CbArev	234	Worobo *et al.*, 1994
Piscicolin 126	Piscfw-Piscrev	253	Jack *et al.*, 1996
Nisin	Nisfw-Nisrev	163	/
Sakacin A	Sakfw-SakArev	124	Holck *et al*., 1992
Sakacin P	Sakfw-SakPrev	125	Eijsink *et al*., 1996
Sakacin G	SakG2fw-SakG2rev	492	Simon *et al*., 2002
Sakacin Q	Sakfw-SakArev	131	Cocolin *et al*., 2005

For the *C. maltaromaticum* CWBI-B1369 and CWBI-B1436 strains, primers for the carnobacteriocins A, BM1 and B2, as well as for piscicolin 126 genes, were used. For the *Lb. sakei* strain CWBI-B1365, primers for sakacin P, Q, G and A genes were used. For strain *Lc. lactis* subsp. *lactis* strain CWBI-B1437, primers for the detection of the nisin A or Z genes were used.

The reference strains used as controls for bacteriocin genes included *C. piscicola* (now *C. maltaromaticum*) 8A which produces carnobacteriocins BM1 and A, *Lb. sakei* Lb 706 which produces sakacin A (Diep *et al.*, 2000) and *Lb. sakei* 2521, which produces sakacin G (Simon *et al.*, 2002).

3.5.6.2 Genetic localisation of the structural gene

Plasmidic DNA was extracted from the different bacteriocin producing lactic acid bacteria strains. The extracted plasmids were restricted using *Xba* I and *Hin*d III restriction enzymes at 37°C for 2 h. The unrestricted and restriction enzyme treated products were

analysed by gel electrophoresis using 1% agarose gels. After electrophoresis at 100 V, DNA fragments were visualised by UV exposure.

For Southern blot hybridisation, the DNA was restricted as follows: for *C. maltaromaticum* CWBI-B1369 and CWBI-B1436, the plasmid DNA was cut using a combination of *Xba* I and *Hin*d III, while the total DNA was cut using a combination of *Eco*RI and *Hin*dIII. For *Lb. sakei* CWBI-B1365, the plasmid DNA was cut with either *Hin*d III or *Eco*RI in separate reactions. The reactions mixtures were incubated at 37°C for 2 h.

For Southern blot, the restriction products were separated on 1% agarose gels using a 500 bp marker (Biorad, Munich, Germany) and the Big Dye Marker (Roche, Penzberg, Germany). Southern transfers were performed using positively charged nylon membrane (Hybaid). The DIG-labelled oligonucleotide probes for bacteriocin structural genes were synthesised by using the PCR DIG Probe synthesis Kit (Roche, Penzberg, Germany) using DNA of the strains of this study. Prehybridisation was performed for 6 hours at 65-69°C with prehybridisation solution [5X SSC (0.75 mol l^{-1} NaCl, 0.075 mol l^{-1} sodium citrate), N-laurosylsarcosine 0.1%, SDS 0.02%, blocking reagent (Herring sperm DNA) 1%]. Hybridisation was performed during 20 h at 65°C. Unbound probe was removed and the membrane was washed once for 5 min in 2 x SSC containing 0.1% SDS and twice for 15 min with 0.1% SDS and 0.1 % SSC at 68°C. Bands were visualised using the DIG DNA Labelling and Detection kit (Boehringer, Mannheim, Germany).

3.5.7 Growth of the selected strains and bacteriocin production

MRS plates were inoculated with the selected strains by streaking and incubated for 24 h at 30°C. 150 ml of MRS broth were inoculated from the plates and incubated at 30°C without agitation for 48 h. Optical density readings (λ=600 nm) and antilisterial activity quantification were monitored every 2 h. Three repetitions were done. For the quantification of bacteriocin activity, *L. monocytogenes* CWBI-B715 was used as sensitive indicator strain. From the growth curves, the maximal growth rate and the generation time were calculated during the exponential growth phase.

3.5.8 Expression studies of the bacteriocin structural gene

The LAB producer strains were grown on MRS plates at 30°C for 24 h. They were then inoculated into 50 ml MRS broth and incubated for 15 h at 30°C. Based on previous correlations with optical density and cell counts, a sample containing 10^9 cells was harvested during the end exponential growth phase by centrifugation at 9500xg. The total RNA was then isolated as described before.

For reverse transcription-PCR, RT-PCR beads (Amersham, Pharmacia, Freiburg, Germany) were used. The primers used were the same as used previously for amplification of the bacteriocin genes (see Table 3). The *phe*S (phenylalanyl t-RNA synthase) housekeeping gene was used as a positive control, using the primers PheSfw and PheSrev. For optimal reaction, 200 ng of RNA were used for each RT-PCR reaction. To assess the absence of DNA in the isolated RNA samples, 200 ng of RNA were treated with RNase for 30 minutes and this was used as a negative control in a further RT-PCR reaction with the primers for the *phe*S.

3.5.9 Meat application of the bacteriocin-producing lactic acid bacteria

Pieces without skin of fresh poultry meat were bought in a supermarket in Germany (Karlsruhe). The meat was cut into 100 g portions. LAB cultures were grown in MRS broth for 16 h at 30°C. Overnight cultures of *L. monocytogenes* strain were grown in M17 broth at 30°C.

Poultry meat was surface inoculated with 10^6 cfu g^{-1} of the LAB and 10^3 cfu g^{-1} of *L. monocytogenes* after appropriate ten-fold dilution of the culture in saline-peptone water. The reference was poultry meat inoculated only with *L. monocytogenes*. Samples were stored at 4°C in sealed bags. Samples of 10 g were aseptically taken every 7 days. 10 ml of peptone water was added and samples were homogenised in a stomacher for 180 s. Cell concentration of *L. monocytogenes* and LAB were determined after decimal ten-fold dilutions in saline peptone water by plating the appropriated dilution on PALCAM and MRS agar plates, respectively. Plates were incubated for 48 h at 37 and 30°C, respectively. Results were analysed by Student-t test analysis (p=0.95).

3.6 Study of the antilisterial bacteriocin from *Lactobacillus sakei* CWBI-B1365 and improvement of its production

3.6.1 Purification and characterisation of the bacteriocin produced by *Lactobacillus sakei* CWBI-B1365

3.6.1.1 Purification of the bacteriocin

After 20 hours culture in MMRS medium at 30°C, bacteriocin present in cell-free supernatant was purified by hydrophobic chromatography on an Amberlite XAD-4 column (Across Organics, Geel, Belgium) followed by an ion-exchange chromatography on a SP-Sepharose Fast Flow column (GE healthcare, Upsalla, Sweden). Active fraction, were further purified by reverse phase high pressure liquid chromatography on a semi-preparative Chromspher 5 C18 column (250 x 10mm, 7 µm packing, Varian Technology, Walnut Creek, CA) and on an analytical LiChroCART column (250 x 4, 5µm packing, Merck, Darmstadt, Germany).

3.6.1.2 Molecular weight determination

The molecular mass of the purified bacteriocin was determined in 10-20 % Tris-Tricine-SDS gel (BioRad Laboratories, Ivry-sur-Seine, France) as previously described (Schägger *et al.*, 1987) using ultra-low range molecular weight standard (Sigma-Aldrich, St Louis, Il). For matrix-assisted laser desorption ionization-time of flight mass spectrometry (MALDI-TOF), measurement was performed on a Bruker Ultraflex tof (Bruker Daltonics) equipped with a pulsed nitrogen laser (λ= 337 nm). The analyser was used at an acceleration voltage of 10 kV and samples were measured in the reflectron mode.

3.6.1.3 Activity spectrum determination

Activity spectrum against a wide range of bacteria was determined as described previously using 100 µl of a 400 AU ml^{-1} active culture supernatant.

3.6.1.4 Mode of action determination

The membrane electrical potential ($\Delta\psi$) was measured by monitoring the distribution of the fluorescent probe 3,3'-dipropylthiadicarbocyanine iodide [$DiSC_3(5)$] on intact listerial cells. Cells were harvested in the exponential growth phase by centrifugation, washed twice with 10 mM Tris-HCl at pH 5, 5.5 or 6 and resuspended in the same buffer to a final $OD_{600\ nm}$ of 0.1, and stored on ice until use. Fluorescence was measured with a Perkin-Elmer LS50 spectrofluorometer at 25°C in a stirred cuvette. An excitation wavelength of 643 nm and an emission wavelength of 666 nm were used with a slide width of 10 mm. The pH gradient (ΔpH) was depleted by addition of the H^+/K^+ exchanger nigericin to a final concentration of 5 µM. After a stable-state $\Delta\psi$ was reached, 10 µl of a 400 AU ml^{-1} active culture supernatant were added to the reaction mixture. The potassium ionophore valinomycin was used at a final concentration of 5 µM to obtain control sample with no $\Delta\psi$. Dissipation of the $\Delta\psi$ for a given strains was express as a percentage obtained compared to that of valinomycin.

3.6.2 Characterization of the bacteriocin gene cluster in *Lb. sakei* CWBI-B1365

For the partial sequencing of the Sakacin G gene cluster, an inverse polymerase chain reaction (IPCR) (Ochman *et al*., 1988) was used. First, the plasmid extracted form *Lb. sakei* CWBI-B1365 (pCWBI01) was *KpnI* digested. Secondly, a 6 kb fragment, containing the sakacin G structural genes determined by Southern Blot was self-ligated and used as a template for DNA walking using primer sakimm5fw, sakstruc4rev, saktrans2fw, saktrans1rev, sakimm6rev, saltrans3fw, salG2fw, sakG2rev, sagCrev, sakgCfw and sakimmfw. In an attempt to identify the immunity genes of sakacin A, P and Q, PCR amplification were performed using primers IMMArev-IMMAfw, OrfYr-OrfYf, SppiAf-SppiAr and IMMQrev-IMMQfw respectively. Identification and sequencing of the genes involved in Sakacin P synthesis was performed using the PCR primers described by Moretro *et al.* (2005).

3.6.3 Expression analysis

Total RNAs were extracted from 10^9 cells and subjected to reverse transcription using RevertAid H Minus First Strand cDNA synthesis kit (Fermentas). cDNAs were used as a template to amplify *skgA1-skgA2*, *skgC*, *skgI*, *skgD* and *orfY* genes using primers pairs sakG2fw/sakG2rev, sagCrev/sakgCfw, sakimmfw/sakimm6rev, saktrans3fw/saktrans1rev and OrfYf/OrfYr, respectively. The 16SrRNA gene, used as positive control, was amplified using primers pairs16sp0/R3seq18. To assess the absence of DNA contamination in RNA samples, 200 ng of extracted RNAs were used as a template to amplify the 16SrRNA gene by PCR.

3.6.4 Study of the effect of various factors on cell growth and bacteriocin production

3.6.4.1 Cultures conditions

Flask cultures, referred below as "static culture", were performed in 100 ml of medium adjusted to an initial pH as stipulated in the text. They were inoculated at an $OD_{600\ nm}$ of 0.01 and incubated without agitation. Culture in bioreactors were performed in a 20 l LSL Biolafite fermentor (Monze-sur-le-Mignon, France) with a working volume of 16 l and equipped with an automatic pH regulation system. KOH 1.5 N was added to maintain a constant pH value. Cultures were conducted at a stirring speed of 80 rpm without medium aeration. Pre-cultures were carried out successively at 25°C for 16 hours in 100 ml and 800 ml of the final culture medium.

3.6.4.2 Optimum pH and temperature for growth and bacteriocin production

To determine the optimal temperature and pH for growth and bacteriocin production, static cultures were performed in MRS medium adjusted to an initial pH of 6.5 and incubated at 20, 25, 30 and 37°C. Cell density and bacteriocin titre were determined at various time periods for 48 h. Similarly, the effect of the pH on the bacteriocin production was investigated in non regulated condition at 25 °C in MRS standing culture adjusted at an initial pH of 5, 5.5, 6 and 6.5. Cultures under pH controlled conditions were performed in bioreactor at a regulated pH value of 5.0, 5.5 and 6.0. All cultures were performed in duplicate.

3.6.4.3 Effects of sugars and nitrogenous sources on bacteriocin activity

Cell density and bacteriocin titre were determined during static cultures for 48 h in either MRS, MRSS, MRST, MRSM and MRSSM medium inoculated with *Lb. sakei* CWBI-B1365. Culture with feeding of either glucose, sucrose, glucose and meat extract, sucrose and meat extract were performed in bioreactor at 25°C with pH regulated at 5.5. Feeding with concentrated solution was performed between 6 and 10 h of culture at a constant flow rate of 10 g l^{-1} h^{-1} for both glucose and sucrose and 5 g l^{-1} h^{-1} for meat extract. All cultures were performed in duplicate.

3.7 Control of *Listeria monocytogenes* in raw meat

3.7.1 Identification of sakacin bacteriocin gene in *Lb. curvatus* CWBI-B28 and *Lb. sakei* LMG17302

PCR amplification of the structural gene of sakacin A, Q, P and G were made using primers pairs described in table 3. PCR products were analysed by agarose gel electrophoresis. If present, they were purified and commercially sequenced.

3.7.2 Meat applications

Fresh beef and chicken meat was bought in a supermarket in Belgium (Liege). The meats were cut into 100 g portions. The LAB strains were grown in 100 ml MRS broth at 30°C for 16 hours. Overnight cultures of *L. monocytogenes* CWBI-B715 were grown in M17 broth at 30°C.

Beef and chicken meat was surface inoculated with 10^2 cfu g^{-1} of *L. monocytogenes* after appropriate ten-fold dilutions of the culture in saline peptone water. The meats were surface inoculated with 10^6 cfu g^{-1} of *Lb. sakei* CWBI-B1365, *Lb. curvatus* CWBI-B28, *Lb. sakei* LMG17302, or a combination of *Lb. sakei* CWBI-B1365 and *Lb curvatus* CWBI-B28 at a concentration of 5 x 10^5 cfu g^{-1} each. LAB strains were inoculated after appropriate ten-fold dilutions of the culture in saline-peptone water. Meats inoculated only with *L. monocytogenes* were used as controls.

Samples were stored at 5°C in sealed bags. Samples of 10 g were aseptically taken every 7 days. 10 ml of peptone water were added and samples were homogenised in a stomacher for 180 s. *L. monocytogenes* and LAB counts were determined after decimal ten-fold dilutions of the suspension in saline peptone water, by plating the appropriated dilutions onto PALCAM (Fluka) and MRS agar plates for 48 h at 37 and 30°C, respectively. Each trial was done in three repetitions. Results were analysed by Student-t test analysis (p = 0.95).

Chapitre 4 Results

4.1 Selection of bacteriocin producing lactic acid bacteria for applications in meat and genetic characterization of the bacteriocin

The aim of this study was to select bacteriocin-producing LAB with antilisterial and/or anti-*Salmonella* activity, to identify the mechanisms involved, to characterize their bacteriocins at the genetic level and to evaluate their potential for meat application. The main tasks are:

- The isolation of lactic acid bacteria from different food products. To increase the probability of isolating original strains, we chosen to study food products from different origin and different environments that has not been widely studied before.
- The selection of the lactic acid bacteria strains that produce antimicrobial compounds active towards *Listeria monocytogenes* or *Salmonella* Typhimurium.
- The identification of the antimicrobial compounds that are responsible for the inhibitory activity.
- The selection of the bacteriocin-producing LAB strains and their polyphasic identification.
- The genetic identification of the bacteriocin produced.
- The study of the growth and bacteriocin production of the bacteriocin-producing strains.
- The study of the application of the bacteriocin-producing strains in raw poultry meat.

4.1.1 Collection of samples and isolation of lactic acid bacteria

The microbial count on MRS plates of salted fish and shells samples studied in Senegal varied between 10^2 and 10^5 cfu g^{-1} and between 10^4 and 10^7 cfu g^{-1} respectively. For the Tunisian food samples, counts on MRS plates were determined to be $1x10^9$ cfu g^{-1} for the two minced beef samples and $3x10^8$ cfu g^{-1} for the poultry intestine sample. The Belgian chicken meat samples were contaminated at levels of $6x10^6$ cfu g^{-1}, while the contamination level were higher at $7x10^8$ cfu g^{-1} for chicken meat stored for 3 weeks at 4°C under vacuum, and ranging between $2x10^7$ and $5x10^8$ cfu g^{-1} for chicken faeces.

Predominant colonies were isolated from the plates and purified on MRS-$CaCO_3$ agar plates. After preliminary characterization, 101 catalase-negative, Gram-positive strains which showed good acidification on MRS-$CaCO_3$ agar plates after 48 h of incubation at 30°C were selected for further studies. Based on these properties, these strains were identified as presumptive LAB. To reduce the selection of clonally related strains, the number of colonies selected from each samples was limited to a maximum of five.

4.1.2 Antimicrobial activity assay

The 101 selected presumptive LAB strains were screened for antagonistic activity against *L. monocotogenes* CWBI-B715, *L. innocua* and *S.* Typhimurium. Of these, 92 inhibited the growth of at least one of these pathogens tested.

After ruling out that the inhibitory activity was a result of either organic acid or hydrogen peroxide action, four strains remained which showed antimicrobial activity against *L. monocytogenes* CWBI-B715 and *L. innocua*. These strains were the strains CWBI-B1436, CWBI-B1437, CWBI-B1365 and CWBI-B1369. No inhibitory activities against *S.* Typhimurium could be detected after eliminating hydrogen peroxide or organic acid activity. The MIC of lactic acid against this pathogen was determined to be between 5 and 10 g l^{-1} at pH 4.0 and between 10 and 15 g l^{-1} at pH 4.5. For hydrogen peroxide, the MIC was between 1 and 2 mmol l^{-1} for *S.* Typhimurium. The lactic acid concentrations in the supernatants of the strains showing inhibitory activity against this pathogen varied between 7.1 and 15.6 g l^{-1} while the pH measured varied between 3.8 and 4.7. The hydrogen peroxide concentration in the supernatant of cultures was not determined.

The proteinaceaous nature of the inhibitory activity was assessed by the action of different proteases on the neutralised cell-free supernatants. The activity against *L. monocytogenes* was lost after treatment with different proteases, thus confirming the proteinaceaous nature of the inhibitory activity. The bacteriocins produced by strain CWBI-B1365 were sensitive to all the proteases tested. Bacteriocins produced by strain CWBI-B1437 were sensitive only to pronase. Bacteriocins produced by strains CWBI-B1369 and CWBI-B1436 were sensitive to all the proteases but the inhibitory activity of the bacteriocins only decreased, but was not completely abolished, by pepsin.

The strain CWBI-B1437 originated from salted fish from Senegal, strains CWBI-B1369 and CWBI-B1436 were both isolated from one sample of chicken meat from Belgium, while the strain CWBI-B1365 was isolated from another Belgian chicken meat sample.

4.1.3 Identification of the strains

Selected strains were identified using both phenotypic and genotypic methods. The strains CWBI-B1369 and CWBI-B1436 were characterised as *Carnobacterium* spp., while strain CWBI-B1437 could be characterised as *Lc. lactis* subsp. *lactis* and strain CWBI-B1365 as a *Lactobacillus* using phenotypic methods. The sequencing of the 16SrRNA genes confirmed these results. The strains CWBI-B1437 was characterised as *Lc. lactis* subsp. *lactis,* and the two strains CWBI-B1369 and CWBI-B1436 as *C. maltaromaticum* strains, while the strain CWBI-B1365 was identified as *Lb. sakei*. The identification of the strain CWBI-B1365 was also confirmed by DNA-DNA hybridisation with the type strains belonging to the closely related *Lb. sakei* and *Lb. curvatus* species group. The DNA-DNA hybridisation with *Lb. sakei* DSM 20017 showed 89% reassociation, while a lower reassociation value of 21% was obtained with the *Lb. curvatus* DSM 20019^{T} type strain, confirming that the CWBI-B1365 strain is a *Lb. sakei*.

4.1.4 Effect of pH and temperature on the bacteriocin activity

The activity of all bacteriocins was pH-dependent, as activity decreased when pH increased (table 4). Heat-treatment of supernatants at 72°C for 15 min did not affect bacteriocin activity, while treatment of 121°C during 20 min completely destroyed the inhibitory activity of the bacteriocin from strain *Lb. sakei* CWBI-B1365, at pH values > 5 for bacteriocin from strains *C. maltaromaticum* CWBI-B1436, pH > 6 for *C. maltaromaticum* CWBI-B1369 and pH > 4 for the bacteriocin from *Lc. lactis* CWBI-B1437.

Table 4: Effect of pH and thermal treatment on the activity of the bacteriocins produced by the selected strains

Strains	Thermal treatment	Remaining activity after heat stress at different pH					
		4	5	6	7	8	10
CWBI-B1436	None	++	++	++	++	++	+
	72°C – 15’	++	++	++	++	+	+
	121°C – 20’	++	+	-	-	-	-
CWBI-B1369	None	++	++	++	++	++	+
	72°C – 15’	++	++	++	++	++	+
	121°C – 20’	++	+	+	-	-	-
CWBI-B1365	None	++	++	++	++	++	+
	72°C – 15’	++	++	++	++	++	+
	121°C – 20’	-	-	-	-	-	-
CWBI-B1437	None	++	++	++	++	++	++
	72°C – 15’	++	++	++	++	++	++
	121°C – 20’	++	-	-	-	-	-

++: No decrease of activity after treatment comparing to the reference
+: Intermediate inhibitory activity after treatment comparing to the reference
-: No inhibitory activity after treatment.

4.1.5 Genetic characterization of the bacteriocin

To determine if the selected strains carry the structural genes of known bacteriocins produced by other strains of the same species, PCR analysis using specific primers for individual bacteriocin genes was done. PCR products corresponding to the expected sizes of known bacteriocin genes are shown in figure 7.

