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Muhammad Inam AFZAL

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Sujet

Physiologie et aspects technologiques de *Carnobacterium maltaromaticum* LMA 28 en biopréservation alimentaire

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Rapporteurs :

Mme Marie-France PILET, Maître de Conférences, HDR, SECALIM, Nantes, France
M. Samuel LUBBERS, Maître de Conférences, HDR, AgroSupDijon, Dijon, France

Examinateurs :

M. Pascal DEGRAEVE, Professeur, IUT Lyon 1, Bourg-en-Bresse, France
Mme Anne-Marie REVOL-JUNELLES, Professeur, UL, Nancy, France
M. Joël SCHER, Professeur, UL, Nancy, France
Mme Catherine CAILLIEZ-GRIMAL, Maître de Conférences HDR (Directeur de thèse), UL, Nancy, France

Invités :

Mme Françoise IRLINGER, Ingénieur-Docteur en Microbiologie, Agro-Paris Tech, Thiverval-Grignon, France
M. Stéphane DELAUNAY, Professeur, (Co-directeur de thèse), UL, Nancy, France

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Liste des abréviations

°C	:	degré celsius
α-KG	:	alpha cétoglutarate
AAs	:	acides aminés
ACK	:	acylkinase
AlcDH	:	alcool déshydrogénase
AldDH	:	aldéhyde déshydrogénase
AOP	:	Appellation d'Origine Protégée
AraT	:	aminotransférase spécifique des acides aminés aromatique
AT	:	aminotransférase spécifique des acides aminés branchés
A_w	:	activité de l'eau
B2	:	carnobactériocine B2
BET	:	bromure d'éthidium
BLBVB	:	bouillon lactosé bilié vert brillant
BM1	:	carnobactériocine BM1
bp	:	paire de base
C	:	cytosine
Ca	:	calcium
Cbn A	:	carnobactériocine A
Ccn A	:	carnocycline A
CFE	:	extrait acellulaire
CFU	:	unité formant colonie
CIP	:	collection de l'Institut Pasteur
CitP	:	citrate perméase
CitL	:	citrate lyase
cm, mm, µm	:	centimètre, millimètre, micromètre
CNRZ	:	centre national de la recherche zootechnique,
CTAS	:	rouge de crésol saccharose acétate de thallium
CTSI	:	rouge de crésol saccharose acétate de thallium inuline
D	:	détecté
DGGE	:	électrophorèse sur gel en gradient dénaturant
DNA	:	acide désoxyribonucléique
DOC	:	concentration d'oxygène dissous
DSM	:	deutsche sammlung von mikro-organismen und zellkulturen
Dvn 750	:	divergicine 750
Dvn A	:	divergicine A
EBRER	:	bouillon à base de ribose d'esculine et de rouge de phénol
EDTA	:	acide éthylène diamine tétra acétique
E_h	:	potentiel redox
EMC	:	enzyme modified cheese powder
ENSAIA	:	Ecole Nationale Supérieure d'Agronomie et des Industries Alimentaires
FT-IR	:	spectroscopie infrarouge à transformée de Fourier
G	:	guanine

g, mg, µg	:	gramme, milligramme, microgramme
GDH	:	glutamate déshydrogénase
GRAS	:	generally recognized as safe
h	:	heure
HADH	:	acide hydroxy déshydrogénase
HCl	:	acide chlorhydrique
HS-GC	:	chromatographie en phase gazeuse
INPL	:	Institut National Polytechnique de Lorraine
INRA	:	Institut National de Recherche Agronomique
KADC	:	cétoacide décarboxylase
KADH	:	cétoacide déshydrogénase
L, mL, µL	:	litre, millilitre, microlitre
LAB	:	bactérie lactique
LB	:	lysogénie bouillon
LDH	:	lactate déshydrogénase
LMA	:	Laboratoire de Microbiologie Alimentaire
M, mM, µM	:	mole, millimole, micromole
min	:	minute
NaCl	:	chlorure de sodium
NAD	:	nicotinamide adénine dinucléotide
NADP	:	nicotinamide adénine dinucléotide phosphate
ND	:	non détecté
OD	:	densité optique
PCR	:	réaction en chaîne par polymérase
PDH	:	pyruvate déshydrogénase
PFGE	:	électrophorèse en champ pulsé
PTA	:	phosphotransférase
rRNA	:	acide ribonucléique ribosomique
Rpm	:	rotation par minute
s	:	seconde
SLCC	:	collection spéciale de cultures de *Listéria*
sp	:	species
ssp	:	subspecies
T_m	:	temps maximal
TRIS	:	2-amino-2-hydroxyméthyl-1, 3-propanediol
TS	:	tryptone sel
TSA	:	agar trypcase soya
TSB	:	bouillon trypcase soya
TTGE	:	temporal temperature gradient gel electrophoresis
UV	:	ultraviolet
v/v	:	volume par volume
V	:	volt
V_m	:	vitesse maximale
YE	:	extrait de levure

Les genres bactériens

B	:	*Brochothrix*
C	:	*Carnobacterium*
E	:	*Escherichia*
E	:	*Enterococcus*
L	:	*Listeria*
L/Lb	:	*Lactobacillus*
L/Lc	:	*Lactococcus*
P	:	*Propionibacterium*
P	:	*Penicillium*
Ps	:	*Pseudomonas*
S	:	*Staphylococcus*
S	:	*Streptococcus*

Introduction

Introduction

Le projet scientifique du Laboratoire d'Ingénierie des Biomolécules (LIBio) concerne la science pour **l'élaboration, la structuration et la stabilisation de biomolécules d'intérêt**. Le choix des molécules actives repose sur leur intérêt nutritionnel ou sur leur importance dans la sécurité des aliments. C'est dans ce domaine que s'inscrivent les recherches de l'équipe de microbiologie du LIBio. Celles-ci sont axées sur la « compréhension des mécanismes d'action des bactériocines » produites par des bactéries lactiques ainsi que sur l'étude de « **l'adaptation de flores d'intérêt »** aux procédés agro-alimentaires. L'utilisation de souches d'intérêt technologique productrices de bactériocines permettra d'étudier leur impact sur la dynamique de diverses communautés microbiennes au sein de matrices alimentaires.

Les travaux entrepris ces dernières années au laboratoire ont permis de mettre en évidence le genre *Carnobacterium* dans des fromages à pâte molle en fin d'affinage, après stockage au froid. Ce genre bactérien a fait l'objet de plusieurs travaux dont les travaux de thèse de Edima (2007) et Jasniewski (2008) au sein du laboratoire et est, de ce fait, devenu pour le LIBio un modèle d'étude des flores d'intérêt dans des matrices alimentaires de type fromages à pâte molle.

La microbiologie du fromage ainsi que le rôle des bactéries lactiques dans la fermentation du lait ont fait l'objet de nombreuses recherches. Aujourd'hui, cette fermentation est largement réalisée par l'inoculation de levains commerciaux. En outre, il est reconnu depuis plus de cinquante ans que de nombreux fromages contiennent une population élevée (10^6-10^9 ufc.mL^{-1}) de micro-organismes qui ne sont pas des bactéries lactiques ajoutés comme starters de la fermentation lactique (Fleet, 1999). Ces bactéries lactiques dites « sauvages », naturellement présentes dans le lait de fabrication du fromage ou apportées par l'ambiance des ateliers de fabrication, sont généralement mésophiles homo- ou hétérofermentaires. Cette flore tolère l'environnement hostile de l'affinage, caractérisé par un faible taux d'humidité dans le produit (61 à 68 % de matière sèche), 1 à 2 % de sel, un pH variant de 4,9 à 5,3 et une déficience en nutriments (Morea et al., 2007). Cette flore lactique fortuite, généralement désignée sous le nom de Non Starter Lactic Acid Bacteria (NSLAB) est habituellement présente suite à une

contamination après pasteurisation, mais constitue également une partie de la microflore du lait qui résiste à cette pasteurisation. De plus, de nombreuses levures et/ou des moisissures colonisent la surface du fromage. Ces micro-organismes sont généralement considérés comme agissant positivement sur le procédé de maturation car ils apportent des flaveurs spécifiques au fromage (Irlinger et Mounier, 2009). Ainsi, la plupart des fromages hébergent un écosystème complexe, caractérisé par une succession de groupes microbiens différents apparaissant pendant la fermentation du lait, la maturation du caillé ou le stockage.

Il a été démontré au LIBio que *Carnobacterium maltaromaticum* fait partie de cette microflore complexe des fromages. Elle constitue une part des NSLAB et présente à des concentrations élevées (10^8 ufc.mL^{-1}) en fin de fabrication, elle jouerait un rôle dans l'affinage des fromages à pâte molle (Millière et Lefebvre, 1994; Millière et al., 1994; Cailliez-Grimal et al., 2005, 2007).

Présente dans le lait cru, mais peu acidifiante, l'espèce *C. maltaromaticum* résiste à l'étape d'égouttage. Sa croissance est ensuite favorisée lors de la remontée de pH, suite à la désacidification de la pâte. La diversification des fromages repose sur la possibilité d'utilisation de souches conférant une nouvelle typicité. *Carnobacterium maltaromaticum* élabore dans certaines conditions des composés volatils (flaveur maltée) et pourrait exprimer ses potentialités aromatiques pendant l'affinage puis le stockage au froid, étapes où l'activité métabolique des bactéries lactiques traditionnelles est ralentie ou inhibée. Cette espèce possède donc des propriétés inhabituelles, pour des bactéries lactiques, qui pourraient s'avérer avantageuses. La connaissance de ses performances pourrait ainsi offrir la possibilité aux industriels d'élargir la gamme des levains et des fromages et d'accroître la sécurité des aliments.

Toutefois, cette espèce d'intérêt, connue dans la filière viande, n'est pas utilisée en technologie fromagère en raison de sa quasi méconnaissance par la profession. Il s'avère donc indispensable de confirmer l'importance et l'impact de cette espèce en production fromagère et d'analyser ses potentialités technologiques.

La présence de micro-organismes d'altération ou pathogènes dans certains aliments constitue un problème majeur pour les industriels de l'agro-alimentaire, malgré des précautions

hygiéniques toujours plus rigoureuses prises lors des différentes étapes de fabrication et de conservation des aliments. Les toxi-infections alimentaires collectives (TIAC), dues à la présence de bactéries pathogènes dans les aliments, demeurent un problème majeur de santé publique. La période 2006-2009 a été marquée par un quasi doublement du nombre de déclarations de foyers de TIAC par rapport à la période 1998-2005 (Delmas et al., 2010). De récents problèmes en restauration collective ont d'ailleurs fait les gros titres de l'actualité française.