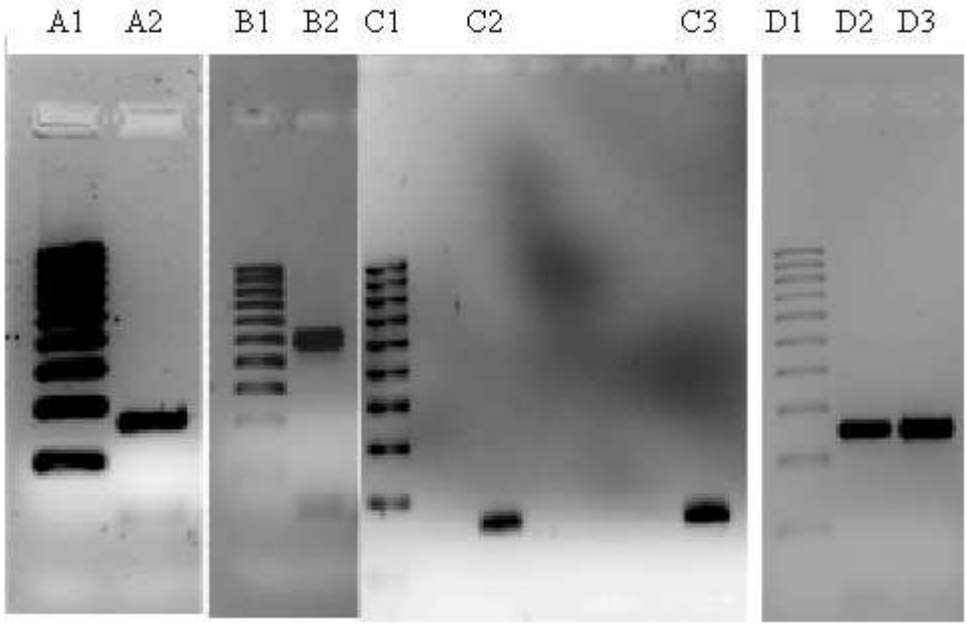

Figure 7 : Agarose-gel electrophoresis of PCR product amplified with specific primers for know bacteriocins. Lane A1, B1, C1 and D1: 100 bp molecular weight marker (Biorad), Lane A2: nisin from CWBI-B1437, Lane B2: Sakacin G from CWBI-B1365, lane C2: Carnobacteriocin Bm1 from CWBI-B1369, lane C3: Carnobacteriocin Bm1 from CWBI-B1436, Lane D2: Piscicolin 126 from CWBI-B1369 and Lane D3: Piscicolin 126 from CWBI-B1436.

In *Lb. sakei* CWBI-B1365, the genes *SkgA1* and *SkgA2* encoding sakacin G are present, whereas the bacteriocin structural genes for sakacin P, Q and A could not be amplified. Sequencing of the PCR products shows that there was 100 % sequence similarity to the *SkgA1* and *SkgA2* sequences as described by Simon *et al* (2002). The nucleotide sequence of the amplified PCR product from *Lc. lactis* subsp. *lactis* CWBI-B1437 was identical to that of the nisin Z gene. Both *C. maltaromaticum* strains were shown to harbour the genes for carnobacteriocin BM1 and piscicolin 126, but not the genes for carnobacteriocin A or carnobacteriocin B2. Again the nucleotide sequence of the PCR products was 100% identical to those reported for carnobacteriocin BM1 and piscicolin 126 by Quadri (1994) and Jack (1996), respectively.

After plasmids extraction, a single band was observed on agarose gel after electrophoresis for strains *Lb. sakei* CWBI-B1365, *C. maltaromaticum* CWBI-B1369 and CWBI-B1436 indicating that a single plasmid is probably present in these isolates. No plasmid DNA could be isolated from *Lc. lactis* subsp. *lactis* CWBI-B1437 indicating that the structural gene for nisin is probably located on the chromosome. Southern blot analysis

showed that the sakacin G structural genes are located on a plasmid in *Lb. sakei* CWBI-B1365. Restriction of the plasmid, gel electrophoresis and analysis of the molecular weight of the fragment shows that it was an approximately 25-kb plasmid. The gene for piscisolin 126 was located on a plasmid in *C. maltaromaticum* CWBI-B1436 and CWBI-B1369, while the genes for carnobacteriocin BM1 were located on chromosomal DNA in *C. maltaromaticum* CWBI-B1436 and CWBI-B1369. Restriction of the plasmid and analysis of the molecular weight of the fragments after gel electrophoresis showed that this was an approximately 35-kb plasmid.

4.1.6 Growth of the selected strains and bacteriocin production

Figure 8 shows the optical density of the culture and inhibitory activity against *L. monocytogenes* during growth in MRS flasks at 30°C. Results show that the inhibitory activities of the supernatants obtained from the two *C. maltaromaticum* cultures were higher than for the two others strains (CWBI-B1365 and CWBI-B1437). Table 5 shows the cell concentration at the end of the exponential phase of growth, the maximal growth rate and generation time of the strains in these culture conditions. The generation time was noticeably longer for the *C. maltaromaticum* strains. For all the strains, the bacteriocins appear to be produced mainly at the end of the exponential phase and during the stationary phase of growth.

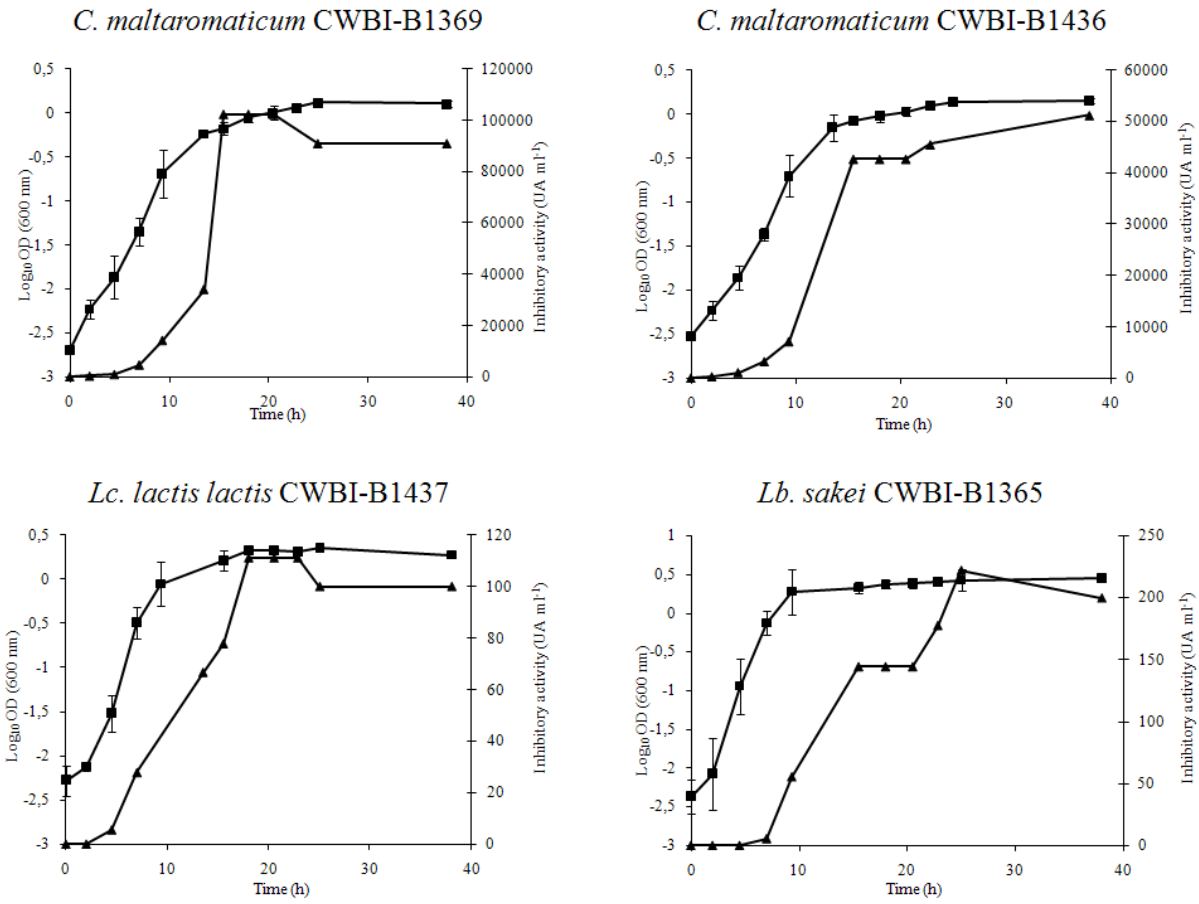

Figure 8 : Growth and bacteriocin production of the selected strains in MRS broth at 30°C during 38 hours. (■) Optical density evolution, (▲) Inhibitory activity evolution.

Table 5: Cell concentration at the end of the exponential growth phase, maximal growth rate and generation time during growth in MRS broth at 30°C without agitation.

Strain	Cell concentration (cfu ml^{-1})	Maximal growth rate (h^{-1})	Generation time (min)
C. maltaromaticum CWBI-B1369	$1.4x10^9$	0.54	77
C. maltaromaticum CWBI-B1436	$2.1x10^9$	0.55	75
Lc lactis lactis CWBI-B1437	$3.2x10^9$	0.84	49
Lb. sakei CWBI-B1365	$6.8x10^8$	0.73	57

4.1.7 Expression studies of the bacteriocin structural gene

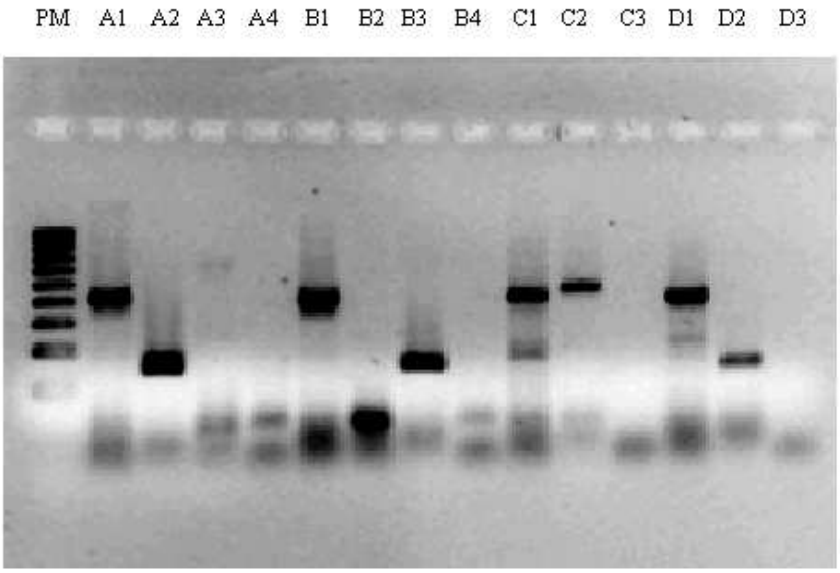

Figure 9 : Agarose gel electrophoresis of RT-PCR product amplified from isolated RNA with specific primer for structural bacteriocin gene and house keeping gene. PM: 100 bp molecular weight marker (Biorad), A: *Cb. maltaromaticum* CWBI-B1436, B: *Cb. maltaromaticum* CWBI-B1369, C: *Lb. sakei* CWBI-B1365, D: *Lc. lactis lactis* CWBI-B1437. A1, B1, C1 and D1: House keeping gene, A2 and B3: Carnobacteriocin Bm1, A3 and B2: piscicolin 126, C2: sakacin G, D2: nisin, A4, B4, C3 and D3: negative reference (RNase treated RNA).

To determine that the bacteriocins identified are indeed actively produced by the selected strains, we studied the expression of these bacteriocin genes at the RNA level. Total RNA was extracted after 16 h of growth, which corresponded to the end of the exponential growth phase. Figure 9 shows the amplified products after RT-PCR. It shows that all the bacteriocins were expressed at the end of the exponential growth phase, except for the structural gene for piscicolin 126. Indeed, no RT-PCR product could be obtained for this structural gene.

4.1.8 Meat application of the bacteriocin producing LAB

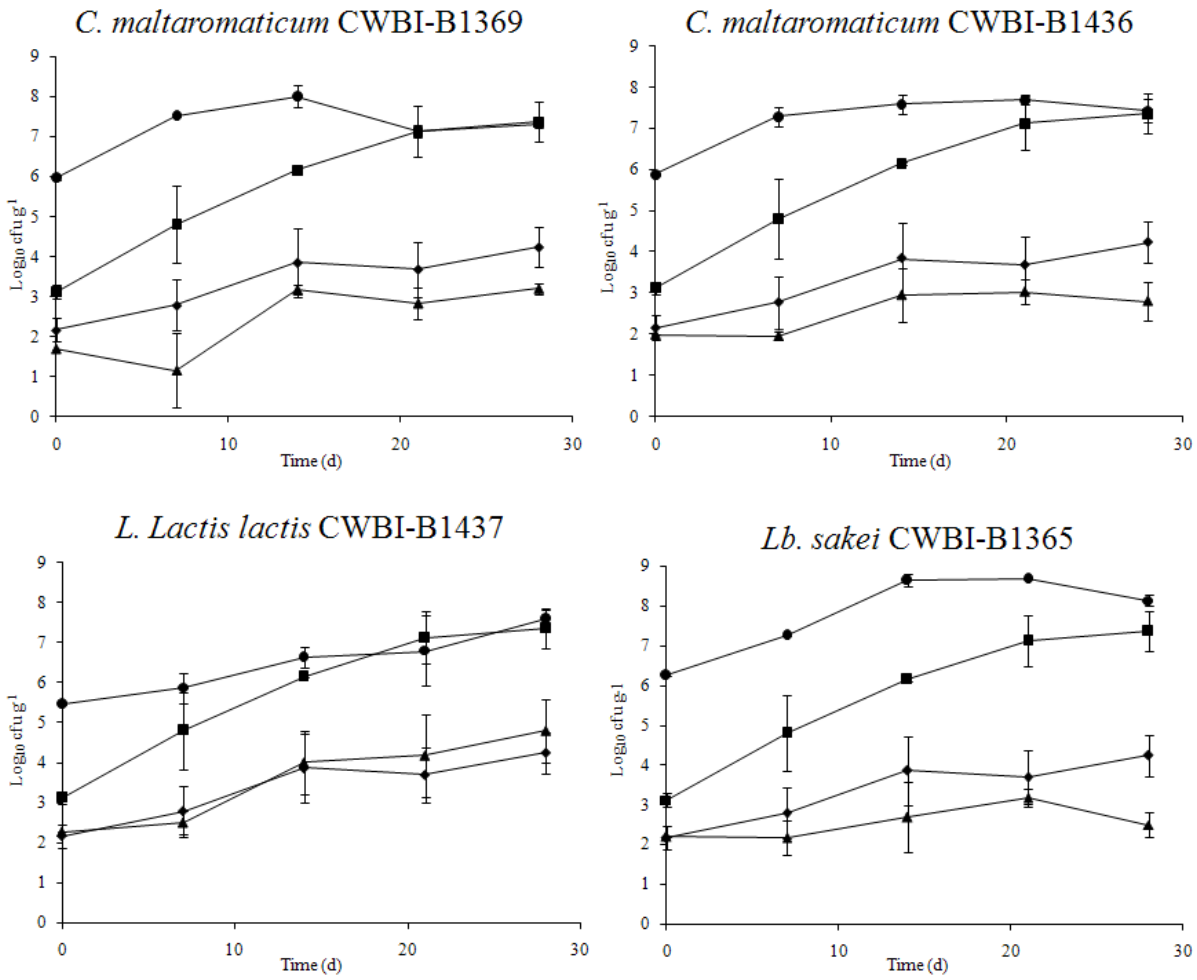

Figure 10: Growth of *Listeria monocytogenes* (▲, ♦) and lactic acid bacteria (●, ■) in chicken meat stored during 28 days at 4°C. (♦, ■) Reference (no inhibitory strains added); (▲, ●) with added inhibitory bacteria. Each value represents the mean of triplicates.

L. monocytogenes cell counts and LAB concentration were assessed in chicken meat stored at 4°C under vacuum during 28 days (figure 10). The inhibitory strains grew on chicken meat at 4°C under vacuum, and increased by 1 log cfu g^{-1} in 7 days, with the exception of *Lc. lactis* subsp. *lactis* CWBI-B1437. This strain did not show as good growth as the other strains and increased only by 0.5 $\log_{10}$ cfu g^{-1} in 7 days. In the reference, growth of other LAB occurred from the beginning to approximatively 10^7 cfu g^{-1} on day 21.

L. monocytogenes grew on the meat, increasing by 1.6 log from day 0 to day 14. Addition of the strains CWBI-B1369, CWBI-B1436 or CWBI-B1365 inhibited slightly but

not significantly (Student-t test (p=0.95)) the growth of *L. monocytogenes* during the first 7 days. After 14 days, the concentration increased slightly, but was still 1 log unit lower than in the control without bacteriocin-producing bacteria. At the end of the storage period, the concentration of *L. monocytogenes* was still 1 to 2 log lower than the reference. The addition of the strain CWBI-B1437 did not affect the growth of *L. monocytogenes* as the cell numbers were similar to those of the control samples throughout storage.

4.1.9 Discussion

The activity spectrum of many bacteriocins is limited to Gram-positive strains, including Gram-positive pathogens such as *L. monocytogenes*. Because of the impermeability of the Gram-negative outer membrane, bacteriocins are generally not active against Gram-negative pathogens such as *Salmonella* species. Nevertheless, as we were able to show in this study, organic acids and possibly also hydrogen peroxide in combination with organic acids, sufficed to inhibit the Gram-negative pathogens *in vitro*. Thus the MIC, pH and organic acid concentration values determined here showed that the amount of organic acids present in the culture supernatants of the investigated strains were sufficient to inhibit *S.* Typhimurium. The MIC value of hydrogen peroxide was between 1 and 2 mol l^{-1}. Previous studies have shown that the hydrogen peroxide concentration in the supernatants of hydrogen peroxide-producing *Lactobacillus* strains grown in MRS broth ranged from 1.6 to 8 mol l^{-1} (Annuk *et al*., 2003; Otero *et al.*, 2006). In our study, we could find no other mechanism associated with the inhibition of *S.* Typhimurium by culture supernatant of LAB strains. Inhibition of Gram-negative bacteria is generally known to be due to the production of organic acid and/or hydrogen peroxide, while inhibition by bacteriocin is unusual and has thus far only been reported for a few structurally unusual bacteriocins produced by LAB (Kwaadstniet *et al.*, 2005; Makras *et al*., 2006).

The selected bacteriocin-producing LAB were isolated from poultry or fish samples. *Lb. sakei* is commonly associated with meat, while *Carnobacteria* are commonly associated with fish, poultry and meat samples (Quadri, 1994; Duffes *et al.*, 1999; Nilsson *et al*., 2002; Nilsson *et al*., 2004; Tahiri *et al.*, 2004; Chaillou *et al*., 2005; Gursky *et al*., 2006). *Lc. lactis*

strains have previously been isolated from milk, fish and meat products (Olasupo *et al*., 1999; Palludan-Muller *et al*., 1999; Noonpakdee *et al*., 2003; Nga, 2005; Zamfir *et al.*, 2006).

Our results clearly showed that the activity of the bacteriocin is affected by exposure to alkaline conditions, as well as to heat treatment at 121°C for 20 min at pH values higher than 4.0 to 5.0. Most of the bacteriocins are known to be heat tolerant, especially at low pH (Kwaadsteniet *et al*., 2005). Nisin produced by *Lc. lactis* WNC20 was inactivated after 15 min at 121°C at pH 7.0, but remained stable at this temperature when in solution at pH 3.0 (Noonpakdee *et al*., 2003). Piscicolin 126 remained stable after treatment at 100 °C for 120 min at low pH, but was completely inactivated at pH higher than 6 (Jack, 1996).

In previous times when data on bacteriocins were still scarce, new bacteriocins were characterised by purification from the culture supernatants, chromatography and Edman degradation sequencing. These methods of course are time consuming and success is dependent on many factors. First, it is necessary to have a high production of the bacteriocin. Nevertheless, bacteriocin production is often an unstable trait, complex and strongly dependent of the growth conditions (Nes *et al*., 2004) as it may also be regulated by a quorum sensing system dependent on cell density. Furthermore, the purification yield is usually very low. For example, the yield of divergicin M35, a bacteriocin produced by *C. divergens,* upon purification was very low at 10%. Most of the bacteriocin losses occurred during ion exchange and reverse phase HPLC steps (Tahiri *et al*., 2004). The loss during purification may also result in the loss of one specific bacteriocin, if different bacteriocins are produced by the same strain. For example, purification of sakacin P from *Lb. sakei* Lb674 didn't allow co-purification of sakacin Q, a bacteriocin which was identified only by the use of genetic methods (Eijsink *et al.,* 1998; Mathiesen *et al*., 2005).

In recent years, many new bacteriocins have been described and these have been often well characterised at the genetic level. This, together with the previously described problems of low production and losses during purification, led us to adopt the strategy to identify unidentified bacteriocins in newly identified antimicrobial strains by using bacteriocin-specific PCR primers. Using this approach, we could identify the genes for nisin z, piscicolin 126 and carnobacteriocin BM1, as well as sakacin G in strains of *Lc. lactis* subsp. *lactis*, *C. maltaromaticum* and *Lb. sakei*, respectively. Southern blot analyses showed that the sakacin G gene was located on a 25-kb plasmid in *Lb. sakei* CWBI-B1365, while it was located on a 35-kb plasmid in *Lb. sakei* 2512 (Simon *et al.,* 2002). The piscicolin 126 gene was located on a 35-kb plasmid in *C. maltaromaticum* CWBI-B1369 and CWBI-B1436. The structural gene

for the carnobacteriocin BM1 was located on the chromosomal DNA in both strains. These results suggest that these two strains are multiple isolate of the same strains. Quadri *et al.* (1997) have previously shown that the strain *C. piscicola* LV17B produces a plasmid-encoded carnobacteriocin B2 and a chromosomally encoded carnobacteriocin BM1, and that the genes coding for the regulation of the production of carnobacteriocin BM1 are located on the plasmid. Gursky *et al*. (2006) reported the production of carnobacteriocin BM1 and piscicolin 126 by *C. maltaromaticum* UAL26. In *C. maltaromaticum* UAL26, the genes for the regulation, export, structure and immunity for both these bacteriocins were located on the chromosomal DNA. Some single nucleotide differences in the immunity, histidine kinase, ABC-transporter and accessory gene in the strain *C. maltaromaticum* UAL26 were present comparing to the strains *C. maltaromaticum* JG126 (Gursky *et al.,* 2006; Rohde *et al*., 2006). The nisin Z gene was probably located on the chromosome of *Lc. lactis* subsp. *lactis* strain CWBI-B1437. It has been shown several times already that nisin is encoded by a conjugative transposon that is capable of integrating into the host chromosome (Kim *et al*., 1997), and thus this possibility cannot be ruled out also in our case.