Pour certains types de produits, la réduction ou l'élimination des flores indésirables repose sur l'utilisation de conservateurs chimiques traditionnels (nitrates, nitrites, sulfites) ou de différents systèmes inhibiteurs dits "naturels", ou bio-conservateurs. D'autres voies sont envisageables pour maîtriser le risque de développement microbien dans les produits alimentaires. Les procédés de fermentation, en favorisant la croissance des bactéries positives, génèrent un effet barrière vis-à-vis des flores indésirables et permettent ainsi de garantir la sécurité alimentaire et la qualité microbiologique des aliments.

Utilisant la même niche écologique que *Listeria monocytogenes* et possédant des caractéristiques culturales communes, comme la psychrotrophie et la croissance à des valeurs élevées de pH, *C. maltaromaticum* pourrait empêcher l'implantation de cette espèce pathogène dans le fromage, par compétition de flores et par production de bactériocines.

Les travaux développés au cours de cette thèse portent sur l'aptitude de *Carnobacterium maltaromaticum* LMA 28 à jouer un rôle de barrière vis-à-vis de l'implantation de flores indésirables dans des produits laitiers fermentés de type fromage à pâte molle, sur la définition des conditions de maintien de cette flore positive ainsi que sur ses potentialités à apporter une note aromatique originale aux produits.

Ce travail s'inscrit dans le cadre du CPER 2007-2013 du pôle Agro-Alimentaire Forêt FABELOR, projet **AGRIVAL**, dont les thématiques de recherche portent sur les **transferts dans la chaîne alimentaire et la sécurité du consommateur** et notamment sur **la sécurité et la qualité microbiologiques des aliments** avec **l'adaptation de flores d'intérêt aux procédés agro-alimentaires.**

Ce manuscrit se décline en trois chapitres.

La revue bibliographique présente l'état de l'art sous forme de 3 articles de revue :

- le premier article présente les caractéristiques principales des bactéries du genre *Carnobacterium*, les outils d'identification et l'importance de ce genre en industrie alimentaire ;
- le second article se focalise particulièrement sur l'espèce *C. maltaromaticum*, son isolement, son identification, ainsi que la place de cette espèce en technologie laitière ;
- le troisième article donne une vue d'ensemble du rôle du composé aromatique, le 3-méthylbutanal, ainsi que les voies principales d'élaboration de ce composé chez les bactéries lactiques et plus particulièrement chez *C. maltaromaticum*.

Les résultats sont présentés sous forme de 3 parties comprenant chacune une publication scientifique acceptée ou soumise :

- la première partie porte sur l'impact de *C. maltaromaticum* sur la dynamique de la microflore lors d'une fabrication de fromage à pâte molle ;
- la seconde partie est axée sur le catabolisme de la leucine et l'identification des voies métaboliques impliquées dans la biosynthèse du 3-méthylbutanal chez *C. maltaromaticum* LMA 28 ;
- la troisième partie montre l'influence de la concentration d'oxygène dissous sur la production du 3-méthylbutanal en réacteur.

Les conclusions générales et les perspectives que laissent entrevoir ce travail sont présentées dans le dernier chapitre de cette thèse.

Publications

Catherine Cailliez-Grimal, **Muhammad Inam Afzal** and Anne-Marie Revol-Junelles. 2012. The genera *Carnobacterium.* Encyclopedia of Food Microbiology, second edition. In press

Muhammad Inam Afzal, Thibaut Jacquet, Stéphane Delaunay, Frédéric Borges, Jean Bernard Millière, Anne-Marie Revol-Junelles and Catherine Cailliez-Grimal. 2010. *Carnobacterium maltaromaticum*: Identification, isolation tools, ecology and technological aspects in dairy products. Food Microbiology. 27, 573-579. IF_{2011} : 3.283

Muhammad Inam Afzal, Kenza-Amel Boulahya, Citlalli Celeste Gonzàlez-Ariceaga, Muriel Jacquot, Stéphane Delaunay and Catherine Cailliez-Grimal. Biosynthesis and role of 3-methylbutanal in cheese: Major metabolic pathways, enzymes involved and strategies for control. Journal of Dairy Research. Submitted. IF_{2011} : 1.566

Muhammad Inam Afzal, Emilie Lhomme, Nehal Kamel Ali, Sophie Payot, Claire Gaiani, Frédéric Borges, Anne-Marie Revol-Junelles, Stéphane Delaunay and Catherine Cailliez-Grimal. *Carnobacterium maltaromaticum*: Impact and interaction as a barrier species against the undesired flora of soft cheese. Food Microbiology. Submitted. IF_{2011} : 3.283

Muhammad Inam Afzal, Stéphane Delaunay, Cédric Paris, Frédéric Borges, Anne-Marie Revol-Junelles and Catherine Cailliez-Grimal. 2012. Identification of metabolic pathways involved in the biosynthesis of flavor compound 3-methylbutanal from leucine catabolism by *Carnobacterium maltaromaticum* LMA 28. International Journal of Food Microbiology. 157, 332-339. IF_{2011} : 3.327

Muhammad Inam Afzal, Kenza-Amel Boulahya, Cédric Paris, Stéphane Delaunay and Catherine Cailliez-Grimal. 2012. Effect of oxygen on the biosynthesis of flavour compound 3-methylbutanal from leucine catabolism during batch fermentation in *Carnobacterium maltaromaticum* LMA 28. Journal of Dairy Science. 96, 352-359. IF_{2011} : 2.564

Catherine Cailliez-Grimal, Stéphane Chaillou, Jamila Anba-Mondoloni, Valentin Loux, **Muhammad Inam Afzal**, Abdur Rahman, Gilles Kergourlay, Marie-Christine Champomier-Vergès, Monique Zagorec, Paw Dalgaard, Jorgen J Leisner, Hervé Prévost, Anne-Marie Revol-Junelles and Frédéric Borges. 2012. Complete chromosome sequence of *Carnobacterium maltaromaticum* LMA 28. GenomeA, American Society for Microbiology. In press

Communication orale

Muhammad Inam Afzal, Emilie Lhomme, Sophie Payot, Frédéric Borges, Anne-Marie Revol-Junelles and Catherine Cailliez-Grimal. Impact of *Carnobacterium maltaromaticum* LMA 28 as a barrier species against the undesired flora of soft cheese. Ecosystème Microbiens et Bioprotection des Aliments. ONIRIS, Site de la Geraudiere. November 2011, Nantes, France.

Communications affichées

Muhammad Inam Afzal, Stéphane Delaunay, Frédéric Borges, Jean Bernard Millière, Anne-Marie Revol-Junelles and Catherine Cailliez-Grimal. Identification of metabolic pathways involved in leucine catabolism in *Carnobacterium maltaromaticum*. 17ème edition du colloque du " Club des Bacteries Lactiques". 2010, Nancy, France.

Muhammad Inam Afzal, Stéphane Delaunay, Emilie Lhomme, Cédric Paris, Frédéric Borges, Jean Bernard Millière, Anne-Marie Revol-Junelles and Catherine Cailliez-Grimal. Enzymes involved in flavor formation from leucine catabolism by *Carnobacterium maltaromaticum*. Annual seminar of Ecole doctorale RP2E. 2011, Nancy, France.

Muhammad Inam Afzal, Stéphane Delaunay, Frédéric Borges, Anne-Marie Revol-Junelles and Catherine Cailliez-Grimal. Knowledges and technological applications of lactic acid bacterium of the genus *Carnobacterium* in food biopreservation. Annual seminar of Ecole doctorale RP2E. 2012, Nancy, France.

Citlalli Celeste Gonzalez-Ariceaga, Muriel Jacquot, **Muhammad Inam Afzal** and Catherine Cailliez-Grimal. Wax encapsulation of bacteria and flavor compounds to improve sensory properties of cheese. XIX International Conference on Bioencapsulation. Octobre 2011, Amboise, France.

1er Chapitre

Synthèse bibliographique

I. Le genre *Carnobacterium*

The genera *Carnobacterium*

Catherine Cailliez-Grimal, **Muhammad Inam Afzal** and Anne-Marie Revol-Junelles

Encyclopedia of Food Microbiology (2012), second edition. In press.

Chapitre I : Synthèse bibliographique

I. Le genre *Carnobacterium*

The genera *Carnobacterium*

Abstract

Carnobacterium is one of the taxonomic additions to the lactic acid bacteria group. Members of this genus are ubiquitous and frequently predominate in a range of foods, including fish, meat and dairy products. It comprises, actually, ten species isolated from various ecological niches, associated with food of animal origin or from cold environments. In this chapter, the following topics are covered: characteristics of the genus and relevant species, the identification tools, the importance of the genus and individual species in the food industry.

Keywords: *Carnobacterium*, Lactic Acid Bacteria, *Lactobacillus*, cheese, dairy products, meat, shrimp, food spoilage, bacteriocins, classification

1. Introduction

The genus *Carnobacterium* was proposed to clarify the taxonomic position of *Lactobacillus*-like organisms, isolated from common habitats like meat, chicken or fish. Ten species are presently recognized to comprise this genus (Table 1), which inhabit animal or product of animal origin but also non-animal or non-food associated environments. Only *C. divergens* and *C. maltaromaticum* are frequently isolated from foods. The interest of the *Carnobacterium* in food is related to their possible involvement in protective culture and most research has focused on the production of bacteriocins, the regulation on metabolic enzyme pathways, their roles in inhibition of *Listeria monocytogenes* and their impact in spoilage of fish products such as cold-smoked salmon. In natural ecosystem, they may reduce the oxygen levels and may create advantageous condition for the development of obligatory anaerobic microorganism.