Bacteriocin production is influenced by several environmental factors such as pH, temperature and composition of the culture broth (Nilsson *et al.*, 2002). Despite a longer generation time, the bacteriocin production by *C. maltaromaticum* CWBI-B1369 and CWBI-B1436 in MRS broth at 30°C was considered to be very high. These results do not agree with those obtained by Gursky *et al*. (2006), who showed that production is completely ceased at temperatures above 19°C. However, Jack (1996) showed that piscicolin 126 was still produced at 25°C by *C. maltaromaticum* JG126. MRS culture broth is not the best growth medium for *C. maltaromaticum* due to the presence of acetate, but acetate can induce bacteriocin production by carnobacteria (Nilsson *et al.,* 2002; Tahiri *et al.,* 2004). The maximal inhibitory activity reached in the supernatants of *Lb. sakei* CWBI-B1365 was only 320 UA ml^{-1}. A lot of studies have indicated that the highest bacteriocin production by *Lb. sakei* was obtained at pH and temperatures values lower than the optimal conditions for growth (Aasen *et al*., 2000; Mataragas *et al.,* 2003; Héquet *et al.,* 2007). The maximal inhibitory activity reached in the supernatant of a *Lc. lactis lactis* CWBI-B1437 culture in MRS broth was only 112.5 UA ml^{-1}, while production of nisin Z by *Lc. lactis* WNC20 in MRS broth at 30°C was at 18 000 UA ml^{-1} (Noonpakdee *et al*., 2003). All the bacteriocins in this study were produced at the end of the exponential growth phase and in the early stationary phase, as it has already been described also for other bacteriocins. Our bacteriocin

expression studies showed that not only the genes for these bacteriocins were present, but also that these were being actively expressed under the growth conditions used, except for the piscicolin 126 gene. In the strain *C. maltaromaticum* UAL26, the structural gene for the two bacteriocins seems to be co-transcribed (Gursky *et al*., 2006).

Our poultry meat applications studies showed that the *C. maltaromaticum* CWBI-B1369 and CWBI-B1346 and *Lb. sakei* CWBIB-1365 strains could slightly inhibit the growth of *L. monocytogenes* in poultry meat, while *Lc. lactis subsp. lactis* CWBI-B1437 did not show an effect. In the case of nisin, it has been shown that in meat the activity is very low, probably as a result of inactivation of the bacteriocin by glutathione S-transferase in fresh meat (Rose *et al*., 2002). The inhibitory effect was more pronounced for the *Carnobacterium* strains than for the *Lb. sakei* CWBI-B1365 strain. It has been reported that the production of two bacteriocins may result in a synergistic effect and thus these can exhibit a stronger inhibitory effect against sensitive strains when occurring together (Gursky *et al*., 2006). Bacteriocins from carnobacteria or bacteriocin producing *Carnobacterium* strains themselves have already been applied for the biopreservation of fish such as smoked-salmon and meat (Azuma *et al*., 2007; Gursky *et al.*, 2006; Nilsson *et al.*, 1999; Schöbitz *et al*., 1999;). The results of this study have led to the successful detection and application of bacteriocin-producing strains.

The results obtained urged us to focus our next investigations on *Lb. sakei* CWBI-B1365. Indeed, this strain has the sakacin G structural gene and expresses it. These genes are located on a smaller plasmid than in strain *Lb. sakei* 2512 (Simon *et al*., 2002). The properties and the factors affecting the application and production of sakacin G have not been widely studied until now. In addition, *Lb. sakei* is a species that is recognized as non-pathogenic and commonly found and applied in meat products. Its metabolism is well adapted to the fresh meat environment (Chaillou *et al*., 2005).

4.2 Study of the antilisterial bacteriocin from *Lactobacillus sakei* CWBI-B1365 and improvement of its production

We showed previously that *Lb. sakei* CWBI-B1365 was able to inhibit *L. monocytogenes* growth in raw poultry meat. The well known role of *Lb. sakei* in meat preservation and fermentation was attributed to the production of lactic acid and/or antibacterial compounds active on food-borne pathogenic or spoilage bacteria. Among those compounds, several bacteriocins known as sakacin P (Holck *et al.*, 1994; Tichaczek *et al*., 1994), sakacin A (Holck *et al*., 1992), sakacin Q (Mathiesen *et al.*, 2005) and sakacin G (Simon *et al*., 2002) have been described to date. Sakacin P, A and G belong to the subclass IIa bacteriocins, while sakacin Q belongs to the subclass IIc. Different studies have already demonstrated the effectiveness of different bacteriocins producing *Lb. sakei* strains to inhibit growth of *L. monocytogenes* or food spoilage bacteria in meat, poultry or fish products (Castellano *et al.*, 2006; Ghalfi *et al*., 2006; Héquet *et al*., 2007; Katikou *et al.*, 2005; Katla *et al.*, 2001; Katla *et al*., 2002). However, a better understanding of the factors affecting the bacteriocin production is still required in order to increase the application and the commercial use of the *Lb. sakei* bacteriocins as food preservative. As explained in chapter 1, the production of class IIa bacteriocin is most of the times regulated by a three-component system consisting of an induction factor, a histidine kinase and a response regulator. Two additional gene clusters are required for bacteriocin production. The first one consisting of the bacteriocin structural gene and a cognate immunity gene, and the second encoding a dedicated ABC transporter and an accessory transport protein.

Bacteriocin production is growth associated but the level of their production is not necessary directly correlated with cell growth (De Vuyst *et al.*, 1996; Kim *et al.*, 1997; Aasen *et al.*, 2000). Culture parameters such as pH, temperature, nature and availability of carbon and nitrogenous sources were found to modulate the level of bacteriocin production in *Lb. sakei* and *Lb. curvatus*, its closer related phylogenic species (Leroy *et al.*, 1999; Aasen *et al.*, 2000; Diep *et al.*, 2000; Moretro *et al.*, 2000; Leroy *et al.*, 2001; Messens *et al.*, 2002; Messens *et al.*, 2003; Verluyten *et al.*, 2004; Héquet *et al.*, 2007). However, it is admitted that those regulation factors are, in most of the case, strain and bacteriocin dependent (Moretro *et al.*, 2000).

Therefore, in the present chapter, the antilisterial compound produced by *Lb. sakei* CWBI-B1365 was partially purified and characterized. Its mode of action on sensitive cells was determined. A part of the genes involved in the bacteriocin production in *Lb. sakei* CWBI-B1365 were sequenced. Finally, factors affecting the growth of *Lb. sakei* CWBI-B1365 and the bacteriocin biosynthesis were investigated during batch and fed-batch fermentations.

4.2.1 Characterization of the bacteriocin produced by *Lactobacillus sakei* CWBI-B1365

The antimicrobial properties of cell-free culture supernatants of *Lb. sakei* CWBI-B1365 were investigated toward an array of 23 bacteria by the well diffusion method. The spectrum of activity seems to be focus on the *Listeria* genus. However, a growth inhibition was also observed for *Enterococcus faecalis* and *Carnobacterium maltaromaticum* (Table 6).

Table 6: Inhibitory spectrum of the bacteriocin produced by *Lb. sakei* CWBI-B1365

Indicator strain	Inhibition zone diam (mm)
Staphylococcus aureus ATCC43300	-
Staphylococcus epidermis ATCC1228	-
Listeria monocytogenese CWBI-B715	12
Listeria monocytogenes LMG21263	12
Listeria monocytogenes LMG23905	12
Listeria innocua ATCC33090	12
Enterococcus faecalis JH22	13
Enterococcus faecium LMG11423	-
Bacillus cereus [b]	-
Bacillus megaterium [b]	-
Clostridium perfingens [b]	-
Micrococcus luteus [b]	-
Carnobacterium maltaromaticum CWBI-B1369	15
Lactobacillus curvatus CWBI-B28	-
Weissella paramesenteroides BFE7601	-
Weissella paramesenteroides BFE7608	-
Campylobacter jejuni LGM6446	-
Campylobacter coli LGM6640	-
Salmonella typhi [b]	-
Escherichia coli K12	-
Pseudomonas putida BTP1	-
Pseudomonas putida KT2240	-
Pseudomonas aeruginosa[b]	-

[a] Wells (6 mm in diameter) were filled with 100 µl of an active 400 AU ml^{-1} supernatant from *Lb. sakei* CWBI-B1365 culture.

[b] Laboratory stock

- no inhibitory activity

The purification of the bacteriocin was conducted by a three-step procedure. After adsorption and subsequent desorption on Amberlite XAD-4 resin, the concentrated antibacterial agent was purified by ion exchange and reverse phase chromatography. The fraction corresponding to the major peak collected during the last step of purification and active against *L. monocytogenes,* was used for further characterisation (figure 11A). The

molecular mass of the purified molecule was approximately 3.5 kDa according to Tris-Tricine-SDS PAGE (data not shown). MALDI-TOF mass spectrometry of the semi-purified molecule showed a signal at 3835.14 Da whereas after dithiotreitol treatment, a signal at 3838.92 Da was obtained, indicating the presence of two possible disulfide bonds in the molecule (figure 11B).

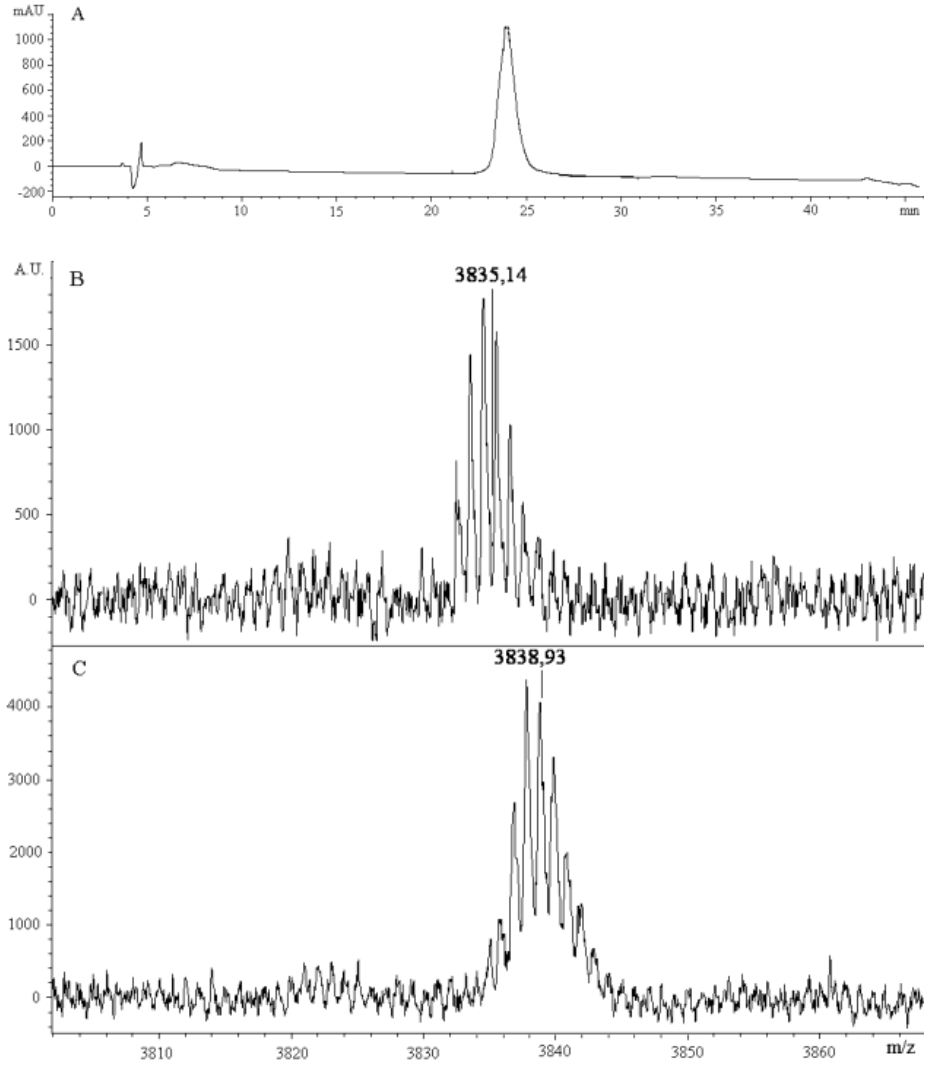

Figure 11: Reverse-phase high performance liquid chromatography (A) and MALDI-TOF analysis of the 23-min major peak before (B) and after reduction treatment with dithiotreitol (C).

The biological mode of action of the bacteriocin was investigated by measuring its ability to dissipate the transmembrane electrical potential (ΔΨ) for three different *L. monocytogenes* isolates. This was performed by measuring the fluorescence of the ΔΨ-sensitive dye $DiSC_3(5)$ upon addition of the bacteriocin to a *L. monocytogenes* cell suspension at different pH. An important fluorescence signal, comparable to that obtained with the ionophore valinomycin, could be observed upon addition of the bacteriocin to the cell suspension for the three strains tested (figure 12I). This traduces the disruption of the ΔΨ following the formation of transmembrane pore by the peptide. No significant differences could be observed for the three pH values tested indicating that the activity is not pH dependent in those conditions (figure 12II).

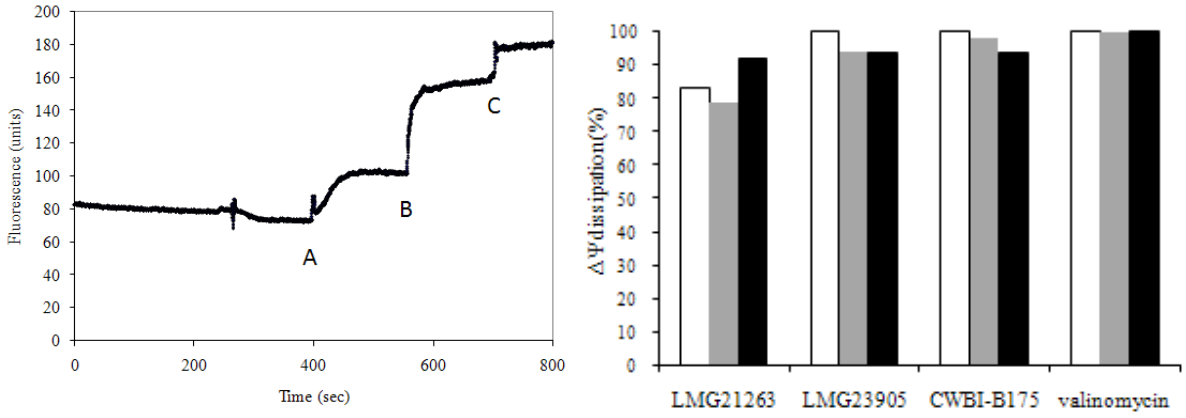

Figure 12: (I) Evolution of the fluorescence after addition of the H^+/K^+ exchanger nigericin (A), addition of the bacteriocin containing culture supernatants of *Lb. sakei* CWBI-B1365 (B) and addition of the potassium ionophore valinomycin (C) to a 0.1 OD_{600nm} suspension of *L. monocytogenes* LMG21263 at pH 5,5. (II) Dissipation of the transmembrane electrical potential (ΔΨ) of *L. monocytogenes* LMG21263, LMG23905 and CWBI-B175 upon addition of 4 AU of sakacin G at pH 5 (white), 5.5 (grey) and 6 (black). Values were normalised to that obtained with 1 µM of valinomycin normalised to the fluorescence signal obtained with 1 µM valinomycin.

All these informations (*i.e.* molecular mass, two disulfides bonds, a narrow inhibition spectrum, a mode of action similar to that of the class IIa bacteriocin and the presence of sakacin G structural gene in this strain) suggest that the bacteriocin produced by *Lb. sakei* CWBI-B1365 could correspond to sakacin G.

4.2.2 Characterization of the Sakacin gene cluster in *Lb. sakei* CWBI-B1365

We located previously by Sounthern hybridation the structural genes coding for sakacin G on the 25 kb plasmid of *Lb. sakei* CWBI-B1365 (pCWBI01). To further characterise this genetic locus, the sequence of a 3 kb fragment was determined by an inverse PCR technique (Ochman *et al*. 1988). For a 2.1-kb fragment of this sequence, Blast analysis showed a 100% homology with partial *skg* locus published by Simon and collaborators. A 531 bp ORF coding for a putative 176-residues protein was also identified that could correspond to skgC (figure 13). Indeed, this latter present good similarity with papC (54 %), an accessory factor involved in pediocin PA-1 excretion (Miller *et al*., 2005). This sequencing also led to the determination of the 5' termini of *skgI* and a part of the 3' termini of *skgD*, respectively. The entire sequence was deposited in the Gene Bank database under the accession number EU570253.

Figure 13: Schematic representation of the sequenced 2.9-kb fragment from plasmid pCWBI01.

Despite the apparent absence of the structural gene of the other known sakacin in *Lb. sakei* CWBI-B1365 on the chromosome and the pCWBI01 plasmid, PCR amplifications were performed in an attempt to point out the presence of their immunity gene. A 273 bp fragment was shown to present 98% homology with *orfY*, an immunity gene of the sakacin P gene cluster in *Lb. sakei* LTH673 (Brurberg *et al*., 1997). Based on this observation, the sakacin P gene cluster was further caracterised in *Lb. sakei* CWBI-B1365 by PCR amplification using the primer described by Moretro *et al.* (2005). Two fragments of 553 bp and 692 bp presenting, respectively, 97 % and 96% homology with 3'-end of *sppK* and *sppR* gene of *Lb.*

sakei LTH673 were obtained. A fragment of 496 bp presenting homology of 56 % with the 5'-end of *sppK* of *Lb. sakei* LTH673 was also detected. By contrast *sppT* and *sppE* homologue were not detected. The sequence corresponding to *orfY*, *sppK* and *sppR* were deposited in the GeneBank database under the accession number EU 676003 and EU 676002.

The expression analysis of the sakacin G (*skg*) gene cluster and *OrfY* was determined during growth in sucrose-meat extract fed-batch fermentation (see below) after 26 and 50 h corresponding to the maximal bacteriocin production and to the late stationary phase, respectively. RT-PCR were performed with the specific primers for *skgC*, *skgI*, *skgA2*, *skgA1*, *skgD* and *OrfY* amplification. All the *skg* genes tested were expressed at both times culture whereas *OrfY* was never expressed.

4.2.3 Regulation of sakacin G production by temperature and pH

The effect of various culture temperatures on both cell growth and bacteriocin production was monitored during 48 h of *Lb. sakei* CWBI-B1365 culture in MRS broth. The lower doubling time was found at 37°C whereas the maximal bacteriocin titre and specific production were obtained at 25 °C (table 7). This indicates that there is a discrepancy between the optimal temperature for cell growth and bacteriocin production.

The optimal pH value for bacteriocin production was investigated during both pH non-regulated and regulated cultures. After 14 h of growth, the production of lactic acid led to an acidification of the medium to pH 4.1 for the non-regulated culture whatever the initial pH value (data not shown). However, an initial pH value of 6 and 6.5 led to the highest bacteriocin titre and specific production. pH-controlled cultures were performed in a 20-litres fermentor with a maximal pH variation of 0.1 unit. As shown in table 7, a maximal inhibitory activity of 400 AU ml^{-1} was found at pH 5.5. This value is similar to that obtained for the non-regulated cultures performed at an initial pH of 6 or 6.5. However, the specific production is clearly lower in this case. For the culture regulated at pH 5 and 6, a lower antilisterial activity was obtained. For the different pH value tested, a slight modulation of doubling time could be observed during pH non-regulated culture whereas no differences were observed during cultures in bioreactor. For the pH controlled culture, a 3-fold increased cell concentration was obtained compared to non regulated culture. The formation of a biofilm was observed during growth in standing flask cultures.

Table 7: Effect of various factors on growth and bacteriocin production by *Lb. sakei* CWBI-B1365

Factor	Doubling time (min)	Antilisterial activity (AU ml^{-1})	Specific production (AU ml^{-1}x OD^{-1})
Growth temperature (°C) under non-controlled pH cultures[1]			
20	78	50	44
25	84	400	151
30	73	200	94
37	47	100	46
Growth pH during non-controlled pH cultures [2]			
5.0	63	200	109
5.5	87	200	116
6.0	80	400	182
6.5	84	400	151
Growth pH during controlled pH cultures[3]			
5.0	85	200	31
5.5	84	400	59
6.0	85	25	3
Carbon source[4]			
MRS	84	400	151
MRST	73	400	107
MRSS	66	800	174
MRSM	59	400	123
MRSSM	63	600	138

[1] Static cultures were performed at an initial pH of 6.5
[2] Static cultures were performed at 25°C
[3] Culture were performed in 20-l fermentor at 25°C in MRS medium
[4] Static cultures were performed at an initial pH of 6.5 and at 25°C

4.2.4 Influence of carbon and nitrogen sources on bacteriocin production

Various carbon and nitrogen sources were tested for their capacity to support bacteriocin production and cell growth during static culture of *Lb. sakei* CWBI-B1365 (table 7).