In this article, the following topics are covered: characteristics of the genus and relevant species, the identification tools, the importance of the genus and individual species in the food industry

2. Characteristics of the genus and relevant species

Taxonomy

The genus *Carnobacterium* belongs to the Lactic Acid Bacteria (LAB), a definition which groups Gram-positive, catalase-negative bacterial species able to produce lactic acid as main end-product of the fermentation of carbohydrates. According to the Bergey's Manual of Systematic Bacteriology, the genus *Carnobacterium* belongs to the phylum *Firmicutes*, Class *Bacilli*, order, *Lactobacillales* , family *Carnobacteriaceae* with *Carnobacterium* the genus type, and its close relatives, being grouped within the same family, consisting of twelve genera (*Alkalibacterium*, *Allofustis*, *Alloiococcus*, *Atopobacter*, *Atopococcus*, *Atopostipes*, *Desemzia*, *Dolosigranulum*, *Granulicatella*, *Isobaculum*, *Marinilactibacillus* and *Trichococcu*s). On the basis of 16S rRNA similarity, the *Carnobacterium* species form a phylogenetically coherent group. Based on their habitats, two ecological groups can be defined, which is not correlated with the phylogenetic groups. Six species have been isolated from food or animal origin and four species from cold environments such as antartic ice lakes and permafrost (Table 1).

Table 1. Characteristics useful in differentiating *Carnobacterium* species.

Characteristic	*C. alterfunditum*	*C. divergens*	*C. funditum*	*C. gallinarum*	*C. inhibens*	*C. jeotgali*	*C. maltaromaticum*	*C. mobile*	*C. pleistocenium*	*C. viridans*
Major Isolation source	fish, polar lakes, deep sea sediment	Dairy, Meat, fish, shrimp, Intestine of fish	Polar lakes, intestine of fish, marine sponge	Meat fish	Atlantic salmon	Jeotgal shrimp	Dairy, meat, fish, shrimp	meat, shrimp fish	permafrost	meat
Ecological group	II	I	II	I	I	I	I	I	II	I
Growth at :										
Temperature range	0-20	0-40	0-20	0-37	0-30	4-37	0-40	0-35	0-28	2-30
NaCl range (%) (required)	NT (0.6)	0-10	NT (1.7)	NT	0-6	0-5	0-5	NT	0.1-5.0	0-4
pH range (optimum)	NT (7.0-7.4)	5.5-9	NT (7.0-7.4)		5.5-9	5.5-9.0	5.5-9.5	NT	6.5-9.5	5.5-9.1
Motility	+	-	+	-	+	-	-	+	+	-
Voges-Prokauer test	-	+	-	+	-		+	-	NT	-
Aesculin hydrolisis	±	+	-	+	+	+	+	+	+	+
DNA G+C content (mol %)	33-34	33-36.4	32-34	34.3-35.4	NT	43.9	33.7-36.4	35.5-37.2	42	NT

Biochemical and physiological attributes

This genus is composed of non-spore-forming, Gram-strain-positive rods or coccobacilli (Figure 1), motile or not. They are fermentative, usually facultatively anaerobic, some species grow aerobically or microaerophilically. They were unable to grow on acetate medium. Species may be psychrotolerant and grow at 0 °C but not at 45 °C. They are halotolerant until 8 % NaCl and alkaliphilic with growth at pH 9. Some species exhibit catalase activity in the presence of heme. The peptydoglycan contains *meso*-diaminopimelic acid. The genomic G+C contents of *Carnobacterium* vary from 33 to 44 %. They do not reduced nitrate to nitrite.

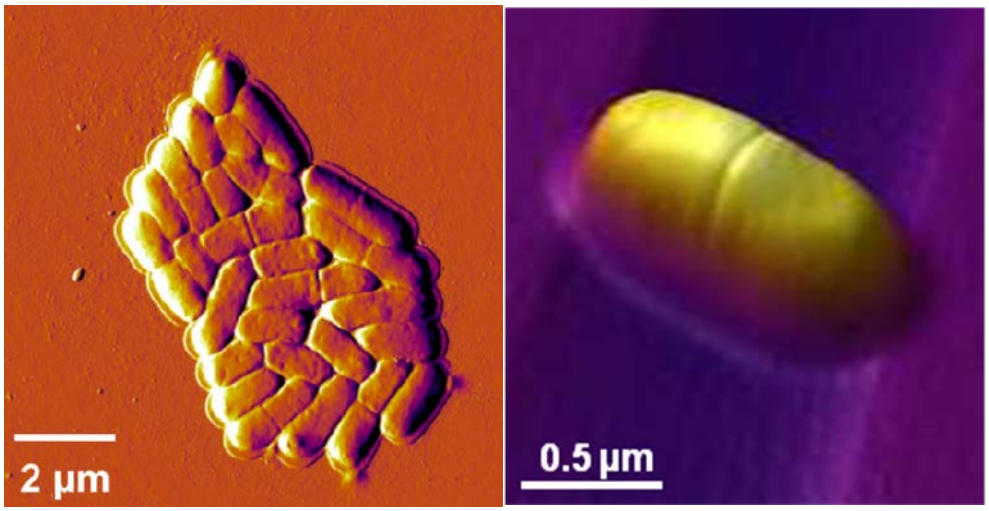

Figure 1. Atomic force microscopy imaging of height (left panels) and deflection (right panels) of *Carnobacterium maltaromaticum* DSM207302.

The metabolism of all the species is fermentative and they are capable of reducing rezazurin in aerobic media during growth. Respiration might occur in the presence of hematin, increasing oxygen consum. Initially described as heterofermenters, carnobacteria could be considered to be homofermentative organisms that produce lactic acid from glucose (except for the species *C. pleistocenium*) or facultative heterofermentative. They are able to catabolise a range of carbohydrate, although there are considerable interspecies and intraspecies heterogeneities. Some species could use hexoses and pentoses metabolized to L(+)-lactate and, depending of the access of oxygen, may produces acetic acid, ethanol, CO_2, formic acid in varying amounts. According to the Voges-Proskauer test, some species can produce acetoin from pyruvic acid (Table 1).

For *C. alterfunditum* and *C. funditum*, glycerol is fermented with no gas to mainly acetic acid and formic acid and traces of ethanol are produced. For *C. pleistocenium* metabolic end products of culture on glucose were acetate and ethanol with traces of CO_2.

Metabolites resulting from the degradation of amino acids are well known for food species with generation of branched alcohols and aldehydes. Production of NH_4^+ from arginine is a result of its catabolism catalyzed by the arginine deaminase pathway. Some species possess the ability of converse tyrosine to tyramine.

Genomic

At present, entire genomes of *Carnobacterium* sp. 17-4 isolated from permanent cold seawater, *C. maltaromaticum* ATCC 35586 isolated from diseased salmon and *Carnobacterium* sp. 7, a piezophilic strain isolated from the Aleutian trench are sequenced. The genome size of *Carnobacterium* species is estimated ranging from 1.9 (For *C. alterfunditum*) to 3.7 Mb (for *C. maltaromaticum*). Knowledge on the genes and DNA sequences of *Carnobacterium* concern mainly bacteriocin-related genes or genes involved in metabolism in the species *C. divergens* and *C. maltaromaticum*. Genes for bacteriocin production may be encoded on the chromosome or on plasmids. For example *C. maltaromaticum* LV17 produces three bacteriocins Carnobacteriocins A (Cbn A), Cbn B2, Cbn BM1. Carnobacteriocins CbnA and CbnB2 are encoded on different and compatible plasmids pCP49 (72 kb) and pCP40 (61 kb), respectively. The Cbn BM1 structural gene and its immunity gene are localized on the chromosome whereas activation and export of Cbn BM1 depend on genes located on plasmid pCP40 encoding Cbn B2. The plasmid of *Carnobacterium* sp. 17-4 encoded three putative carbohydrate phosphotransferase systems.

In *C. maltaromaticum* ATCC 35586, a range of putative virulence genes was identified including genes encoding products involved in adhesion, capsule synthesis, haemolysis invasion and resistance to toxic compounds. This strain carries putative virulence genes that may explain its reported ability to infect fish. This finding gives no reason for concern regarding human health by the presence of this species in food products.

Isolation and cultivation procedures

Depending on their isolation origin, the *Carnobacterium* species do not require the same growth conditions.

Species isolated from foods and products of animal origin (group I) do not grow on acetate rich media and the omission of acetate from conventional *Lactobacillus* media is commonly used for their isolation. Similarly, the use of neutral to alkaline pH media promotes the growth of carnobacteria at the expense of the *Lactobacillus* and could be used as enrichment method. Non selective media (universal media like Tryptone Soy, complex media, like Brain Heart Infusion) can be used for isolation when the microbial population is dominated by carnobacteria. Even if there is a better growth at 30-37 °C, incubation under psychrotrophic conditions (10 days at 7 °C) permit the selection of *Carnobacterium* species.

Species isolated from cold environment are less fastidious. The strains do not grow at 30°C and are psychrotrophic. At 20 °C, *C. alterfunditum* and *C. funditum* strains grow better anaerobically than aerobically whereas *C. pleistocenium* isolate grows well under aerobic or anaerobic conditions. For general cultivation, universal media with neutral or alkaline pH can be used. Conservation could be done by freezing with cryopreservation or by lyophilisation.

Enumeration of carnobacteria in foods

Various media are available for the nonselective, semi-selective or selective recovery of carnobacteria of group I. Table 2 described some of them. Mane Rogosa and Sharpe (MRS) is a medium commonly used for the isolation of LAB from foods but because of its acetate content, carnobacteria are poorly recovered with this medium. So it was modified (D-MRS) by increasing the pH to 8.5, omitting acetate and substituting sucrose by glucose. All *Carnobacterium* species of the group II grow on this medium.

Different media used antibiotics, alone or in association in their components. Nalidixic acid is used to inhibit most of the Gram-negative microorganisms, vancomycin and gentamicin to inhibit most of Gram-positive bacteria. Cresol Red Thallium Acetate Sucrose (CTAS) agar was previously devised for the selective recovery of *Carnobacterium* spp., however problems with low recovery and interference by other microorganisms prevented this medium from becoming widely accepted. The selectivity of this medium is based on its high pH and the presence of thallium acetate, nalidixic acid and a relatively high concentration of sodium citrate (15 $g.L^{-1}$). Good growth of *Enterococcus* spp. is also observed. *Listeria* spp. grows sparsely. The Cresol Red Thallium Acetate Sucrose Inulin (CTSI) agar like medium of numeration of the four principal species of the *Carnobacterium* kind does not satisfied because of its inhibiting capacity

against strains to be selected. On these two media, the periphery of *Carnobacterium* colonies is often characterized by a yellow colour due to media acidification with a red button in the center due to the reduction of TCC. The elective EBRER medium is composed of ribose, aesculin and phenol red as principal components with the addition of amphotericin and nalidixic acid. The incubation is more selective during ten days at 7 °C than at 30 °C. Moreover, this medium also allows the growth of enterococci.