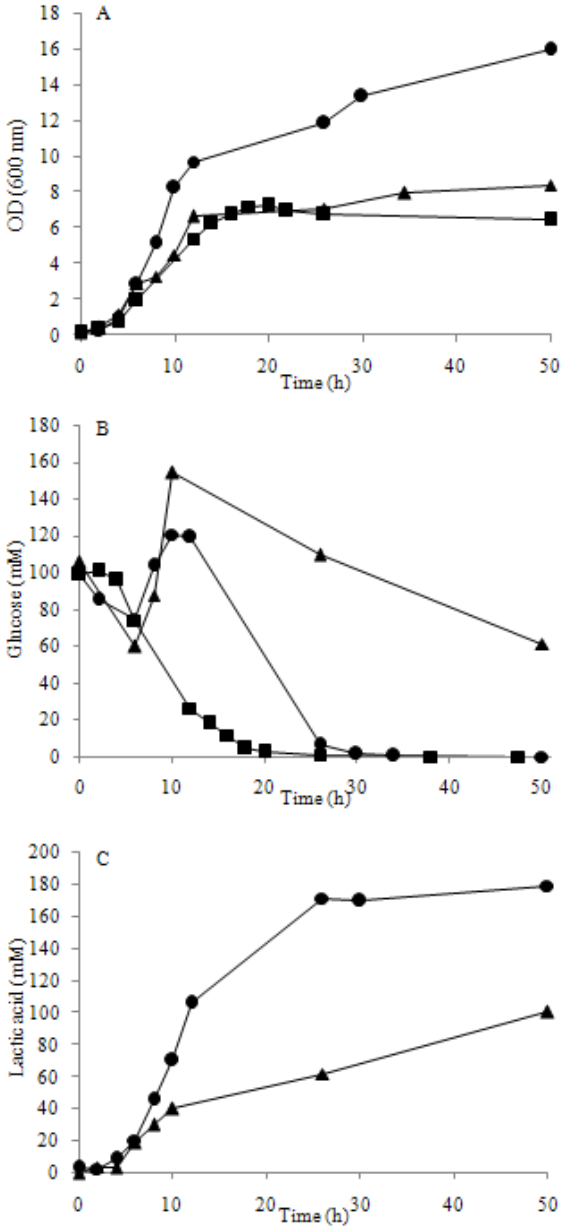

Figure 14: Time course of the cell growth (A), glucose (B) and lactic acid (C) concentration during batch culture (■), glucose fed-batch (▲) and both glucose and meat extract fed batch (●) in MRS medium. OD 600 nm, optical density at 600 nm. Data are mean value of two independent experiments.

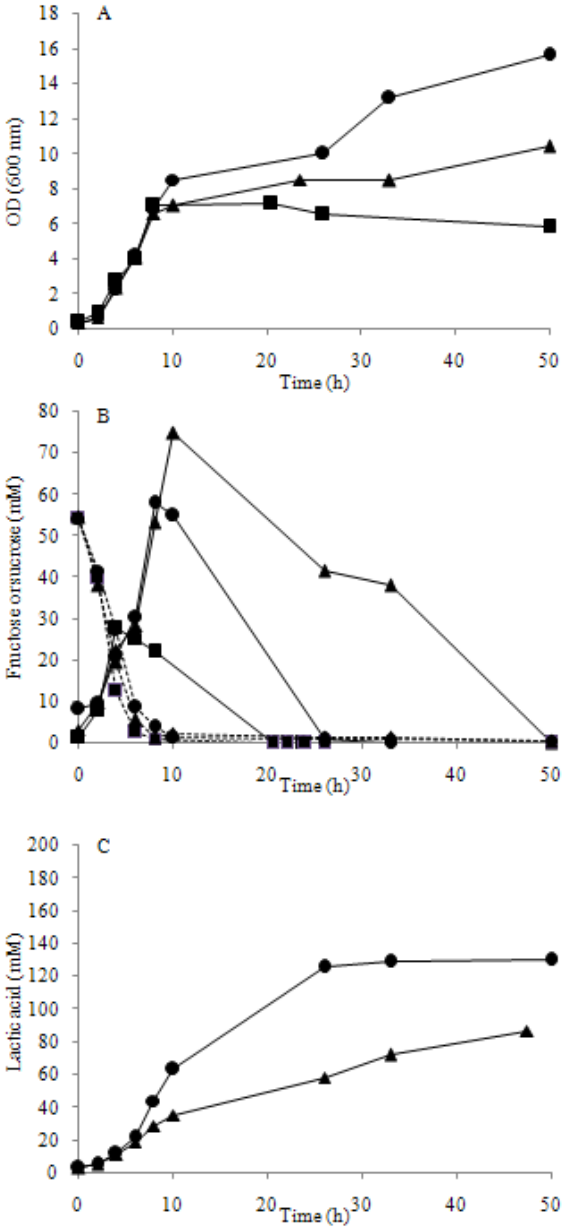

Figure 15: Time course of the cell growth (A), sucrose (---), fructose (——) (B) and lactic acid (C) concentration during batch culture (■), sucrose fed-batch (▲) and both sucrose and meat extract fed batch (●) in MRSS medium. OD 600 nm, optical density at 600 nm. Data are mean value of two independent experiments.

The highest bacteriocin titres (800 AU ml^{-1}) and specific production (174 AU ml^{-1} OD^{-1}) were obtained in MRSS, indicating that sucrose is the carbon source of choice for bacteriocin production. By contrast, the use of trehalose or glucose as primary carbon source led to lower bacteriocin titres and specific productions. Addition of meat extract in MRS or MRSS medium led to similar or lower antilisterial activities. In both case, the specific productions were lower compared to the corresponding non-supplemented culture. Glucose, sucrose and meat extract influences on bacteriocin production were further investigated during batch and fed-batch cultures.

Feedings with concentrated solutions of either glucose, sucrose or both carbon source and meat extract were performed during the exponential growth phase at a constant feeding rate to obtain a final carbon source and meat extract concentration of 60 g l^{-1} and 25 g l^{-1}, respectively. By contrast to the static culture, no significant differences in both the antilisterial activity and specific production could be observed for glucose or sucrose media during batch cultures (table 8). However, the specific production obtained in sucrose medium (61 AU ml^{-1} OD^{-1}) is approximately 3-fold lower than that obtained during the static culture without pH regulation (174 AU ml^{-1} OD^{-1}). During batch cultures, sugar and lactic acid concentration were monitored for 50 h. As shown in fig. 14, glucose was totally consumed within 20 hours of culture whereas sucrose was almost completely depleted after 8 hours of culture (fig. 15). Surprisingly, in sucrose medium, glucose could never been detected in the culture broth (data not shown) whereas fructose tended to first accumulate in the culture broth before being finally metabolised then after (fig. 15). Lactic acid concentration increased to a value of 60 mM during the first 26 hours of growth, corresponding to the fructose consumption phase. No further variation in lactic acid concentration could be observed then after (data not shown).

Table 8: Effect of batch cultures in MRS or MRSS medium (B glucose or B sucrose), fed batch addition of glucose or sucrose during cultures in MRS or MRSS medium (F glucose or F sucrose) and fed batch addition of glucose or sucrose and meat extract during culture in MRS or MRSS medium (F glucose + ME and F sucrose + ME) on growth, bacteriocin production, specific production and yield coefficient for the conversion of the substrate to lactic acid ($Y_{L/S}$) at pH controlled at 5.5 and 25°C.

Factor	Doubling time (min)	Antilisterial activity (AU ml^{-1})*	Specific production (AU ml^{-1} x OD^{-1})*	$Y_{L/S}$ g lactic acid x (g substrate)$^{-1}$
B glucose	84	400	59	Nd
B sucrose	89	400	61	0.56
F glucose	86	400	47	0.43
F sucrose	90	1600	201	0.28
F glucose + ME	86	800	61	0.57
F sucrose + ME	84	4800	478	0.41

* Calculated when the maximal inhibitory activity is reached during the culture

During glucose and sucrose fed-batch culture, cell densities at the end of the growth phase were not significantly different compared to batch culture (Fig. 14 and 15). Addition of sucrose led to a significant increase in the antilisterial activity and specific production (1600 AU ml^{-1} and 201 AU ml^{-1} OD^{-1}, respectively) compared to batch culture. In addition, a decrease in the $Y_{L/S}$ coefficient could be observed, indicating that the lactic acid production decreases in those conditions. By contrast, for glucose fed-batch culture, the specific production was equal to 47 AU ml^{-1} OD^{-1}, indicating that addition of glucose had a negative influence on the production yield. However, no effect on the antilisterial activity could be observed in those conditions. Similarly to that observed during sucrose batch culture, the carbon source was completely depleted in the medium after 8 h of culture whereas glucose was never detected during the feeding period. Fructose also tended first to accumulate in the fermentor vessel before being completely consumed at a low rate in a second step (Fig. 15). During glucose fed batch culture, the carbon source was consumed at the same rate than for batch culture within the first 6 h of culture (4.2 mM h^{-1}); it then accumulates during the feeding period before being consumed at a lower rate then after (2.3 mM h^{-1}). At the end of the culture, glucose concentration in the fermentor vessel was equal to 60 mM. In both type of fed-batch, lactic acid accumulates during both exponential and stationary phase of growth. However, the $Y_{L/S}$ was higher in glucose medium indicating that higher amount of lactic acid

were produced. During the stationary phase, a slight increase of the optical density could be observed while the inhibitory activity remains constant.

Feeding with both meat extract and glucose or sucrose did not lead to significant differences in the doubling time compare to glucose and sucrose fed-batch cultures. However, an increase in the cell densities during the stationery phase could be observed (fig. 14 and 15). During sucrose and meat extract feeding; sucrose was rapidly depleted in the culture broth leading to an accumulation of fructose during the first 10h. The latter was then totally consumed after 26 hours of culture (fig. 15). As previously observed, glucose was never detected in the culture broth. Similarly, during glucose and meat extract feeding, glucose tended first to accumulate during the first 10 h before being totally consumed after 26 hours. In both types of cultures, the $Y_{L/S}$ coefficient increased compare to glucose or sucrose fed batch culture. As shown in fig. 14 and 15, lactic acid was produced during the first 30 hours and accumulates in the medium. Addition of both glucose and meat extract led to a two-fold increased bacteriocin activity compare to glucose fed-batch and to a slight increase in specific production (table 8). By contrast, addition of both sucrose and meat extract, led to a significant increased bacteriocin titre and specific production. Indeed, a bacteriocin titre of 4800 AU ml^{-1} and a productivity of 478 AU ml^{-1} OD^{-1} were obtained in those conditions.

During all these cultures, the maximal antilisterial activity was reached at the beginning of the stationary phase of growth and remained constant then after. The acetate concentration and proteolytic activity were determined as a time function. No variation in the acetate concentration could be measured whereas no proteolytic activity could be detected (data not shown).

4.2.5 Discussion

Development of alternative solutions to chemical preservatives and heat treatments for the suppression of food-borne pathogens has become a major issue in food science. Bacteriocin and especially, those from the subclass IIa, presents a very promising perspectives in the field due to their antibacterial activity against the major food pathogen *L. monocytogenes*. However, development of cost effective bacteriocin production process are still limited due to a lack of knowledge on the factors controlling their biosynthesis.

Lb. sakei CWBI-B1365 was shown to express sakacin G structural genes. Based on biochemical evidences, we demonstrated here that the antilisterial compound produced by *Lb. sakei* CWBI-B1365 correspond to sakacin G. It was also shown that the sakacin G biological mode of action consist in the formation of transmembrane pores similarly to what was reported for several pediocin-like bacteriocin. The genetic characterisation of the strain permitted to complement the sequence previously reported by Simon and collaborators. The sequence of *skgI* encoding an immunity protein was completed and a putative papC homologue involved in membrane translocation, named skgC, was identified. This latter confirm the original organization of the sakacin G genetic determinant (Simon *et al.*, 2002). Indeed, *skgC* is not located upstream of the ABC transporter encoding gene (i.e. *skgD*) as it is usually the case in most of class IIa bacteriocin gene cluster. *SkgC* was found on a different transcriptional unit, on the opposite orientation downstream *skgI*. The high similarity between the sakacin G genetic determinant found in strains *Lb. sakei* 2512 and *Lb. sakei* CWBI-B1365 suggest that these genes could be conserved among the *Lb. sakei* sakacin G producer. Such a conservation of bacteriocin genes have already been observed in *Lb. sakei* for sakacin P and sakacin Q (Cocolin *et al.*, 2007; Moretro *et al*., 2005; Urso *et al*., 2006a).

Expression analysis showed that all the *skg* genes are expressed at the beginning and late stationary phase. Nevertheless, the antilisterial activity does not increase during this period. These results are similar to that observed for *sppA*, responsible for sakacin P production in *Lb. sakei* I151. In this latter strain, *sppA* was found to be expressed during the late stationary phase of growth without increased antilisterial activity in the culture supernatants (Urso *et al*., 2006b).

It is well established that sakacin production is strongly regulated by pH and temperature and that optimal temperature for bacteriocin production do not necessary correlates with optimal growth conditions (Diep *et al.*, 2000; Messens *et al*., 2002; Moretro *et al*., 2000). Our results agree with those observations. However, optimal conditions for bacteriocin production are slightly different in *Lb. sakei* CWBI-B1365 compare to that of *Lb. sakei* 2512 although no significant differences were found in the sakacin G genetic determinant. This suggests that trans-acting regulator could differ between those two strains. The strain dependence for the optimal condition of bacteriocin production was also reported for sakacin A, sakacin P and for nisin, suggesting that this phenomenon is not restricted to sakacin G (Diep *et al*., 2000; Moretro *et al.*, 2000, Pongtharangkul *et al*., 2006). The pH regulation of the bacteriocin production is a complex phenomenon which is not well

understood. Some authors have suggested that low pH (i) have a positive effect on the posttranslational modification and transport process, (ii) lead to a less stringent binding of the bacteriocin to the cell wall of the producer strains and/or (ii) have a reducing effect on the bacteriocin degradation (Guerra *et al*., 2003; Messens *et al*., 2002; Moretro *et al*., 2000, Nel *et al*., 2001; Yang *et al*., 1994). Our results suggest that the pH drop of the culture, due to the production of lactic acid, could also be a key parameter in those regulations. Indeed, for the non-regulated cultures, a free medium acidification from an initial pH of 6 or above led to the higher bacteriocin titre whereas culture regulated at pH 6 led to very low level of production. In addition, pH value of 5.5 also seems to be important for the obtainment of high level of production. Indeed, culture starting from pH of 5,5 or below (and thus decreasing rapidly to lower pH) led to lower antilisterial activity whereas culture at a regulated pH of 5.5 led to high bacteriocin titre.

Carbon and nitrogenous sources were reported to modulate the level of bacteriocin production in *Lb. sakei* but they are in most of the case, strain and bacteriocin dependant. This study demonstrated that sucrose increased the specific production of sakacin G in *Lb. sakei* CWBI-B1365. However, values obtained for static culture were significantly higher than those obtained in agitated cultures. Another characteristic trait of static cultures is the formation of an immerged biofilm. Therefore, we made a correlation between those two observations and hypothesized that the formation of biofilm could have a positive effect on the bacteriocin productivity. This hypothesis is reinforced by the fact that increased production yield has already been reported for the production of nisin in biofilm bioreactor compare to free culture (Pongtharangkul *et al*., 2006b).

Cultures with medium containing meat extract highlight that this latter could play a role in sakacin G production. However, its beneficial effect seems to depend of the carbon source used and how it is added in the culture broth. Indeed, no significant effect could be observed either in glucose or in sucrose batch culture. By contrast, significant increase in bacteriocin productivity was observed when meat extract addition was fractionated during sucrose based culture. This suggests that sucrose in combination with meat extract could act as an activator of the bacteriocin synthesis.

During this chapter, the sakacin G gene cluster was further characterized and was found to display an unusual genetic organization. This study highlights that Sakacin G production is subjected to a complex mechanism of regulation in which the culture conditions,

the nature of carbon and nitrogenous source used as well as the strain physiology seems to play a crucial role.

The next study will focus on the evaluation of the bacteriocin production and activity in raw poultry and beef.

4.3 Control of *Listeria monocytogenes* in raw meat

Lb. sakei is commonly associated with the meat environment and is recognized as one of the most important components of starter cultures used for production of fermented meat products (Chenol *et al.*, 2006, Hüfner *et al*., 2007). As explained previously, it may inhibit the growth of unwanted microorganisms in the raw meat environment by nutrient competition, acid production and pH reduction, as well as bacteriocin production (Chaillou *et al*., 2005; Hüfner *et al.*, 2007; Katla *et al.*, 2002, Vermeiren *et al.*, 2006a).

Lactobacillus curvatus is phylogenetically closely related to *Lb. sakei*. The bacteriocins produced by *Lb. curvatus* which have been described include curvacin A, which is identical to sakacin A, sakacin P and sakacin Q (Cocolin *et al*., 2007; Tichaczek *et al.*, 1993).

Lb. sakei and *Lb. curvatus* may be applied as a protective culture in meat (Vermeiren *et al.*, 2004). For example, sakacin P and sakacin P-producing *Lb. sakei* have proven anti-listerial activity in cold smoked salmon and chicken cold cuts (Katla *et al.*, 2001; Katla *et al*., 2002), while sakacin G producing *Lb. sakei* has activity against *L. monocytogenes* on slicked cooked ham (Héquet *et al*., 2007). Bacteriocin-producing *Lb. curvatus* strains have also proven activity in pork meat and vacuum-packaged sliced beef (Castellano *et al.*, 2006; Ghalfi *et al*., 2006; Katikou *et al.*, 2005). Non-bacteriocin producing *Lb. sakei,* on the other hand, were also described to be able to extend the shelf-life of a model cooked ham and cooked meat products (Vermeiren *et al.*, 2006a, Vermeiren *et al.*, 2006b).

The objective of this study was to evaluate the effect of *Lb. sakei* CWBI-B1365 on *L. monocytogenes* CWBI-B715. The strain *Lb. curvatus* CWBI-B28 and *Lb. sakei* LMG17302 were chosen as references. Indeed, *Lb. curvatus* CWBI-B28 has already proven good antilisterial activity in various meat products (Ghalfi *et al*., 2006a). As it will be shown in this chapter, *Lb. sakei* LMG17302 do not produce bacteriocin and exhibits the same growth rate and acidification properties than *Lb. sakei* CWBI-B1365 in standard growth medium. During meat application, it allows the evaluation of the effect of the nutrient competition and acidification properties compared to the bacteriocin activity on *L. monocytogenes* growth.

4.3.1 PCR amplification of the sakacin structural genes

Lb. curvatus CWBI-B28 has already been show to produce bacteriocins (Ghalfi *et al*., 2006b). Nevertheless, the bacteriocin has not been identified. We performed PCR amplifications of the sakacin structural genes with specific primers. A 120-bp PCR product was obtained with primers used to specifically amplify the sakacin P structural gene in *Lb. curvatus* CWBI-B28. Sequencing of the resulting PCR product showed that the nucleotide sequence is identical to that of the *sppA* gene in *Lb. curvatus* L442 and LTH1174 (Cocolin *et al*., 2007). Other structural sakacin genes were not detected in this strain.

No antilisterial activity could be detected in the culture supernatants of *Lb. sakei* LMG17302 in the culture conditions used (static cultures in 100 ml MRS medium at 25°C during 48 hours). Nevertheless, since the culture conditions may affect bacteriocin production, we checked by PCR amplification that the structural sakacin genes were not present. No PCR products were obtained from DNA isolated from *Lb. sakei* LMG17302, indicating that this strain does not carry any of the known sakacin structural genes.

4.3.2 Spectrum of activity of the neutralised cell-free supernatants

The activity spectrum of *Lb. sakei* CWBI-B1365 and *Lb. curvatus* CWBI-B28 against 16 strains of *L. monocytogenes* from various food origins was evaluated. As show in table 9, the neutralised cell-free supernatant (NCFS) of strain *Lb. curvatus* CWBI-B28 had activity against all the strains tested, while the NCFS of *Lb. sakei* CWBI-B1365, which produces sakacin G, had activity against only 9 of the tested strains. The sensitivities of the *L. monocytogenes* strains to the NCFS fractions varied greatly between the strains.

Table 9: Activity spectrum of neutralised cell-free culture supernatants of *Lb. sakei* CWBI-B1365, which produces sakacin G, and *Lb. curvatus* CWBI-B28, which has the structural gene of sakacin P, against several *L. monocytogenes* strains

L. monocytogenes strain	Zone of inhibition (diameter in mm)[a]	
	Lb. sakei CWBI-B1365	*Lb. curvatus* CWBI-B28
BFE 181	0	11
BFE 104	8	20
BFE 250	0	12
BFE 172	10	19
BFE 188	9	20
BFE 411	0	11
BFE 286	0	10
BFE 171	10	18
BFE 384	0	12
BFE 236	6	12
BFE 422	10	19
BFE 227	0	15
BFE 60	0	11
CWBI-B715	10	20
LMG21263	10	20
LMG23905	10	20

[a] Spot of 10 µl of NCFS were made in the surface of standard-I agar inoculated by the *L. monocytogenes* strain

4.3.3 Effect of *Lb. sakei* CWBI-B1365, *Lb. curvatus* CWBI-B28 and *Lb. sakei* LMG17302 on *L. monocytogenes* growth in raw beef and chicken meat

Lb. sakei CWBI-B1365 and *Lb. sakei* LMG17302 exhibited similar acidification properties in MRS broth during growth at 25 °C for 24 hours. In both cultures, the pH dropped to pH 4.0 ± 0.1. Since *Lb. sakei* LMG17302 did not produce any bacteriocin, this strain was chosen to compare the effect of the bacteriocin production to the acid production

and nutrient competition effects, on the survival and growth of *L. monocytogenes* in raw beef or chicken meat. Statistical analysis was done using the student-t test (p = 0.95).