A selective medium based on TS-YE agar at pH 8.8 supplemented with antibiotics (nalidixic acid, vancomycin and gentamicin) was proposed, named CM medium. It is highly specific to *C. maltaromaticum* with a recovery of 100 %, along with *C. mobile* and *Desemzia incerta* and do not permit growth of others carnobacteria. Although, the standard approach to enumerate carnobacteria has involved differential counting by simultaneously plating on two media using an acetate-containing agar and plate count agar (PCA).

Table 2. Media used for quantitative isolation of *Carnobacterium* sp.

Media	pH	Principal agents ($g.l^{-1}$)	Culture condition
D-Man Rogosa Sharpe (D-MRS)	8.5	without acetate sucrose	24 -72 h at 25 °C
Cresol Red Thallium Acetate Sucrose (CTSA)	9.1	Sucrose 20 Nalidixic acid 0,04 Cresol red 0,004 Thallium acetate 1 Triphenyl-tetrazolium chloride 0,01	24 - 48 h at 30 °C 3 - 4 days at 25 °C
Cresol Red Thallium Acetate Sucrose Inulin (CTSI)	9.1	Idem CTSI with Sucrose 10 Inulin 10 Nisin 0,00125 Vancomycin 0,001 Thallium acetate 0,5	2 days at 25 °C 2 days at 8 °C
CM medium	8.8	TS-YE 15 Gentamicin 0.005 Vancomycin 0.0035 Nalidixic acid 0.02	36 - 46 h at 25 °C

3. Identification tools

Identification

Phenotypic and genotypic tools were developed and applied to *Carnobacterium* species isolated from various environments. The current taxonomic classification of this genus using a numerical phenetic study has been revisited using traditional biochemical reactions, carbohydrate fermentation and inhibition tests (temperature, pH, salt, chemical preservatives, antibiotics, bacteriocins). Simple identification key must be used with caution. However, when a larger number of phenotypic tests were used and data evaluated by numerical taxonomy, the same degree of confidence was obtained as with genotypic tools.

During isolation and identification procedures, the acetate sensitivity, the ability to grow at alkaline pH and at psychrotrophic temperature may serve as routine tests for the recognition of carnobacteria among the rod-shaped LAB.

Whole cells lysates were used for the detection of meso-diaminopimelic acid (meso-DAP) in the peptydoglycan. Analysis of whole cells protein by SDS-PAGE permitted to differentiate *C. maltaromaticum* from *C. divergens*. Fourier Transform Infrared spectroscopy could allow the differentiation of *Carnobacterium* strains.

The determination of carbohydrate fermentation pattern can be obtained with automated systems API 50 CH. All species produce acid from cellobiose, fructose, glucose, maltose, mannose, salicin but not from adonitol, dulcitol, glycogen, inositol, raffinose, rhamnose and sorbitol.

About genotypic identification, sequence analysis of 16S rRNA permit a differentiation of all *Carnobacterium* species and with DNA-DNA hybridization may be the best way to differentiate phenotypically very similar species. Different techniques using primers specifics or not can be used (RFLP, AFLP, RAPD). Digestion of DNA followed by PFGE also permits identification of species

4. Importance of the genus and individual species in the food industry

In meat, meat products and poultry

In 1987, the genus *Carnobacterium* was proposed as the genus accommodating the species *Lactobacillus divergens* and *L. piscicola* which has been isolated from refrigerated meats. These two species are the most isolated from food products. Meats, meat products and

poultry are substrates rich in nutrients with water activity (A_w) and neutral pH favourable for the growth of carnobacteria, reaching high levels (10^6-10^8 cfu.g^{-1}). They are found in vacuum-packaged meat and related products stored at low temperatures. The five species (*C. divergens*, *C. gallinarum*, *C. maltaromaticum*, *C. mobile* and *C. viridians*) are commonly associated with the spoilage of these products. For instance, *C. viridians* is responsible of the green discoloration of refrigerated vacuum packed bologna sausage. In cooked minced sausage, *C. maltaromaticum* could be responsible of the fatty odour.

In fish and seafood

Carnobacterium maltaromaticum was first isolated from diseased fishes (rainbow trout, salmon) and has been described as a fish pathogen organism. At a later stage, it was associated with healthy fish. In fact, carnobacteria belong to the normal gastrointestinal flora of healthy fish and others species, *C. divergens* and *C. inhibens* also inhabit fishes.

Among the ten species, only *C. divergens* and *C. maltaromaticum* are frequently isolated from seafood. They can resist to high pressure, cold temperature, modified atmosphere, high concentration of NaCl. So in vacuum-packed cold smoked seafood, they are able to grow to high concentration (10^6-10^8 cfu.g^{-1}). These species were able to form tyramine which can represent a hazard. Depending on the seafood product, they can be considered as spoilage or not.