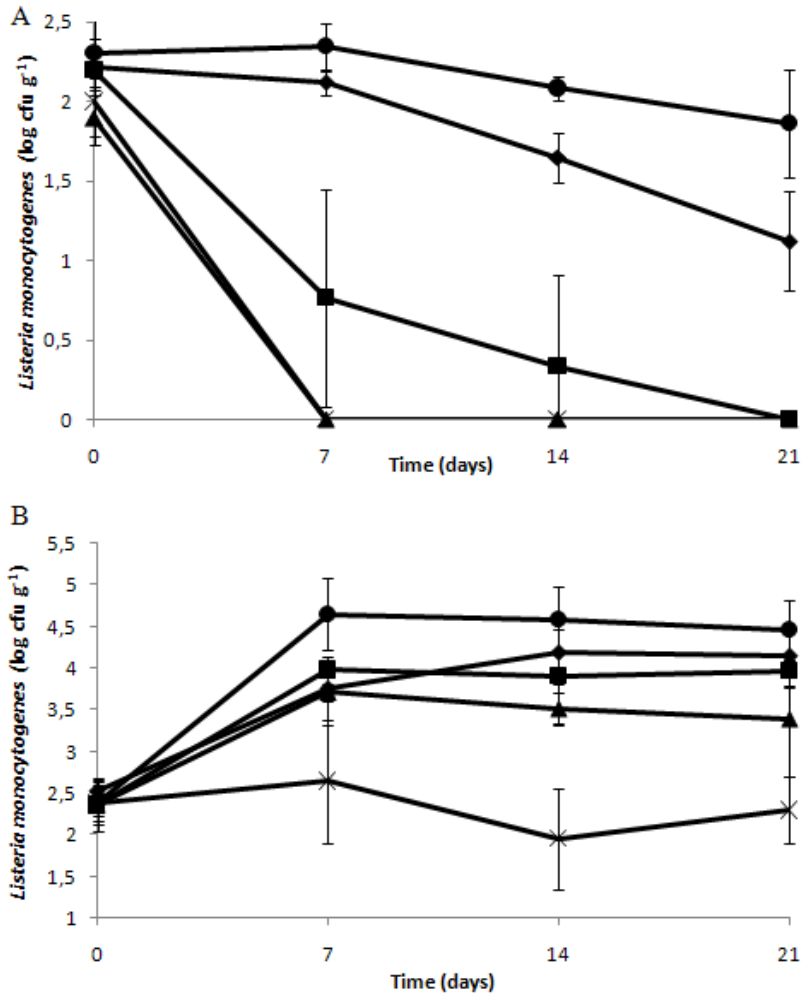

Figure 16: Effect of inhibitory culture on the growth of *L. monocytogenes* during storage at 5°C in beef meat (A) or poultry meat (B). ■: *Lb. sakei* CWBI-B1365, ▲: *Lb. curvatus* CWBI-B28, ×: *Lb. sakei* CWBI-B1365 + *Lb. curvatus* CWBI-B28, ♦: *Lb. sakei* LMG17302, ●: Control. Data are mean and standard deviation from three replicates. Counts below the detection limit ($2*10^1$ cfu g^{-1}) were consider as equal to zero.

In both meats, the developments of the LAB concentrations in the inoculated samples were very similar, irrespective of the matrix and which LAB strains were used. The concentrations of the LAB strains increased from 10^6 to 10^7 cfu g^{-1} at day 0 to 10^8 to 5 x10^8 cfu g^{-1} after 14 days of incubation, and remained stable up to 21 days. The numbers of *L.*

monocytogenes during meat applications is showed in Figure 16. In the raw beef inoculated with an initial concentration of approx. 10^2 *L. monocytogenes* cfu g^{-1}, the concentration of *L. monocytogenes* in the control decreased slightly during storage from 3.1 x 10^2 cfu g^{-1} to 1 x 10^2 cfu g^{-1} after 21 days. The addition of *Lb. sakei* CWBI-B1365 led to a significant ($p = 0.95$) reduction of the *L. monocytogenes* concentration by 1.5 log units after 7 days of storage, to 2 log units after 14 days, and finally to below the detection limit (2 x 10^1 cfu g^{-1}) at the end of the storage period. The addition of *Lb. curvatus* CWBI-B28 allowed a reduction of numbers of *L. monocytogenes* to below the detection limit after 7 days and up to 21 days. The addition of *Lb. sakei* LMG17302 led to a lower, yet significant ($p = 0.95$) reduction of *L. monocytogenes* numbers. These counts were only 0.2 log units lower than those of the control after 7 days, and 0.5 and 0.7 log units lower than the control after 14 and 21 days, respectively. The addition of *Lb. curvatus* CWBI-B28 and *Lb. sakei* CWBI-B1365 together decreased *L. monocytogenes* counts to below the detection limit after 7 days and up to 21 days of storage. The application of the bacteriocin-producing LABs led to a significantly ($p = 0.95$) better inhibition of *L. monocytogenes* than the application of the non-bacteriocinogenic *Lb. sakei* LMG17302.

The *L. monocytogenes* concentration in the poultry meat inoculated at a concentration of 2 x 10^2 cfu g^{-1} increased to 5 x 10^4 cfu g^{-1} after 7 days of storage, and remained stable at this level up to 21 days in the control. The addition of *Lb. sakei* CWBIB-1365, *Lb. curvatus* CWBI-B28 or *Lb. sakei* LMG17302 separately did not significantly reduce *L. monocytogenes* numbers during the storage of poultry meat. The combined application of *Lb. sakei* CWBI-B1365 and *Lb. curvatus* CWBI-B28 was able to inhibit the growth of *L. monocytogenes* through the storage period. The reduction of the growth of *L. monocytogenes* was significant ($p = 0.95$) under these conditions when comparing to the control and the separate application of the LAB strains.

4.3.4 Discussion

The sakacin P structural gene (*sppA*) was shown to be present in *Lb. curvatus* CWBI-B28, while the structural genes for sakacin Q, A and G could not be detected. The presence of the sakacin P gene in *Lb. curvatus* was previously described for strains LTH1174 and L442 (Cocolin *et al*., 2007). However, in these two strains, *sppQ* (encoding sakacin Q) was also detected, which is not the case in *Lb. curvatus* CWBI-B28. This is the third *Lb. curvatus*

strain where a nucleotide mismatch at position 88 of *sppA* is described, in which a cytosine is substituted with a thymine. This substitution is resulting in a modification at protein level where a histidine is replaced by a tyrosine (Cocolin *et al*., 2007). It is thus common among *Lb. curvatus* strains which possess the structural genes for sakacin P. No sakacin structural genes could be detected in *Lb. sakei* LMG17302. This fits well with the observation that no bacteriocin activity against *L. monocytogenes* could be detected.

The common feature of class IIa bacteriocins is their antilisterial activity. However, the present study shows natural variation in the resistance of *L. monocytogenes* strains. The level of resistance of *L. monocytogenes* against class IIa bacteriocin may vary according to the type of resistance mechanism present, i.e. the level of expression of the *mpt* operon or cell surface modification (Dalet *et al.,* 2001; Héchard *et al.,* 2001; Vadyvaloo *et al.,* 2004a; Vadyvaloo *et al*., 2004b).

The inhibition of *L. monocytogenes* in meat may be due to several factors like bacteriocin activity, nutrient competition and acid production. In raw beef, the success of the bacteriocin-producing strains was significantly higher for inactivation of *L. monocytogenes*, when compared to the non-bacteriocin-producing *Lb. sakei* LMG17302. Therefore, it is clear that the antilisterial effect is predominantly due to bacteriocin, rather than to nutrient competition and/or acid production. Bacteriocin produced by *Lb. curvatus* CWBI-B28 has already proven activity in smoked salmon, pork meat and bacon (Ghalfi *et al*., 2006a; Ghalfi *et al*., 2007b), while sakacin G-producing *Lb. sakei* 2512 were successfully used for biopreservation in sliced cooked ham (Héquet *et al*., 2007). A sakacin P-producing *Lb. sakei* strain has also been successfully applied in various products like fermented sausages (Urso *et al*., 2006b), poultry meat and cold smoked salmon (Katla *et al*., 2001; Katla *et al*., 2002). The results obtained in this study demonstrate the effectiveness of sakacin G producing strains for biopreservation in various meat products.

In poultry meat, the application of the LAB strains separately could not decrease the *L. monocytogenes* concentration in our study. Under these conditions, the bacteriocins produced did not seem to have noticeable activity. Since the LAB concentrations were the same in the chicken samples, it was hypothesised that the bacteriocin activity was negligible compared to the antimicrobial effects of nutrient competition or acid production. This could be due to the absence of production of bacteriocin in the poultry meat, to its inactivation through binding to food ingredient or possibly by the activity of endogenous protease (Aasen *et al*., 2003; Galvez *et al.,* 2007; Katikou *et al*., 2005; Katla *et al*., 2002; Vermeiren *et al*., 2006b).

The application of the bacteriocin producing *Lb. curvatus* and *Lb. sakei* in combination in raw chicken meat led to the successful inhibition of the *L. monocytogenes* growth, but not to a reduction below the detection limits. Since the LAB concentration during this trial was the same than that of the trials with the bacteriocin-producing strains employed singly, it seems that this effect is due to the synergistic interaction between the bacteriocins produced by the two strains. This increased effect on *L. monocytogenes* when different bacteriocins are applied together has already been demonstrated for nisin and curvaticin 13 (Bouttefroy *et al.,* 2000) and lactocin 705, nisin and enterocin CRL35 (Vignolo *et al.,* 2000). To our knowledge, this is the first example of a synergistic effect between a sakacin G producing *Lb. sakei* strain and a probable sakacin P-producing *Lb. curvatus* strain.

This study demonstrated that *Lb. sakei* CWBI-B1365 and *Lb. curvatus* CWBI-B28, which produce sakacin G, and sakacin P, respectively, may be applied in raw beef to avoid *L. monocytogenes* contamination. In poultry meat, the inhibitory effect is strongly reduced. However, the combined application of the two strains results in a successful growth inhibition of *L. monocytogenes*.

Chapitre 5 : General discussion

At the beginning of this thesis, we showed that there are a lot of data about bacteriocins from lactic acid bacteria. Nevertheless, numerous factors still have to be understood like:

- The phylogenetic origin of the genes necessary for bacteriocin production
- The impact of the sequence and the structure on the bacteriocin and the immunity protein activities
- The impact of nutritional and environmental factors on the bacteriocin production
- The limits, applicability and legislation of the bacteriocin applications

During this discussion, we will evaluate how our study allows answering these questions.

Phylogenetic origin of the genus necessary for bacteriocin production

The objective during the LAB selection was to isolate strains of unknown species and/or producing original antimicrobial compounds. We had then chosen to isolate the LAB from products originating from different environments. We also studied African products that have not been widely studied. After the selection, only strains producing bacteriocins with anti-*Listerial* activity were selected. It was not possible to select LAB that produce anti-*Salmonella* compounds other than organic acid and/or hydrogen peroxide. In addition, the selected strains belong to well known species and have the structural genes for already described bacteriocins i.e. sakacin G in *Lb. sakei* CWBI-B1365, nisin Z in *Lc. lactis lactis* CWBI-B1437, piscicolin 126 and carnobacteriocin Bm1 in *Cb. maltaromaticum* CWBI-B1369 and CWBI-B1436. For *Lb. sakei* CWBI-B1365, it was also shown that the bacteriocin present in the culture supernatants was sakacin G, but that part of the genes necessary for the production of sakacin P (*sppK* and *sppR*), as well as an immunity gene (*OrfY*), commonly associated to the sakacin P gene cluster, were present.

As shows in table 10, these bacteriocins and genes have already been identified in LAB isolated from various food products from around the world. The genes coding for the same bacteriocins in different strains present a high degree of similarity. They are localized on the chromosome like for nisin and carnobacteriocin Bm1, on the chromosome or on a plasmid like for sakacin P and piscicolin 126 or only on a plasmid like for sakacin G. Some parts of the sakacin P gene cluster have been detected in all the *Lb. sakei* strains studied until now (16 strains from different food environments).

Table 10 : LAB strains isolated from all around the world and producing sakacin G, sakacin P, piscicolin 126, carnobacteriocin Bm1 or nisin Z

Strain	Food product	Country	Genetic localization	Reference
Sakacin G				
Lb. sakei 2512	Nd	Nd	Plasmid (35kb)	Simon *et al.*, 2002
Lb. sakei CWBI-B1365	Poultry meat	Belgium	Plasmid (25kb)	This study
Sakacin P				
Lb. sakei LTH673	Fermented sausages	Germany	Chromosome	Tichaczek *et al.*, 1994
Lb. sakei Lb674	Nd	Nd	Chromosome	Hühne *et al.*, 1996
Lb. sakei CCUG 42687	Nd	Nd	Nd	Aasen *et al.*, 2000
Lb. sakei 5	Malted barley	Ireland	Plasmid	Vaughan *et al.*, 2001
Lb. sakei I151	Fermented sausages	Italy	Nd	Urso *et al.*, 2006
Lb. curvatus L442	Fermented sausages	Greece	Chromosome	Cocolin *et al.*, 2007
Lb. curvatus LTH1174	Nd	Italy	Chromosome	Cocolin *et al.*, 2007
Lb. curvatus CWBI-B28	Raw meat	Belgium	Nd	Ghalfi, 2006b
Sakacin P non producer containing part of the sakacin P gene cluster				
Lb. sakei CWBI-B1365	Poultry meat	Belgium	Nd	This study
Lb. sakei -15 different strains	Meat and fish	Nd	Nd	Moretro *et al.*, 2005
Piscicolin126				
Cb. piscicola JG126	Ham	Australia	Nd	Jack *et al*., 1996
Cb. maltaromaticum UAL26	Minced beef	Canada	chromosome	Gursky *et al*., 2006
Cb. maltaromaticum CWBI-B1369	Poultry meat	Belgium	Plasmid (35kb)	This study
Carnobacteriocin Bm1				
Cb. maltaromaticum UAL26	Minced beef	Canada	Chromosome	Gursky *et al*., 2006
Cb. piscicola LV17B	Meat	Canada	Chromosome	Quadri *et al.*, 1994
Cb. piscicola V1	Fish	France	Nd	Buhgaloo-Vial *et al.*, 1996
Cb. piscicola CP5	Cheese	France	Plasmid	Herbin *et al.*, 1997
Cb. maltaromaticum CWBI-B1369	Poultry meat	Belgium	Chromosome	This study
Nisin Z				
Lc. lactis NIZO2186	Nd	Nd	Nd	Mulders *et al.*, 1991
Lc. lactis IO-1	Nd	Nd	Nd	Matsusaki *et al.*, 1996
Lc. lactis UL719	Cheese	France	Nd	Meghrous *et al.*, 1997
Lc. lactis HPB1688	Beans	Canada	Chromosome	Cai *et al.*, 1997
Lc. lactis N8	Nd	Nd	Chromosome	Immonen *et al.*, 1998
Lc. lactis BFE1500	Wara (fermented milk product)	Nigeria	Chromosome	Olasupo *et al.*, 1999
Lc. lactis INIA415	Cheese	Spain	Nd	Garde *et al.*, 2002
Lc. lactis WNC20	Nham (Fermented sausages)	Thailand	Chromosome	Noonpakdee *et al.*, 2003
Lc. lactis	Kimchi (Fermented cabbage)	Japan	Chromosome	Park *et al.*, 2003
Lc. lactis IPLA729	Cheese	Spain	Nd	Martinez *et al.*, 1995
Lc. lactis	Cheese	Tunisia	Nd	Ghrairi *et al.*, 2004
Lc. lactis CM1	Dahi (fermented milk)	India	Nd	Mitra *et al.*, 2007
Lc. lactis CWBI-B1437	Fermented fish	Senegal	Chromosome	This study

Nd : not determined

A specified bacteriocin is only produced by strains belonging to the same species or to close phylogenetically related species. Nevertheless, the amino acid sequence of the different

bacteriocins may have a high degree of similarity, especially for class IIa bacteriocins like already described in chapter III. The mechanisms for the regulation of the production, the immunity, the maturation and the excretion of bacteriocin are similar in most of the class IIa bacteriocin producing LAB. Nevertheless, the genetic organisation and position of the bacteriocin gene cluster may vary. Since the presence of two structural genes and the position of *SkgC* in the sakacin G gene cluster, the genetic organisation differs from those of other class IIa bacteriocin. Piscicolin 126 is another example. The prebacteriocin gene of piscicolin 126 is located on a plasmid in *Cb. maltaromaticum* CWBI-B1369 and CWBI-B1436 while Carnobacteriocin Bm1 prebacteriocin gene is on the chromosome. These positions seem to have an impact on the bacteriocin production. Indeed, in *Cb. maltaromaticum* UAL26 (Gursky *et al*., 2006), the piscicolin 126 prebacteriocin gene is cotranscribed with the carnobacteriocin Bm1 prebacteriocin gene, which is not the case in *Cb. maltaromaticum* CWBI-B1369 and CWBI-B1436.

Given that the genes coding for bacteriocins with closed structural characteristics are highly varied, we can hypothesize about their phylogenetic origin. They are only a few studies about this. Nevertheless, the genomes of 18 LABs have been sequenced recently. These data allow understanding the evolution of the *Lactobacillales*. The common ancestor of *Lactobacillales* had lost 600 to 1200 genes and gain <100 genes after divergence from the Bacilli ancestor. The gains have been made by horizontal gene transfer (HGT) through bacteriophage or conjugation. This evolution seems to be related to the transition made by the LAB to the existence in nutritionally rich medium. In the genome of seven of the sequenced LAB, gene for putative bacteriocins and associated proteins were identified, these novel putatives bacteriocins are all homologues to pediocin-like bacteriocins (Makarova *et al*., 2007). Bacteriocin-production-related genes seem to be among those that are often transfer via HGT as indicated by the analysis of the phylogenetic trees and differences in the operon organization, even in closely related genomes. Their presence is the result of their long term existence in complex microbial communities. Bacteriocins allowed increasing the LAB competitivity in rich environments like food products (Makarova *et al.*, 2007).

Sakacin P is a good example of the transmission of bacteriocin genes. Indeed, parts of the sakacin P gene cluster are present in all the *Lb. sakei* strains studied until now (*sppK* 3'-end and *sppR*), even if they do not produce the bacteriocin. The genes organization is varied in the non-producer strains. The presence of a sakacin P producer common ancestor and the loss of the genes or their recombination through evolution has thus been suggested (Moretro

et al., 2005). The presence of genes for bacteriocin production in non-bacteriocin producer has also been described in *Lactococcus lactis* (Moschetti *et al.,* 1996; Venema *et al.,* 1996). In conclusion, we can presume that the genes for bacteriocins have been acquired via HGT followed by vertical transfer to obtain the actual diversity.

If the bacteriocin genes are located on a plasmid, the transfer of this genetic information between closely related bacteria through plasmids conjugation could be interesting for industrial application. Indeed, strains that have interesting technological properties but that do not produce bacteriocins may then acquire this property. The plasmid stability in the strains has to be carefully evaluated.

Impact of the sequence and the structure on class IIa bacteriocin and immunity proteins activities

Even if the sequences of class IIa bacteriocin have a high degree of similarity, the activity spectrum of these bacteriocins is highly variable. Nevertheless, they all have activity against *L. monocytogenes*. As described in chapter III, the N-terminal part of the peptide interacts with the hydrophilic external surface of the cell membrane while the C-terminal part interacts with the internal hydrophobic part. The mannose permease, a membrane receptor, is involved in this interaction. Two hypotheses have been suggested to understand the activity spectra. The first one suggests that the second disulfide bridge present in the C-terminal part of some bacteriocins give them a higher activity and a wider inhibitory spectrum by stabilisation of the C-terminal tertiary structure (Eijsink *et al.,* 1998; Fimland *et al.,* 2000; Richard *et al*., 2006). The second suggests that the sequence situated at the C-terminal side of the hinge region is the main factor determining the activity spectrum. The C-terminal tertiary structure is stabilised by a second disulfide bridge or by a tryptophan residue, which are both important for the activity (Johnsen *et al*., 2005; Fimland *et al.*, 2002).

As show in figure 17, class IIa bacteriocin may be classified in different sub-group on the basis of their sequence similarity. The bacteriocin of sub-group 2 have a narrow inhibitory spectrum almost limited to *L. monocytogenes*. Like mesentericin Y105, sakacin G has a narrow inhibitory spectrum, despite the presence of a second disulfide bridge in its C-terminal part. Like previously suggested (Simon *et al.,* 2002), our study confirms the second hypothesis highlighting that C-terminal sequence of the bacteriocin is the major factor determining the inhibitory spectrum. Nevertheless, the mechanism behind has not been

explained until now. Diep *et al.* (2007) have recently suggested that the specific activity is due to the interaction with the mannose permease. Indeed, even if this phosphotransferase system is widely spread in bacteria, it may vary according to the genus, the species and the strain. The bacteriocin recognition could then be specific.

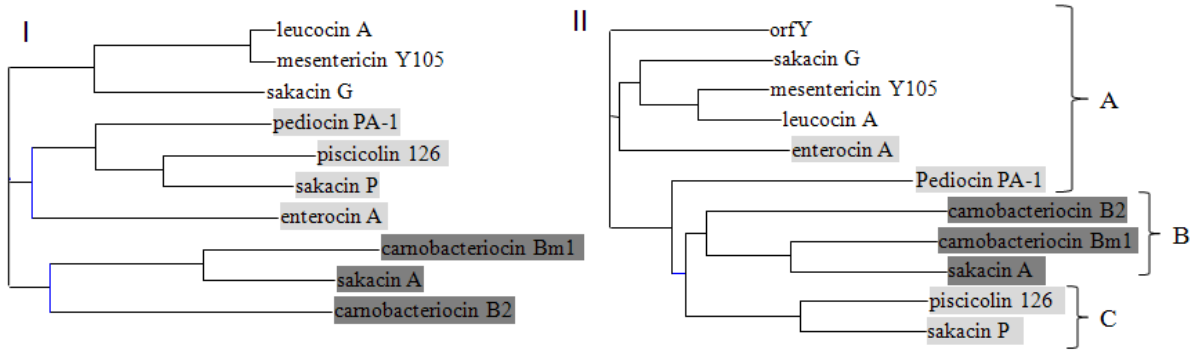

Figure 17: Classification on the basis of sequence similarity of class IIa bacteriocin (I) and of their immunity proteins (II). On the basis of the similarity of their C-terminal part, the class IIa bacteriocins may be sub-classified in three sub-groups (in light grey: sub-group 1; in white: sub-group 2, in dark-grey: sub-group3) (Fimland *et al.*, 2002). The immunity proteins may also be classified in three subgroups (A, B and C) (Drider *et al.*, 2006).

On the basis of their sequence similarities, the immunity proteins are also classified in three sub-groups (figure 17). The specificity and the functionality of the immunity protein have been attributed to the specific recognition of the C-terminal part of the bacteriocin by the C-terminal part of the immunity protein and to the strains were this process arise (Johnsen *et al.*, 2004; Johnsen *et al.*, 2005). Indeed, it has been suggested that the sequence and the structure of the mannose permease have a strong influence on the interaction with the immunity protein (Diep *et al.*, 2007). The sakacin G immunity protein belongs to the same group than the immunity protein of the sub-group 2 bacteriocins. During this study, it has been showed that *Cb. maltaromaticum* CWBI-B1369, which has the piscicolin 126 and carnobacteriocin Bm1 prebacteriocin gene, is sensitive to sakacin G. The immunity proteins associated have thus no protective activity against sakacin G. The strain *Lb. curvatus* CWBI-B28 that produce sakacin P is not sensitive to sakacin G. It is probable that *OrfY*, which is part of the *spp* cluster, is present in this strain and gives it the resistance. Although, the strain could be naturally resistant. It has already been showed that *OrfY* may gives resistance to leucocin A and enterocin A. Their immunity proteins belong also to the sub-group A (Fimland

et al., 2002). Results obtained with sakacin G confirm the impact of the sequence on the specificity of the immunity proteins.

The narrow inhibitory spectrum of sakacin G, which is limited to food pathogenic or spoilage bacteria, could be useful for food applications. Indeed, one type of application of the bacteriocin producer strains or the bacteriocin itself is the application in fermented products in combination with a starter culture that will be able to carry out the desired fermentation process optimally (Galvez *et al.,* 2007). The adjunct cultures do not need to contribute to the fermentations, but they must not interfere with the primary function of the starter cultures. The bacteriocin should then specifically inhibit food spoilage or pathogenic bacteria without affecting the starter strains. For this reason, bacteriocin resistance of the starter culture may be a key factor. The bacteriocin with a narrow inhibitory spectrum could then be useful.

A problem associated to the use of bacteriocins or the producer strains in food product is the apparition of resistant pathogenic strains like for the resistance to antibiotics. It was showed that 1 to 8 percents of the wild-type strains of *L. monocytogenes* are resistant to bacteriocins. In addition, spontaneous mutations may occur after exposition to the bacteriocins. To avoid contamination of food product by resistant strains, it is important to consider the bacteriocins as an additional food preservation process. In addition, the successive use of bacteriocin from different class should allow decreasing the level of resistance since the resistance mechanisms are different. Nevertheless, the development of cross-resistance should be carefully considered.

Impact of nutritional and environmental factors on bacteriocin production

A lot of factors, including pH, temperature and composition of the culture broth, have an impact on bacteriocin production. The growth of the strains is a requirement for bacteriocin production. However, it has been often suggested that optimal conditions for growth do not correlate with optimal conditions for bacteriocin production. The study of the growth and sakacin G production by *Lb. sakei* CWBI-B1365 confirms this. Indeed, the optimal temperature for bacteriocin production is lower than the optimal temperature for growth. pH and composition of the culture broth have also a considerable impact on the sakacin G production without affecting the growth. Small pH variations may strongly affect bacteriocin production. The carbon and nitrogen sources used, as well as their concentration, are also factors of great importance for bacteriocin production.

The different factors limiting bacteriocin production already described are protease production, bacteriocin adsorption to the surface of the producer cells, nutriments limitation and genes repression (Straume *et al*., 2007). For sakacin G, the stop of the apparent production of bacteriocin (evaluated by the inhibitory activity of the cell-free supernatants) seems to be due to nutriments limitation rather than to gene repression, since bacteriocin genes were still expressed in the late stationary phase of growth.

During production optimization, it is also important to consider the possible applications of the bacteriocin and the cost of the product that the users are ready to give. A compromise should be found between the price increased of the growth medium and the resulting additional inhibitory activity. The yield of the down-stream process, which has not been studied here, will also be a critical point.

A successful application of bacteriocins: some essential factors

During chapter 1, we showed that factors affecting the bacteriocins efficacy in a food product are complex. Some of these factors are the product composition, the storage temperature, the transformation process, the pH, the salt concentration, the adsorption of the bacteriocin to the food constituent, the endogenous microflora, the protease present in the product and, if the producer strain is used, the production of the bacteriocin inside the food product (Galvez *et al.*, 2007). The user may then wonder: "How can we increase the probability of a successful application of bacteriocins or the producer LAB to inhibit *L. monocytogenes* in a specific food product?" We will try to point out some important aspects.

One of the conclusions of chapter IV.1 is that *Lc. lactis subsp. lactis* CWBI-B1437 can not inhibit *L. monocytogenes* growth in poultry meat. There are two reasons. Firstly, the strain did not growth in the meat at this temperature. Secondly, nisin may be inactivated in meat as show by Rose *et al.* (2002). *Lc. lactis* colonise mainly milk products while *Lb. sakei* and *Cb. maltaromaticum* are mainly present in the meat environment (Chaillou *et al.*, 2005; Leisner *et al.*, 2007). The selected strains belonging to these species have a good growth in poultry meat and inhibit *L. monocytogenes* growth as showed in chapter IV.1. One of the conclusions of this study is that the first parameter to be considered for successful application of bacteriocin producing LAB is to choose strains that belong to species that are well adapted to the product and to the storage temperature. Since the food applications of LAB are well studied, a lot of informations are present in the scientific literature. The product composition (pH, salt, fat

content, conservator…) and the production process (thermal treatment…) will also have a strong impact. The in vitro evaluation of the impact of such factors (i.e. pH or temperature) on the bacteriocin activity will allow eliminating the LAB and/or the bacteriocins that are not well adapted. That will permit reducing the time-consuming trials that have to be made in the products before industrial applications.

Two application types of the bacteriocins are possible: (i) the semi-purified bacteriocin or (ii) the producer strain. Each one has its own advantages and inconvenient. The advantage of the application of the producer strain is the low cost and the absence of specific legislative agreement. The main inconvenience is the necessary growth of the strain and the bacteriocin production in the food product. Indeed, the trials made in chapter VI.3 show that the bacteriocin produced by *Lb. sakei* CWBI-B1365 and *Lb. curvatus* CWBI-B28 are responsible for the inhibition of *L. monocytogenes* in beef meat. Nevertheless, the LAB cell concentration reached is 10^8 cfu g^{-1}. This is higher than the acceptable limit of 10^6 cfu g^{-1} for non-fermented food products. The impact of growth of the LAB on the organoleptic properties of the food product has not been evaluated here but will be considered for industrial applications. The growth is not always sufficient to guarantee the antilisterial effect of the strains through bacteriocins production. Indeed, despite a good growth of the LAB strains in chicken meat, the bacteriocins produced by *Lb. sakei* CWBI-B1365 and *Lb. curvatus* CWBI-B28 have no antilisterial activity. This could be due to the absence of the bacteriocin production in chicken meat or the degradation of the produced bacteriocins. Surprisingly, the combined application of these two strains allows a good inhibition of *L. monocytogenes*. This could be the result of a synergic effect between the two strains and/or their bacteriocins. To our knowledge, this is the first time that such effect has been reported between two class IIa bacteriocins producing LAB.

The application of the purified bacteriocin has not been tested during this work because it has a main disadvantage: it is considered as a food additive. In addition, the semi-purified bacteriocin may be degraded in the food product by endogenous protease and proteases produced by the endogenous microflora (Kouakou *et al.*, 2008). It is also necessary to apply a sufficient quantity of bacteriocin to inhibit the entire pathogenic flora in the food matrix. This may result to an expensive process or to a modification of the organoleptic properties of the food product. Even if the application of the semi-purified bacteriocin to inhibit *L. monocytogenes* has led to good results, a rebound phenomenon has sometimes been observed. This rebound is due to the growth of an undetectable quantity of *L. monocytogenes*

that has not been destroy at the beginning of the storage and/or to the degradation of the bacteriocin or its inactivation (Ghalfi *et al*., 2006; Kouakou *et al.*, 2008).

Chapitre 6 : General conclusion and perspectives

Bacteriocins from lactic acid bacteria are antimicrobial peptides that kill closely related bacteria. A high number of bacteriocin have an activity against the food pathogenic bacteria *L. monocytogenes* and have been studied for their use as food preservative.

The genes involved in the production of bacteriocin from lactic acid bacteria have been described for numerous bacteriocins. Most of the time, they are organized in three operons. An operon contains the structural and immunity genes. The second contains the ABC transporter and accessory protein genes while the third contains the genes coding for the proteins involved in the regulatory system (the histidine kinase, the response regulator and the inducing peptide). These genes are located on the chromosome or on a plasmid. During this work, we contributed to the identification of the plasmid located genes involved in sakacin G production.

These data may be use to improve the production of bacteriocin by LAB strains through genetic engineering. If the genetic information is plasmid linked, the transfer of this plasmid to another strain of the same species or a closed related species, which have other good technological properties (i.e. a good starter culture or a good resistance to freeze-drying and storage), could be interesting. Indeed, it will allow the industrial to have strains with different useful properties. It has also been suggested that combining the N-terminal part of a bacteriocin and the C-terminal part of another bacteriocin may allow having bacteriocin with a wider inhibitory spectrum and a higher activity. Another improvement is the addition of copies of the genes responsible for the production of bacteriocin to make overproducing mutant. However, even if all these transformations are technologically possible, the main question remains in the use of genetically engineered strains for the food consumption. In Europe, this will be a problem at the legislative and the consumer point of view. Attempts should be done to use a method that will allow the transformation of the bacteria naturally.

Another option to improve the bacteriocin production is the use of an optimized fermentation process. Since the production of bacteriocin is regulated by a quorum sensing system, the cell concentration in the cultures has to be sufficient to allow the induction of the production. It also appears that the formation of a biofilm at the bottom of the flask during static cultures may improve the production. Some other factors have also a great impact on bacteriocin production. Indeed, as show in this study, the pH regulation, the temperature, the carbon source used and its concentration as well as the nitrogen source concentration are some key parameters for bacteriocin production. The use of a fed-batch to add the nutriments, sugar and meat extract for example, during the culture allow also improving the bacteriocin

production. Optimising these parameters for sakacin G production by *Lb. sakei* CWBI-B1365 led to a 12-fold improvement of the bacteriocin production. The impact of the culture conditions on the bacteriocin production seems to be bacteriocin and strains dependent. It should then be studied for each bacteriocin and each bacteria.

In most of the studies, the optimisation of the fermentation condition is made at a small scale (maximum 20 litres fermentors). However, the industrial application of bacteriocin will require a large-scale production. The scale-up of the production of bacteriocin has not been studied here but is a perspective of this work. For an industrial application, the cost of the production process has to be evaluated. Indeed, the optimization of the bacteriocin production may require the addition of expensive nutritional sources like meat extract. The use of by-product from the food industry may perhaps be an alternative.

After the optimization of the production of the bacteriocin, the next challenge will be the purification of the bacteriocin. Several purification techniques are available from the literature. At a laboratory scale, the classical purification process of bacteriocin is the use of ammonium sulphate precipitation followed by reverse phase and ion exchange chromatography and RP-HPLC. Its application at an industrial scale will be difficult and expensive. Another way to purify the bacteriocin is to adsorb them onto the wall of the producer strains. This adsorption is pH dependent. The culture is then centrifuged and the bacteriocin may be recovered after desorption. Then after, the bacteriocin may be concentrated by ultrafiltration for example. After adsorption, the culture may also be heat killed and subsequently centrifuged and freeze-dried. The final product will be a powder containing the bacteriocin but also the heat killed bacteria. The purification process has to be chosen depending of the applications planned. The effect of the different formulation on the survival of the pathogenic bacteria and on the organoleptical properties of the product has to be evaluated. One important factor for the choice of the purification process will also be the cost of the product that the consumer is ready to give.

The use of bacteriocin or the producer bacteria in food products can be an alternative to the use of chemical preservatives. Indeed, the results obtained during this work and those of several studies available in the literature show that bacteriocin may successfully inhibit food pathogenic or spoilage bacteria. Nevertheless, there are several limitations to their use. Indeed, the activity of the bacteriocin in the food product may be limited by the inactivation by the food constituents, the storage condition, the transformation process, the endogenous microflora, the protease present in the product and, if the producer strains is used, the

production of the bacteriocin inside the food product. The activity of the bacteriocin or the producer bacteria is also influenced by the matrix used. The use of strains with a metabolism adapted to the food product is necessary. The in vitro studies of the bacteriocin and the producer bacteria may allow determining the effect of different parameters on the bacteriocin activity and on the producer bacteria. These in vitro tests are important in order to reduce the time-consuming trials in food. As suggested before and as showed in this study, the use of different bacteriocin may lead to a synergic activity, even if they belong to the same class. It could be interesting to study the mechanism leading to this effect.

Depending of the product, a choice should be made between the different formulation possible (i.e. protective culture, purified bacteriocin or the cell adsorbed bacteriocin). In addition to the cost and the impact on the activity and the organoleptic properties, the legislative status of these formulations has also to be considered. The use of purified bacteriocin is considered as a food preservatives and required a specific agreement. The use of the producing bacteria as protective culture should refer to the legislation “novel food”.

Chapitre 7 References

Aasen, I., Moretro, T., Katla, T., Axelsson, L., Storro, I. (2000) Influence of complex nutrients, temperature and pH on bacteriocin production by *Lb. sakei* CCUG 42687. *Appl. Microbiol. Biotechnol*. 53, 159-166.

Abee, T. (1995) Pore-forming bacteriocins of Gram-positive bacteria and self-protection mechanisms of producer organisms. *FEMS Microbiol. Lett.* 129(1), 1-9.

Ammor, S., Tauveron, G., Dufour, E., Chevallier, I. (2006) Antibacterial activity of lactic acid bacteria against spoilage and pathogens bacteria isolated from the same meat small-scale facility. *Food Control* 17, 454-468.

Anastasiadou, S., Papagianni, M., Ambrosiadis, I., Koidis, P. (2008) Rapid quantifiable assessment of nutritional parameters influencing pediocin production by *Pediococcus acidilactici* NRRL B5627. *Bioresour. Technol.* in press.

Annuk, H., Shchepetova, J., Kullisaar, T., Songisepp, E., Zilmer, M., Mikelsaar M. (2003) Characterization of intestinal lactobacilli as putative probiotic candidates. *J. Appl. Microbiol.* 94, 403-412.

Arous, S., Dalet, K., Héchard, Y. (2004) Involvement of the mpo operon in resistance to class IIa bacteriocins in *L. monocytogenes*. *FEMS Microbiol. Lett*. 238, 37-41.

Azuma, T., Bagenda, D.K., Yamamoto, T., Kawai, Y., Yamazaki, K. (2007) Inhibition of *L. monocytogenes* by freeze-dried piscicocin CS526 fermentate in food. *Lett. Appl. Microbiol.* 44, 139-144.

Bauer, R., Dicks, L. (2005) Mode of action of lipid II-targeting lantibiotics. *Int. J. Food Microbiol.* 101, 201-216.

Benkerroum, N., Oubel, H, Ben Mimoun, L. (2002) Behaviour of *L. monocytogenes* and *Staphylococcus aureus* in yogurt fermented with a bacteriocin-producing thermophilic starter. *J. Food Protect*. 65, 799-805.

Bhugaloo-Vial, P., Dousset, X., Metivier, A., Sorokine, O., Anglade, P., Boyaval, P., Marion, D. (1996) Purification and amino-acid sequences of piscicocins V1a and V1b, two class IIa bacteriocins secreted by *Carnobacterium piscicola* V1 that display significantly different levels of specific inhibitory activity. *Appl. Environ. Microbiol*. 62, 4410-4416.

Björkroth, J., Korkeala, H. (1996) rRNA gene restriction patterns as a characterization tool for *Lactobacillus sake* strains producing ropy slime. *Int. J. Food Microbiol*. 30, 293-302.

Bouttefroy, A., Millière, J.B. (2000) Nisin-curvaticin 13 combinations for avoiding the regrowth of bacteriocin resistant cells of *L. monocytogenes* ATCC15313. *Int. J. Food Microbiol.* 62, 65-75.

Brul, S., Coote, P. (1999) Preservative agents in foods: Mode of action and microbial resistance mechanisms. *Int. J. Food Microbiol.* 50(1-2), 1-17.

Brurberg, M., Nes, I., Eijsink, V. (1997) Pheromone-induced production of antimicrobial peptides in *Lactobacillus*. *Mol. Microbiol.* 26, 347-360.

Cai, Y., Ng, L.K., Farber, J.M. (1997) Isolation and characterization of nisin-producing *Lactococcus lactis subsp. lactis* from bean-sprouts. *J. Appl. Microbiol*. 83, 499-507.

Callewaert, T., De Vuyst, L. (2000) Bacteriocin production with *L. amylovorus* DCE471 is improved and stabilized by fed-Batch fermentation. *Appl. Environ. Microbiol*. 66(2), 606-613.

Caplice, E., Fitzgerald, G. (1999) Food fermentations: role of microorganisms in food production and preservation. *Int. J. Food Microbiol.* 50(1-2), 131-149.

Castellano, P. and Vignolo, G. (2006) Inhibition of *L. innocua* and *Brochothrix thermosphacta* in vacuum-packaged meat by addition of bacteriocinogenic *Lb. curvatus* CRL705 and its bacteriocins. *Lett. Appl. Microbiol*. 43, 194-199.

Castellano, P., Vignolo, G., Farias, R.N., Arrondo, J.L., Chelin R. (2007) Molecular view by fourier transform infrared spectroscopy of the relationship between lactocin 705 and membranes : speculations on antimicrobial mechanism. *Appl. Environ. Microbiol*. 73(2), 415-420.

Chaillou, S., Champomier-Vergès, M.C., Cornet, M., Crutz-Le Coq, A.-M., Dudez, A.-M., Martin, V., Beaufils, S., Darbon-Rongère, E., Bossy, R., Loux, V., Zagorec, M. (2005) The complete genome sequence of the meat-borne lactic acid bacterium *Lb. sakei* 23K. *Nature Biotechnol.* 23, 1527-1533.

Chen, Y.S., Srionnual, S., Onda, T., Yanagida, F. (2007) Effects of prebiotic oligosaccharides and trehalose on growth and production of bacteriocins by lactic acid bacteria. *Lett. Appl. Microbiol.* 45, 190-193.

Chenol, E., Macian, M.C., Elizaquivel, P., Aznar, R. (2006) Lactic acid bacteria associated with vacuum-packed cooked meat product spoilage: population analysis by rDNA-based methods. *J. Appl. Microbiol.* 102, 498-508.

Cocolin, L., Comi, G. (2005). Analysis of PCR-based methods for characterization of Listeria monocytogenes strains isolated from different sources. *Int. J. Food Microbiol*. 103, 167-178.

Cocolin, L., Rantsiou, K. (2007) Sequencing and expression analysis of sakacin genes in *L. curvatus* strains. *Appl. Microbiol. Biotechnol*. 76, 1403-1411.

Cotter, P.D., Hill, C. (2003) Surviving the acid test: response of Gram-Positive bacteria to low pH. *Microbiol. Mol. Biol. Rev.* 67(3), 429-453.

Dalet, K., Briand, C., Cenatiempo, Y., Héchard, Y. (2000) The rpoN gene of *Enterococcus faecalis* directs sensitivity to subclass IIa bacteriocins. *Curr. Microbiol*. 41(6), 441-443.

Dalet, K., Cenatiempo, Y., Cossart, P. and Héchard, Y. (2001) A σ^{54}-dependent PTS permease of the mannose family is responsible for sensitivity of *L. monocytogenes* to mesentericin Y105. *Microbiology* 147, 3263-3269.

Deegan, L.H., Cotter, P.D., Hill, C., Ross, P. (2006) Bacteriocins: Biological tools for bio-preservation and shelf-life extension. *Int. Dairy J.* 16,. 1058-1071.

De Kwaadsteniet, M., Fraser, T., Van Reenen, C.A., Dicks, L.M. (2006) Bacteriocin T8, a novel class IIa sec-dependent bacteriocin produced by *Enterococcus faecium* T8, isolated from vaginal secretions of children infected with human immunodeficiency virus. *Appl. Environ. Microbiol*. 72(7), 4761-4766.

De Vuyst, L., Callewaert, R., Crabbé, K. (1996) Primary metabolite kinetics of bacteriocin biosynthesis by *Lb. amylovorus* and evidence for stimulation of bacteriocin production under unfavourable growth conditions. *Microbiology* 142, 817-827.

Diep, D., Axelsson, L., Grefsli, C., Nes, I.F., (2000) The synthesis of the bacteriocin sakacin A is a temperature-sensitive process regulated by a pheromone peptide through a three-component regulatory system. *Microbiology* 146, 2155-2160.

Diep, D., Salehian, Z., Holo, H., Nes, I.F. (2007). Common mechanisms of target cell recognition and immunity for class II bacteriocin. *Proc. Natl. Acad. Sci.* 104, 2384-2389.

Dodd, H.M., Horn, N., Gasson, M.J. (1990) Analysis of the genetic determinant for production of the peptide antibiotic nisin. *J. Gen. Microbiol*. 136, 555-566.

Drider, D., Fimland, G., Héchard, Y., Mc Mullen, L.M., Prevost, H. (2006) The continuing story of class IIa bacteriocin. *Microbiol. Mol. Biol. Rev.* 70 (2), 564-582.

Duffes, F., Leroi, F., Boyaval, P., Dousset, X., (1999) Inhibition of *L. monocytogenes* by *Carnobacterium* spp. strains in a simulated cold smoked fish system stored at 4 deg C. *Int. J. Food Microbiol*. 47, 33-42.

Eijsink, V.G.H., Brurberg, M.B., Middelhoven, P.M., Nes, I.F., (1996) Induction of bacteriocin production in *Lb. sakei* by a secreted peptide. *J. Bacteriol.* 178 (8), 2232-2237.

Eijsink, V.G.H., Skeie, M., Middelhoven, P.H., Brurberg, M.B., Nes I.F. (1998) Comparative studies of class IIa bacteriocins of lactic acid bacteria. *Appl. Environ. Microbiol*. 64(9), 3275-3281.

Eijsink, V.G.H., Axelsson, L., Diep, D.B., Havarstein, L.S., Holo, H., Nes, I.F. (2002) Production of class II bacteriocins by lactic acid bacteria; an example of biological warfare and communication. *Antonie Van Leeuwenhoek* 81(1-4), 639-654.

El-Ziney, M.G., Uyttendaele, M., Debevere, J., Jakobsen, M. (1998) Characterization of growth and metabolite production of *Lb. reuteri* during glucose/glycerol cofermentation in batch and coutinuous cultures. *Biotechnol. Lett.* 20(10), 913-916.

Ennahar, S., Sashihar, T., Sonomoto, K., Ishizaki, A. (2000) Class IIa bacteriocins: biosynthesis, structure and activity. *FEMS Microbiol. Rev.* 24, 85-106.

Fimland, G., Axelsson, L., Brurberg, M.B., Nes, I.F., Eijsink, V.G., Nissen-Meyer, J. (2000) A C-Terminal disulfide bridge in pediocin-like bacteriocins renders bacteriocin activity less temperature dependent and is a pajor determinant of the antimicrobial spectrum. *J. bacterial.* 182, 2643-2648.

Fimland, G., Eijsink, V.G., Nissen-Meyer, J. (2002) Comparative studies of immunity proteins of pediocin-like bacteriocins. *Microbiology* 148, 3661-3670.

Flynn, S., Van Sinderen, D., Thornton, G.M., Holo, H., Nes, I.F., Collins, J.K. (2002) Characterization of the genetic locus responsible for the production of ABP-118, a novel bacteriocin produced by the probiotic bacterium *Lb. salivarius subsp. salivarius* UCC118. *Microbiology* 148, 973-984.

Garde, S., Carbonell, M., Fernandez-Garcia, E., Medina, M., Nunez, M. (2002) Volatile compounds in Hispanico cheese manufactured using a mesophilic starter, a thermophilic starter, and bacteriocin-producing *Lc. lactis subsp. lactis* INIA415. *J. Agri. Food Chem*. 50, 6752-6757.

Gahan, C., Hill, C. (1999) The relationship between acid stress responses and virulence in *Salmonella* Typhimurium and *L. monocytogenes*. *Int. J. Food Microbiol.* 50(1-2), 93-100.

Galvez, A., Abriouel, H., Lopez, R.L., Ben Oma, N. (2007) Bacteriocin-based strategies for food biopreservation. *Int. J. Food Microbiol.* 120(1-2), 51-70.

Gandhi, M., Chikindas, M. L. (2007) *Listeria*: A foodborne pathgen that knows how to survive. *Int. J. Food Microbiol.* 113, 1-15.

Garneau, S., Martin, N.I., Vederas, J.C. (2002) Two-peptide bacteriocins produced by lactic acid bacteria. *Biochimie* 84(5-6), 577-592.

Ghalfi, H., Allaoui, A., Destain, J., Benkerroum, N., Thonart, P. (2006a) Bacteriocin activity by *Lb. curvatus* CWBI-B28 to inactivate *L. monocytogenes* in cold-smoked salmon during 4 degrees C storage. *J. Food Prot.* 69, 1066-1071.

Ghalfi, H. (2006b) Sélection et utilisation des bactéries lactiques productrices de bactériocines anti-listeria comme bio-conservateur et caractérisation de trois bactériocines produites par *Lb. curvatus* CWBI-B28. Dissertation originale présentée en vue de l'obtention du grade de docteur en sciences agronomiques et ingénierie biologique, FUSAGx.

Ghalfi, H., Benkerroum, N., Doguiet, D.D., Delvigne, F.,Thonart, P. (2007a) Comparison of the performances of different fermentation strategies on cell growth and bacteriocin production by *Lb. curvatus* CWBI-B28. *J. Sci. Food Agr*. 87:541-549.

Ghalfi, H., Benkerroum, N., Doguiet, D.D.K., Bensaid, M., Thonart, P. (2007b) Effectiveness of cell-adsorbed bacteriocin produced by *Lb. curvatus* CWBI-B28 and selected essential oils to control *L. monocytogenes* in pork meat during cold storage. *Lett. Appl. Microbiol.* 44, 268-273.

Ghrairi, T., Manai, M., Berjeaud, J.M., Frère, J. (2004) Antilisterial activity of lactic acid bacteria isolated from rigouta, a traditional Tunisian cheese. *J. Appl. Microbiol*. 97, 621-628.

Gravesen, A., Ramnath, M., Rechinger, K.B., Andersen, N., Jänsch, L., Héchard, Y., Hastings, J.W., Knochel, S. (2002a). High-level resistance to class IIa bacteriocins is associated with one general mechanism in *L. monocytogenes*. *Microbiology* 148, 2361-2369.

Gravesen, A., Jydegaard, A., Mendes da Silva, J., Hansen, T.B., Knochel, S. (2002b) Frequency of Bacteriocin resistance development and associated fitness costs in *L. monocytogenes*. *Appl. Environ. Microbiol.* 68(2), 756-764.

Gravesen, A., Diao, Z., Voss, J., Budde, B., Knochel, S. (2004) Differential inactivation of *L. monocytogenes* by D- and L-lactic acid. *Lett. Appl. Microbiol.* 39, 528-532.

Guerra, N.P., Pastrana, L. (2003) Influence of pH drop on both nisin and pediocin production by *Lc. Lactis*, *Pediococcus acidilactici*. *Lett. Appl. Microbiol*. 37, 51-55.

Guerra, N.P., Araujo, A.B., Barrera, A.M., Agrasar, A.T., Macias, C.L., Carballo, J., Pastrana, L. (2005) Antimicrobial activity of nisin adsorbed to surfaces commonly used in the food industry. *J. Food Prot*. 68(5), 1012-1019.

Guinane, C.M., Cotter, P.D., Hill, C., Ross, P. (2005) A review - Microbial solutions to microbial problems; lactococcal bacteriocins for the control of undesirable biota of food. *J. Appl. Microbiol.* 98, 1316-1325.

Guinane, C.M., Cotter, P.D., Hill, C., Ross, P. (2006) Spontaneous resistance in *Lc. lactis* IL1403 to the lantibiotic lacticin 3147. *FEMS Microbiol. Lett.* 260(1), 77-83.

Guinane, C.M., Cotter, P.D., Lawton, E.M., Hill, C., Ross, P. (2007) Insertional mutagenesis to generate lantibiotic resistance in *Lc. lactis*. *Appl. Environ. Microbiol.* 73(14), 4677-4680.

Gursky, L.J., Martin, N.I., Derksen, D.J., van Belkum, M.J., Kaur, K., Vederas, J.C., Stiles, M.E., McMullen L., (2006) Production of piscicolin 126 by *Carnobacterium maltaromaticum* UAL26 is controlled by temperature and induction peptide concentration. *Arch. Microbiol.* 186, 317-325.

Héchard, Y., Pelletier, C., Cenatiempo, Y., Frère, J. (2001) Analysis of sigma(54)-dependent genes in *Enterococcus faecalis* : a mannose PTS permease (EII(Man)) is involved in sensitivity to a bacteriocin, mesentericin Y105. *Microbiology* 147, 1575-1580.

Héquet, A., Laffitte, V., Simon, L., De Sousa-Caetano, D., Thomas., C., Fremaux, C., Berjeaux, J.M., (2007) Characterization of new bacteriocinogenic lactic acid bacteria isolated using a medium designed to simulate inhibition of *Listeria* by *Lb. sakei* 2512 on meat. *Int. J. Food Microbiol*. 113, 67-74.

Herbin, S., Mathieu, F., Brulé, F., Branlant, C., Lefebvre, G., Lebrihi, A. (1997) Characteristics and genetic determinants of bacteriocin activities produced by *Carnobacterium piscicola* CP5 isolated from cheese. *Curr. Microbiol.* 35, 319-326.

Holck, A., Axelsson, L., Birkeland, S.E., Aukrust, T., Blom, H., (1992) Purification and amino acid sequence for sakacin A, a bacteriocin from *Lb. sake* Lb706. *J. Gen. Microbiol.* 138, 2715-2720.

Holck, A., Axelsson, L., Hühne, K., Kröckel, L. (1994) Purification and cloning of sakacin 674, a bacteriocin from *Lb. sake* LB674. *FEMS Microbiol. Lett.* 115:143-149.

Hsiao, C., Siebert, K. (1999). Modeling the inhibitory effects of organic acids on bacteria. *Int. J. Food Microbiol.* 47(3), 189-201.

Hüfner, E., Markieton, T., Chaillou, S., Crutz-Le Coq, A.M., Zagorec, M., Hertel, C. (2007) Identification of *Lb. sakei* genes induced during meat fermentation and their role in survival and growth. *Appl. Environ. Microbiol.* 73 (8), 2522-2531.

Hühne, K., Axelsson, L., Holck, A., Kröckel, L. (1996) Analysis of the sakacin P gene cluster from *Lactobacillus sake* LB674 and its expression in sakacin-negative *Lb. sakei* strains. *Microbiology* 142, 1437-1448.

Huss, V.A.R., Festl, H., Schleifer, K.H., (1983) Studies on the spectrophotometric determination of DNA hybridisation from renaturation rates. *Syst. Appl. Microbiol.* 4, 184-192.

Immonem, T., Saris, P.E. (1998) Characterization of the nisFEG operon of the nisin Z producing *Lc. lactis subsp. lactis* N8 strain. *DNA seq*. 9, 263-274.

Jack, T., Ray, B. (1995) Bacteriocins of Gram-Positive bacteria. *Microbiol. Rev.* 59, 171-200.

Jack, R.W., Wan, J., Gordon, J., Harmark, K., Davidson, B.E., Hillier, A.J., Wettenhall, R.E., Hickey, M.W., Coventery, M.J. (1996) Characterization of the chemical and antimicrobial properties of Piscicolin 126, a bacteriocin produced by *Carnobacterium piscicola* JG126. *Appl. Environ. Microbiol*. 62, 2897-2903.

Jacob, F., Siminovitch, L., Wollman, E. (1993) Comparison between the induced biosynthesis of colicine and of bacteriophage and between their mode of action. *Ann. Inst. Pasteur* 84(1), 313-318.

Janssen, M., Geeraerd, A.H., Cappuyns, A., Garcia-Gonzalez, L., Schockaert, G., Houteghem, N.V., Vereecken, K.M., Debevere, J., Devlieghere, F., Impe, J. (2007) Individual and combined effects of pH and lactic acid concentration on *L. innocua* inactivation: Development of a predictive model and assessment of Experimental Variability. *Appl. Environ. Microbiol.* 73(5), 1601-1611.

Johnsen, L., Fimland, G., Mantzilas, D., Nissen-Meyer, J. (2004) Structure-function analysis of immunity proteins of pediocin-like bacteriocins: C-terminal parts of immunité proteins are involved in specifric recognition of cognate bacteriocins. *Appl. Environ. Microbiol*. 70(5), 2647-2652.

Johnsen, L., Fimland, G., Nissen-Meyer, J. (2005) The C-terminal domain of Pediocin-like antimicrobial peptides (Class IIa bacteriocins) is involved in specific recognition of the C-terminal part of cognate immunity proteins and in determining the antimicrobial spectrum. *J. Biol. Chem.* 280, 9243-9250.

Katikou, P., Ambrosiadis, I., Georgantelis, D., Koidis, P., Georgakis, S.A. (2005) Effect of *Lactobacillus*-protective cultures with bacteriocin-like inhibitory substances' producing ability on microbiological, chemical and sensory changes during storage of refrigerated vacuum-packaged sliced beef. *J. Appl. Microbiol.* 99, 1303-1313.

Katla, T., Moretro, T., Holck, A., Axelsson, L., Naterstad, K. (2001) Inhibition of *L. monocytogenes* in cold smoked salmon by addition of sakacin P and/or live *Lb. sakei* cultures. *Food Microbiol.* 18, 431-439.

Katla, T., Moretro, T., Sveen, I., Aasen, I.M., Axelsson, L., Rorvik, L.M.,Naterstad, K. (2002) Inhibition of *L. monocytogenes* in chicken cold cuts by addition of sakacin P and sakacin P-producing *Lb. sakei*. *J. Appl. Microbiol.* 93, 191-196.

Kim, W.S., Dunn, N.W., (1997) Stabilization of the *Lc. lactis* nisin production transposon as a plasmid. *FEMS Microbiol. Lett.* 146, 285-289.

Klaenhammer, T.R. (1988) Bacteriocins of lactic acid bacteria. *Biochimie* 70, 337-349.

Klaenhammer, T.R. (1993) Genetics of bacteriocins produced by lactic acid bacteria. *FEMS Microbiol. Rev.* 12(1-3), 39-85.

Klaenhammer, T.R., Barrangou, R., Logan Buck, B., Azcarate-Peril, M.A. (2005) Genomic features of lactic acid bacteria effecting bioprocessing and health. *FEMS Microbiol. Rev.* 29, 393-409.

Kleerebezem, M. (2004) Quorum sensing control of lantibiotic production; nisin and subtilin autoregulate their own biosynthesis. *Peptides* 25, 1405-1414.

Kobilinsky, A., Nazer, A.I., Dubois-Brissonnet, F. (2007) Modeling the inhibition of *Salmonella* Typhimurium growth by combination of food antimicrobials. *Int. J. Food Microbiol.* 115, 95-109.

Kouakou, P., Ghalfi, H., Destain, J., Duboisdauphin, R., Evrard, P., Thonart, P. (2008) Enhancing the antilisterial effect of *Lb. curvatus* CWBI-B28 in pork meat and cocultures by limiting bacteriocin degradation. *Meat Sci.* In Press.

Kramer, N.E., van Hijum, S.A., Knol, J., Kok, J., Kuipers, O.P. (2006) Transcriptome analysis reveals mechanisms by which *Lc. lactis* acquires nisin resistance. *Antimicrob. Agents Chemother*. 50(5), 1753-1761.

Kullisaar, T., Zilmer, M., Mikelsaar, M., Vihalemm, T., Annuk, H., Kairane, C., Kilk, A. (2002) Two antioxidative Lactobacilli strains as promising probiotics. *Int. J. Food Microbiol.* 72(3), 215-224.

Kwaadsteniet, M., Todorov, S.D., Knoetze, H., Dicks, L.M.T., (2005) Characterization of a 3944 Da bacteriocin, produced by *Enterococcus mundtii* ST15, with activity against Gram-positive and Gram-negative bacteria. *Int. J. Food Microbiol.* 105, 433-444.

Leal-Sanchez, M.V., Jimenez-Diaz, R., Maldonado-Barragan, A., Garrido-Fernandez, A., Ruiz-Barba, J.L. (2002) Optimization of bacteriocin production by batch fermentation of *Lb. plantarum* LPCO10. *Appl. Environ. Microbiol.* 68, 4465-4471.

Lecuit, M. (2007). Human listeriosis and animal models. *Microbes Infect.* 9, 1216-1225.

Leisner, J.J., Laursen, B.G., Prévost, H., Drider, D., Dalgaard, P. (2007) Carnobacterium: positive and negative effects in the environment and in foods. *FEMS Microbiol. Rev*. 31, 592-613.

Leroy, F., De Vuyst, L. (1999) Temperature and pH conditions that prevail during fermentation of sausages are optimal for production of the antilisterial bacteriocin sakacin K. *Appl. Environ. Microbiol*. 65, 974-981.

Leroy, F., De Vuyst, L. (2001) Growth of the bacteriocin-producing *Lb. sakei* strain CTC494 in MRS broth is strongly reduced due to nutrient exhaustion : a nutrient depletion model for the growth of lactic acid bacteria. *Appl. Environ. Microbiol*. 67, 4407-4413.

Leroy, F., De Winter, T., Adriany, T., Neysens, P., De Vuyst, L. (2006) Sugars relevant for sourdough fermentation stimulate growth of and bacteriocin production by *Lb. amylovorus* DCE 471. *Int. J. Food Microbiol*. 12, 102-111.

Li, H., O'Sullivan, D.J. (2006) Identification of a nisI promoter within the nisABCTIP operon that may enable establishment of nisin immunity prior to induction of the operon via signal transduction. *J. Bacteriol*. 188(24), 8496-8503.

Liu, S. (2003) Practical implications of lactate and pyruvate metabolism by lactic acid bacteria in food and beverage fermentations. *Int. J. Food Microbiol.* 83(2), 115-131.

Luchansky, J.B., Call, J.E. (2004). Evaluation of nisin-coated cellulose casings for the control of *L. monocytogenes* inoculated onto the surface of commercially prepared frankfurters. *J. Food Protec.* 67, 1017-1021.

Lv, W., Zhang, X., Cong, W. (2005) Modelling the production of nisin by *Lc. lactis* in fed-batch culture. *Appl. Microbiol. Biotechnol.* 68, 322-326.

Makarova, K., Slesarev, A., Wolf, Y., Sorokin, A., Mirkin, B., Koonin, E., Pavlov, A., Pavlova, N., Karamychev, V., Polouchine, N., Shakhova, V., Grigoriev, I., Lou, Y., Rohksar, D., Lucas, S., Huang, K., Goodstein, D.M., Hawkins, T., Plengvidhya, V., Welker, D., Hughes, J., Goh, Y., Benson, A., Baldwin, K., Lee, J.H., Diaz-Muniz, I., Dosti, B., Smeianov, V., Wechter, W., Barabote, R., Lorca, G., Altermann, E., Barrangou, R., Ganesan, B., Xie, Y., Rawsthorne, H., Tamir, D., Parker, C., Breidt, F., Broadbent, J., Hutkins, R., O'Sullivan, D., Steele, J., Unlu, G., Saier, M. Klaenhammer, T., Richardson, P., Kozyavkin, S., Weimer, B., Mills, D. (2006) Comparative genomics of the lactic acid bacteria. *Proc. Natl. Acad. Sci.* 103, 15611-15616.

Makarova, K.S., Koonin, E.V. (2007) Evolutionary genomics of lactic acid bacteria. *J. Bacteriol.* 189, 1199-1208.

Makras, L., De Vuyst, L., (2006) The in vitro inhibition of Gram-negative pathogenic bacteria by *Bifidobacteria* is caused by the production of organic acids. *Int. Dairy J.* 16, 1049-1057.

Martinez, B., Suarez, J.E., Rodriguez, A. (1995) Antimicrobials produced by wild lactococcal strains isolated from homemade cheeses. *J. Food Protect*. 58, 118-123.

Mataragas, M., Metaxopoulos, J., Galiotou, M., Drosinos, E.H. (2003) Influence of pH and temperature on bacteriocin production by *Leuconostoc mesenteroides* L124 and *Lb. curvatus* L442. *Meat Sci*. 64, 265-271.

Mataragas, M., Drosinos, E.H., Tsakalidou, E., Metaxopoulos, J. (2004) Influence of nutrients on growth and bacteriocin production by *Leuconostoc mesenteroides* L124 and *Lb. curvatus* L442. *Antonie van Leeuwenhoek* 85, 191-198.

Mathiesen, G., Huehne, K., Kroeckel, L., Axelsson, L., Eijsink, H. (2005) Characterization of a new bacteriocin operon in sakacin P-producing *Lb. sakei*, showing strong translational coupling between the bacteriocin and immunity genes. *Appl. Environ. Microbiol*. 71 (7), 3565-3574.

Matsusaki, H., Endo, N., Sonomoto, K., Ishizaki, A. (1996) Lantibiotic nisin Z fermentative production by *Lc. lactis* IO-1: relationship between production of the lantibiotic and lactate and cell growth. *Appl. Microbiol. Biotechnol*. 45, 36-40.

McAuliffe, O., Hill, C. (2001) Lantibiotics: structure, biosynthesis and mode of action. *FEMS Microbiol. rev.* 25, 285-308.

Meghrous, J., Lacroix, C., Bouksaïm, M., LaPointe, G., Simard, R.E. (1997) Note : Genetic and biochemical characterization of nisin Z produced by *Lc. lactis spp. lactis biovar. diacetylactis* UL719. *J. Appl. Microbiol*. 83, 133-138.

Messens, W., De Vuyst, L., (2002) Inhibitory substances produced by *Lactobacilli* isolated from sourdoughs – a review. *Int. J. Food Microbiol*. 72, 31-43.

Messens, W., Verluyten, J., Leroy, F., De Vuyst, L. (2003) Modelling growth and bacteriocin production by *Lb. curvatus* LTH1174 in response to temperature and pH values used for European sausage fermentation processes. *Int. J. Food Microbiol.* 81, 41-52

Miller, R.W., Ray, P., Steinmetz, T., Hanekamp, T., Ray, B. (2005). Gene organization and sequences of pediocin AcH/PA-1 production operons in *Pediococcus* and *Lactobacillus* plamids. *Lett. Appl. Microbiol*. 40(1), 56-62.

Mitra, S., Chakrabartty, P.K., Biswas, S.R. (2007) Production of nisin Z by *Lc. lactis* isolated from dahi. *Appl. Biochem. Biotechnol*. 143, 41-53.

Morgan, S.M., O'Connor, P.M., Cotter, P.D., Ross, R.P., Hill, C. (2005) Sequential actions of the two component peptides of the lantibiotic lacticin 3147 explain its antimicrobial activity at nanomolar concentration. *Antimicrob. Agents Chemother.* 49(7), 2606-2611.

Moretro, T., Aasen, I.M., Storro, I., Axelsson, L. (2000) Production of sakacin P by *Lb. sakei* in a completely defined medium. *J. Appl. Microbol.* 88(3), 536-545.

Moretro, T., Naterstad, K., Wang, E., Aasen, I.M., Chaillou, S., Zagorec, M., Axelsson, L. (2005) Sakacin P non-producing *Lb. sakei* strains contain homologues of the sakacin P gene cluster. *Res. Microbiol.* 156(9):949-960.

Moschetti, G., Villani, F., Blaiotta, G., Baldinelli, A., Coppola, S. (1996) Presence of non-functional nisin genes in *Lc. lactis subsp. lactis* isolated from natural starters. *FEMS Microbiol. Lett.* 145, 27-32.

Mulders, J.W.M., Boerrigter, I.J., Rollema, S., Siezen, de Vos W.M. (1991) Identification and characterization of the lantibiotic nisin Z, a natural nisin variant. *Europ. J. Biochem*. 201, 581-584.

Naghmouchi, K., Drider, D., Kheadr, E., Lacroix, C., Prévost, H., Fliss, I., (2006) Multiple characterizations of *L. monocytogenes* sensitive and insensitive variants to divergicin M35, a new pediocin-like bacteriocin. *J. Appl. Microbiol*. 100, 29-39.

Naghmouchi, K., Kheadr, E., Lacroix, C., Fliss, I. (2007) Class I/ class IIa bacteriocin cross-resistance phenomenon in *L. monocytogenes*. *Food Microbiol.* 24, 718-727.

Nel, H.A., Bauer, R., Vandamme, E.J., Dicks, L.M. (2001) Growth optimization of *Pediococcus damnosus* NCFB1832 and the influence of pH and nutrients on the production of pediocin PD-1. *J. Appl. Microbiol.* 91, 1131-1138.

Nes, I.F., Johnsborg, O., (2004) Exploration of antimicrobial potential in LAB by genomics. *Curr. Op. Biotechnol*. 15, 100-104.

Nga, B.H., (2005) Genome analysis of lactic acid bacteria in food fermentations and biotechnological applications. *Curr. Op. Biotechnol*. 8, 307-312.

Nilsen, T., Nes, I.F., Holo, H. (2003) Enterolysin A, a cell wall-degrading bacteriocin from *Enterococcus faecalis* LMG2333. *Appl. Environ. Microbiol*. 69(5), 2975-2984.

Nilsson, L., Nielsen, M.K., Ng, Y., Gram, L., (2002) Role of acetate in production of an autoinducible class IIa bacteriocin in *Carnobacterium piscicola* A9b. *Appl. Environ. Microbiol*. 68 (5), 2251-2260.

Nilsson, L., Ng, Y.Y., Christiansen, J.N., Jorgensen, B.L., Grotinum, D., Gram, L., (2004) The contribution of bacteriocin to inhibition of *L. monocytogenes* by *Carnobacterium piscicola* strains in cold-smoked salmon systems. *J. Appl. Microbiol.* 96, 133-143.

Nigutova, K., Morovsky, M., Pristas, P., Teather, R.M., Holo, H., Javorsky, P. (2007) Production of enterolysin A by rumen *Enterococcus faecalis* strain and occurrence of enlA homologues among ruminal Gram-positive cocci. *J. Appl. Microbiol*. 102(2), 563-569.

Noonpakdee, W., Santivarangkna, C., Jumriangrit, P., Sonomoto, K., Panyim, S., (2003) Isolation of nisin-producing *Lc. lactis* WNC20 strain from nham, a traditional thai fermented sausage. *Int. J. Food Microbiol*. 81, 137-145.

Ochman, H., Gerber, A.S., Hartl, D.L. (1988) Genetic applications of an inverse polymerase chain reaction. *Genetics* 120, 621-623.

Olasupo, N.A., Schillinger, U., Narbad, A., Dodd, H., Holzapfel, W. (1999) Ocurrence of nisin Z production in *Lc. lactis* BFE 1500 isolated from wara, a traditional Nigerian cheese product. *Int. J. Food Microbiol*. 53, 141-152.

Oppegard, C., Rogne, P., Emanuelsen, L., Kristiansen, P.E., Fimland, G., Nissen-Meyer, J. (2007) The two-peptide class II bacteriocins: structure, production and mode of action. *J. Mol. Microbiol. Biotechnol*. 13(4), 210-219.

Otero, M.C., Nader-Macias, M.E. (2006) Inhibition of *Staphylococcus aureus* by H_2O_2 producing *Lb.s gasseri* isolated from the vaginal tract of cattle. *Anim. Reprod. Sci.* 96, 35-46.

Paludan-Muller, C., Henrik Huss, H., Lone, G.R. (1999) Characterization of lactic acid bacteria isolated from a Thai low-salt fermented fish product and the role of garlic as substrate for fermentation. *Int. J. Food Microbiol.* 46, 219-229.

Papagiannin, M. (2003) Ribosomally synthesized peptides with antimicrobial properties: biosynthesis, structure, function and applications. *Biotechnol. Adv*. 21(6), 465-499.

Patton G.C., Van Der Donk, W.A (2005). New developments in lantibiotic biosynthesis and mode of action. *Curr. Op. Microbiol.* 8, 543-551.

Parente, E., Ricciardi, A. (1999) Production, recovery and purification of bacteriocins from lactic acid bacteria. *Appl. Microbiol. Biotechnol.* 52, 628-638.

Park, S.H., Itoh, K., Kikuchi, E., Niwa, H., Fujisawa, T. (2003) Identification and characteristics of nisin Z-producing *Lc. lactis subsp. lactis* isolated from Kimchi. *Curr. Microbiol.* 46, 385-388.

Pitcher, D.G., Saunters, N.A., Owen, R.H. (1989) Rapid extraction of bacterial genomic DNA with guanidium thiocyanate. *Lett. Appl. Microbiol.* 8, 151-156.

Pongtharangkul, T. and Demirci, A. (2006a) Effects of pH profiles on nisin production in biofilm reactor. *Appl. Microbiol. Biotechnol*. 71, 804-811.

Pongtharangkul, T., Demirci, A. (2006b) Evaluation of culture medium for nisin production in a repeated-batch biofilm reactor. *Biotechnol. Prog.* 22, 217-224.

Quadri, L.E., Sailer, M., Roy, K.L., Vederas, J.C., Stiles, M.E. (1994) Chemical and genetic characterization of bacteriocins produced by *Carnobacterium piscicola* LV17B. *J. Biol. Chem.* 269 (16), 12204-12211.

Quadri, L.E.N., Kleerebezem, M., Kuipers, O.P., De Vos, W.M., Roy, K.L., Vederas, J.C., Stiles, M.E. (1997) Characterization of a locus from *Carnobacterium piscicola* LV17B involved in bacteriocin production and immunity: evidence for global inducer-mediated transcriptional regulation. *J. Bacteriol.* 179 (19), 616-6171.

Richard C., Canon R., Naghmouchi K., Bertrand D., Prévost H., Drider D. (2006) Evidence on correlation between number of disulfide bridge and toxicity of class IIa bacteriocins. *Food Microbiol.* 23(2), 175-183.

Rodhe, B.H., Quadri, L.E.N. (2006) Functional characterization of a three-component regulatory system involved in quorum sensing-based regulation of peptide antibiotic production in *Carnobacterium maltaromaticum*. *BMC Microbiol.* 6, 93-102.

Rodgers, S. (2001) Preserving non-fermented refrigerated foods with microbial cultures – a review. *Trends Food Sci. Technol.* 12, 276-284.

Rodgers, S. (2003) Potential applications of protective culture in cook-chill catering. *Food Control* 14, 35-42.

Rodgers, S. (2004). Novel approaches in controlling safety of cook-chill meals. *Trends Food Sci. Technol.* 15, 366-372.

Rose, N., Palcic, M.M., Sporns, P., McMullen, L.M. (2002) Nisin: A novel substrate for glutathione S-transferase isolated from fresh beef. *J. Food Sci.* 67, 2288-2293.

Sakamoto, M., Tano, Y., Uchimura, T. Komagata, K. (1998) Aerobic growth of some lactic acid bacteria enabled by the external addition of peroxidase (horseradish) to the culture medium. *J Fermentation and Bioengineering* 85(6), 627-629.

Sambrook, J., Russel, D.W. (2001) Molecular cloning : a laboratory manual, 3rd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

Sanchez, J., Diep, D., Herranz, C., Nes, I., Cintas, L., Hernandez, P. (2007). Amino acid and nucleotide sequence, adjacent genes and heterologous expression of hiracin JM79, a sec-dependent bacteriocin produced by *Enterococcus hirae* DCH5, isolated from Mallard ducks (*Anas platyrhynchos*). *FEMS Microb. Lett*. 270, 227-236

Savijoki, K., Ingmer, H., Varmanen, P. (2006) Proteolytic systems of lactic acid bacteria. *Appl. Microb. Biotech.* 71, 394-406.

Scannell, A.G., Hill, C., Ross, R.P., Marx, S., Hartmeier, W., Arendt, E.K. (2000) Continuous production of lacticin 3147 and nisin using cells immobilized in calcium alginate. *J. Appl. Microbiol.* 89, 573-579.

Schägger, H., Von, Jagow, G. (1987) Tricine-Sodium dodecyl sulfate polyacrylamide gel electrophoresis for the separation of protein in the range from 1 to 100 kDa. *Anal. Biochem*. 166(2):368-379.

Schöbitz, R., Zaror, T., Leon, O., Costa, M., (1999) A bacteriocin from *Carnobacterium piscicola* for the control of *L. monocytogenes* in vacuum-packaged meat. *Food Microbiol.* 16, 249-255.

Schöbitz, R., Suazo, V., Costa, M., Ciampi, L. (2003) Effects of a bacteriocin-like inhibitory substance from *Carnobacterium piscicola* against human and salmon isolates of *L. monocytogenes*. *Int. J. of Food Microb*. 84, 237-244.

Simon, L., Fremaux, C., Cenatiempo, Y., Berjeaud, J.M., (2002) Sakacin G, a new type of antilisterial bacteriocin. *Appl. Environ. Microbiol*. 68 (12) 6416-6420.

Stein, T., Heinzmann, S., Solovieva, I., Entian, K.D. (2003) Function of *Lc. lactis* nisin immunity genes nisI and nisFEG after coordinated expression in the surrogate host *Bacillus subtilis*. *J. Biol. Chem.* 278(1), 89-94.

Straume, D., Kjos, M., Nes, I.F., Diep, D.B. (2007) Quorum-sensing based bacteriocin production is down-regulated by N-terminally truncated species of gene activators. *Mol. Genet. Genomics* 278(3), 283-293.

Strus, M., Gosiewski, T., Kochan, P., Heczko, P.B. (2006) The in vitro effect of hydrogen peroxide on vaginal microbial communities. *FEMS Immuno. Medical Microbiol.* 48, 56-63.

Simon, L., Fremaux, C., Cenatiempo, Y., Berjeaud, J.M. (2002) Sakacin G, a new type of antilisterial bacteriocin. *Appl. Environ. Microbiol*. 68, 6416-6120.

Tagg, J.R., Dajani, A.S., Wannamaker, L.W. (1976) Bacteriocins of Gram-positive bacteria. *Bacteriol. Rev*. 40(3), 722-756.

Tahiri, I., Desbiens, M., Benech, R., Kheadr, E., Lacroix, C., Thibault, S., Ouellet, D., Fliss, I., (2004) Purification, characterization and amino acid sequencing of divergicin M35: a novel class IIa bacteriocin produced by *Carnobacterium divergens* M35. *Int. J. Food Microbiol.* 97, 123-136.

Takala, T.M., Saris, P.E. (2006) C-terminus of NisI provides specificity to nisin. *Microbiology* 152, 3543-3549.

Tichaczek, P.S., Vogel, R.F., Hammes, W.P. (1994) Cloning and sequencing of sakP encoding sakacin P, the bacteriocin produced by *Lb. sake* LTH 673. *Microbiology* 140, 361-367.

Todorov, S.D., Dicks, L.M. (2004) Influence of growth conditions on the production of a bacteriocin by *Lc. lactis subsp. lactis* ST34BR, a strain isolated from barley beer. *J. Basic Microbiol.* 44, 305-316.

Twomey, D., Ryan, M., Meaney, B., Hill, C. (2002) Lantibiotics produced by lactic acid bacteria: structure, function and applications. *Antonie van Leeuwenhoek* 82, 165-185.

Urso, R., Rantisou, K., Cantoni, C., Comi, G., Cocolin, L. (2006a) Sequencing and expression analysis of the sakacin P bacteriocin produced by a *Lb. sakei* strain isolated from naturally fermented sausages. *Appl. Genet. Molec. Biotechnol*. 71, 480-485.

Urso, R., Rantsiou, K., Cantoni, C., Comi, G., Cocolin, L. (2006b) Technological characterization of a bacteriocin-producing *Lb. sakei* and its use in fermented sausages production. *Int. J. Food Microbiol.* 110, 232-239.

Vadyvaloo, V., Hastings, J.W., van der Merwe, M.J., Rautenbach, M. (2002) Membranes of class IIa Bacteriocin-resistant *L. monocytogenes* cells contain increased levels of desaturated and short-acyl-chain phophatidylglycerols. *Appl. Environ. Microbiol.* 68(11), 5223-5230.

Vadyvaloo, V., Arous, S., Gravesen, A., Héchard, Y., Chauhan-Haubrock, R., Hastings, J.W., Rautenbach, M. (2004a) Cell-surface alterations in class IIa bacteriocin-resistant *L. monocytogenes* strains. *Microbiology* 150, 3025-3033.

Vadyvaloo, V., Snoep, J.L., Hasting, J.W., Rautenbach, M. (2004b) Physiological implications of class IIa bacteriocin resistance in *L. monocytogenes* strains. *Microbiology* 150, 335-340.

Van Wely, K., Swaving, J., Freudl, R., Driessen,A. (2001). Translocation of proteins across the cell envelope of Gram-positive bacteria. *FEMS Microbiol. Rev*. 25, 437-454.

Vaughan, A., Eijsink, V.G., O'Sullivan, T.F., O'Hanlon, K., van Sinderen, D. (2001) An analysis of bacteriocins produced by lactic acid bacteria isolated from malted barley. *J. Appl. Microbiol*. 91, 131-138.

Vaughan, A., O'Mahony, J., Eijsink, V.G.H., O'Connel-Motherway, M., van Sinderen, D., (2004) Transcriptional analysis of bacteriocin production by malt isolate *Lb. sakei* 5. *FEMS Microbiol. Lett*. 35, 377-384.

Venema, K., Dost, M.H.R., Beun, P.A.H., Haandrikman, A.J., Venema, G., Kok, J. (1996) The genes for secretion and maturation of lactococcins are located on the chromosome of *Lc. lactis* IL1403. *Appl. Environ. Microbiol.* 62, 1689-1692.

Ventura, M., Elli, M., Reniero, R., Zink, R., (2001) Molecular microbial analysis of *Bifidobacterium* isolates from different environments by the species-species amplified ribosomal DNA restriction analysis (ARDRA). *FEMS Microbiol. Ecol.* 36, 113-121.

Verluyten, J., Leroy, F., De Vuyst, L. (2004) Influence of complex nutrient source on growth of and curvacin A production by sausage isolated *Lb. curvatus* LTH 1174. *Appl. Environ. Microbiol.* 70, 5081-5088.

Vermeiren, L., Devlieghere, F., Debevere, J. (2004) Evaluation of meat born lactic acid bacteria as protective cultures for the biopreservation of cooked meat products. *Int. J. Food Microbiol.* 96(2), 149-164.

Vermeiren, L., Devlieghere, F., Debevere, J. (2006a) Co-culture experiments demonstrate the usefulness of *Lb. sakei* 10A to prolong the shelf-life of a model cooked ham. *Int. J. Food Microbiol.* 108, 68-77.

Vermeiren, L., Devlieghere, F., Vandekinderen, I. Debevere, J. (2006b) The interaction of the non-bacteriocinogenic *Lb. sakei* 10A and lactocin S producing *Lb. sakei* 148 towards *L. monocytogenes* on a model cooked ham. *Food Microbiol.* 23, 511-518.

Vignolo, G., Palacios, J., Farias, M., Sesma, F., Schillinger, U., Holzapfel, W., Oliver, G. (2000) Combined effect of bacteriocins on the survival of various *Listeria* species in broth and meat system. *Curr. Microbiol.* 41, 410-416.

Vollenweider, S. (2004) 3-hydroxypropionaldehyde: applications and perspectives of biotechnological production. *Appl. Microbiol. Biotech.* 64, 16-27.

Wiedemann, I., Böttiger, T., Bonelli, R.R., Wiese, A., Hagge, S.O., Gutsmann, T., Seydel, U., Deegan, L., Hill, C., Ross, P., Sahl, H.G. (2006) The mode of action of the lantibiotic lacticin 3147 - a complex mechanism involving specific interaction of two peptides and the cell wall precursor lipid II. *Mol. Microbiol.* 61(2), 285-296.

Wijaya, A., Neudeker, C., Holzapfel, W., Franz, C.M.A.P. (2006) Influence of bacteriocin-producing *Enterococcus faecalis* BFE 1071 on *Lactobacillus* spp. in the rat gastrointestinal tract. *Proceedings of Food Micro,* Bologna, August 2007, p. 124.

Willey, J.M., van der Donk, W.A. (2007) Lantibiotics: peptides of diverse structure and function. *Annu. Rev. Microbiol.* 61, 477-501.

Worobo, R.W., Henke, T., Sailer, M., Roy, K.L., Vederas, J.C., Stiles, M.E. (1994) Characteristics and genetic determinant of a hydrophobic peptide bacteriocin, carnobacteriocin A, produced by *Carnobacterium piscicola* LV17A. *Microbiology* 140, 517-26.

Xie, L., van der Donk, W.A. (2004) Post-translational modifications during lantibiotic biosynthesis. *Curr. Opin. Chem. Biol*. 8(5), 498-507.

Yang, R., Ray, B. (1994) Factors influencing production of bacteriocins by lactic acid bacteria. *Food Microbiol.* 11, 281-291.

Zalan, Z., Barath, A., Halasz, A. (2005) Influence of growth medium on hydrogen peroxide and bacteriocin production of *Lactobacillus* strains. *Food Technol. Biotech.* 43(3), 219-225.

Zamfir, M., Vancanneyt, M., Madras, L., Vaningelgem, F., Lefebvre, K., Pot, B., Swings, J., De Vuyst, L., (2006) Biodiversity of lactic acid bacteria in Romanian dairy products. *Syst. Appl. Microbiol.* 29, 487-495.

Chapitre 8 Liste des publications et activités scientifiques

8.1 Publications

Réalisées dans le cadre de la thèse

Dortu, C., Franz, C., Kostinek, M., Schillinger, U., Holzapfel, W., Thonart, P. Genetic characterization of bacteriocin production by lactic acid bacteria from foods and application of producer cultures for biopreservation. Submitted in Journal of basic Microbiology

Dortu, C., Fickers, P., Ndagano, D., Bernard, J., Thonart, P. Characterisation of an antilisterial bacteriocin produced by *Lactobacillus sakei* CWBI-B1365 isolated from raw poultry meat and determination of some factors controlling its production. Submitted in Appl. Environm. Microbiol.

Dortu, C., Huch, M., Holzapfel, W., Franz, C., Thonart, P. Anti-Listerial activity of bacteriocin-producing *Lactobacillus curvatus* CWBI-B28 and *Lactobacillus sakei* CWBI-B1365 on raw beef and poultry meat. Submitted in Lett. Appl. Microbiol.

Dortu, C., Thonart, P. Bacteriocin from lactic acid bacteria: characteristics and interest for food preservation. Review article accepted in BASE.

Réalisées en dehors du cadre de la thèse

Kostinek, M. Specht, I., Edward, V.A., Pinto, C., Egounlety, M., Sossa, C., Mbugua, S., Dortu, C., Thonart, P., Taljaard, L., Mengu, M., Franz, C.M., Holzapfel, W.H. 2007. Characterisation and biochemical properties of predominant lactic acid bacteria from fermenting cassava for selection as starter culture. Int. J. Food Microbiol. 114(3), 342-351.

8.2 Communications à des congrès

Réalisées dans le cadre de la thèse

Dortu, C., Thonart, P. 2004. Evaluation of food born lactic acid bacteria for their use as protective cultures in food. Bioforum, Liège, Belgium

Dortu C., Franz, C., Kostinek, M., Holzapfel, W., Thonart P. 2006. Selection and characterization of bacteriocin producing lactic acid bacteria for their use as protective culture on poultry meat. "14° colloque du club des bactéries lactiques", Paris.

Dortu, C., Franz, C., Kostinek, M., Holzapfel, W., Thonart, P. 2006. Characterization of bacteriocins from lactic acid bacteria. "FoodMicro 2006", Bologna, Italy.

Dortu, C., Ndagano, D., Thonart, P. 2007. Impact of culture conditions on growth and bacteriocin production by *Lactobacillus sakei* CWBI-B1365. "15° colloque du club des bactéries lactiques", Rennes.

Dortu, C., Ndagano, D., Thonart, P. 2008. Impact of different factor on bacteriocin production by *Lactobacillus sakei* CWBI-B1365. "9th symposium on lactic acid bacteria" Egmond aan Zee, The Netherland.

Réalisées en dehors du cadre de la thèse

Dortu, C., Thonart, P., Pinto, C., Edward, V., Yao, A., Egounlety, M., Mbugua, S., Kostinek, M., Franz, C., Holzapfel, W., Mengu, M., Aaen, T. 2006. Improving the quality and nutritional status of Gari through the use of starter cultures and fortification with soybean, palm oil and coconut milk. "FoodMicro2006". Bologna, Italy.

Thonart, P., Dortu, C., Yao, A., Edward, V., Pinto, C., Egounlety, M., Kostinek, M., Franz, C., Mbugua, S., Mengu, M., Holzapfel, W. 2006. Selection of *Lactobacillus plantarum* strains for their use as starter cultures during fortified cassava fermentation for the production of gari. "FoodMicro 2006", Bologna, Italy.

Michel B. Diop, Carine M. Dortu, Abib Ngom, Emmanuel Tine, Philippe Thonart. 2006. Selection of bacteriocin-producing lactic acid bacteria from Senegalese traditional fermented food for use as bio-preservative in fish. "FoodMicro2006", Bologna, Italy.

Ndagano, D., Dortu, C., Thonart, P. 2008. Study of antifungal activity of lactic acid bacteria isolated from fermented cassava and mill flour. “9th symposium of lactic acid bacteria”, Egmond aan Zee, The Netherland.

8.3 Diplômes complémentaires, expérience et stages depuis l'obtention du diplôme de second cycle

Diplômes complémentaires

2003-2005 : Diplôme complémentaire en économie et gestion, orientation gestion – Université de Liège.

2002-2003 : Diplôme d'étude approfondie en Agronomie et ingénierie biologique préparatoire au doctorat - FUSAGx

Expérience

Novembre 2004 à Octobre 2008: Assistante de recherche, CWBI: « Développement d'une culture starter pour inhiber *Listeria monocytogenes* dans des produits viandeux ».

Janvier 2003 à Octobre 2004: Assistante de recherche, CWBI : « Augmentation de la qualité hygiénique et nutritionnel du Gari, une farine de manioc fermentée, par l'utilisation d'une culture starter ».

Novembre 2002 à Décembre 2002 : Assistante de recherche, CWBI : « Propriétés industrielles des microorganismes ».

Stages

15 septembre 2005 au 15 Mars 2006 : Stage au « Institut für Hygiene und Toxikologie » Karlsruhe, Allemagne.

Septembre 2004 : Mission d'un mois au « Council for Scientific and Industrial Research » à Johannesburg (Afrique du Sud).