In dairy product

Carnobacterium maltaromaticum was first isolated from milk having developed a distinct malty or chocolate like flavour and aroma which correspond to the presence of aldehydes. This species was also found to be a citrate-fermenting member of the microflora involved in Mozzarella cheese fermentation. Its presence was reported in a variety of French soft-ripened or red-smear cheeses made from cow's, ewe's or goat's milk. It constituted the main psychrotrophic LAB flora and can reached high levels at the end of the storage period. It may appear as a potential ripening flora in soft cheese and contribute to the aroma development. This production depends on various factors, including, intracellular enzymes involved in the catabolism of branched-chain amino acids, i.e., leucine, isoleucine and valine by the bacterial transaminases, oxygen or redox potential. Not much is known about their metabolism during ripening but they did not cause off-flavours in the cheeses tested.

Preservation in food

The genus *Carnobacterium* is well known for its ability to produces bacteriocins. These bacteriocins are effective against spoilage microorganisms and inhibit the pathogenic bacteria *Listeria monocytogenes*. The genera *Listeria* and *Carnobacterium* are both psychrotrophic and have the same behaviour towards pH and temperature variations. The use of bacteriocin-producing *Carnobacterium* strains could prevent the growth of *Listeria* during critical phases on a variety of refrigerated foods. Nevertheless, inactivation of bacteriocin by proteolytic enzymes and the emergence of resistant strains could appear. Inhibition by glucose depletion was demonstrated using a bacteriocin negative strain of *C. maltaromaticum*.

Since 2005, one strain of *C. maltaromaticum* (CB1) has been class as Generally Recognized as Safe (GRn 00159) for use in ready-to-eat meat products.

Further Reading

Afzal I, Jacquet T, Delaunay S *et al*. (2010) *Carnobacterium maltaromaticum:* taxonomy, identification and isolation tools, ecology and technological aspects Food Microbiology 25: 580-585

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II. *Carnobacterium maltaromaticum*: Identification, outils d'isolement, écologie et aspects technologiques dans les produits laitiers

Carnobacterium maltaromaticum: Identification, isolation tools, ecology and technological aspects in dairy products

Muhammad Inam Afzal, Thibaut Jacquet, Stéphane Delaunay, Frédéric Borges, Jean Bernard Millière, Anne-Marie Revol-Junelles and Catherine Cailliez-Grimal

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Review

Carnobacterium maltaromaticum: Identification, isolation tools, ecology and technological aspects in dairy products

Muhammad Inam Afzal [a], Thibaut Jacquet [a], Stéphane Delaunay [b], Frédéric Borges [a], Jean-Bernard Millière [a], Anne-Marie Revol-Junelles [a], Catherine Cailliez-Grimal [a,*]

[a] *Nancy-Université, Institut National Polytechnique de Lorraine, Laboratoire d'Ingénierie des Biomolécules, 2, avenue de la Forêt de Haye, B.P. 172, 54505 Vandoeuvre-lès-Nancy, France*

[b] *Nancy-Université, Institut National Polytechnique de Lorraine, Laboratoire Réaction et Génie des Procédés, UPR CNRS 3349, 2, avenue de la Forêt de Haye, B.P. 172 54505 Vandoeuvre-lès-Nancy, France*

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ABSTRACT

Carnobacterium species constitute a genus of Lactic Acid Bacteria (LAB) present in different ecological niches. The aim of this article is to summarize the knowledge about *Carnobacterium maltaromaticum* species at different microbiological levels such as taxonomy, isolation and identification, ecology, technological aspects and safety in dairy products. Works published during the last decade concerning *C. maltaromaticum* have shown that this non-starter LAB (NSLAB) could present major interests in dairy product technology. Four reasons can be mentioned: i) it can grow in milk during the ripening period with no competition with starter LAB, ii) this species synthesizes different flavouring compounds e.g., 3-methylbutanal, iii) it can inhibit the growth of foodborne pathogens as *Listeria monocytogenes* due to its ability to produce bacteriocins, iv) it has never been reported to be involved in human diseases as no cases of human infection have been directly linked to the consumption of dairy products containing this species.

1. Introduction

Strains from *Carnobacterium maltaromaticum* are facultatively anaerobic, catalase negative, Gram positive rod shaped LAB which produce L(+)-lactic acid from glucose (Collins et al., 1987). Their presence at various levels in food including fish, meat and dairy products has resulted in an increased number of scientific investigations to study their ecology, role and effect on these food products. For decades, the emphasis has been on product developments in the dairy industry with respect to organoleptic properties and/or health protection such as the control of foodborne pathogens. As discussed in this review, the major potential role of *C. maltaromaticum* might be as a ripening flora by producing flavouring compounds and as a biopreservative flora, by inhibiting the growth of foodborne pathogens.

This review focuses on the taxonomy and methods for isolation and identification of *C. maltaromaticum* and its place and role in dairy products.

2. Taxonomy

The genus *Carnobacterium* was proposed for the reclassification of atypical heterofermentative *Lactobacillus* species (group III) isolated from vacuum-packed meat, chicken and fish, and is unable to grow on acetate agar (Collins et al., 1987; Hammes and Hertel, 2006). Based on their habitats, two ecological groups can be defined with group I associated with animal and products of animal origin and group II present in a cold environment (Hammes and Hertel, 2009). These rod shaped LAB produce L(+)-lactic acid from glucose and are characterized by the presence of *meso*-diaminopimelic acid in their cell-wall composition. The *Carnobacterium* species form a phylogenetically coherent group which is quite distinct from other LAB, as shown by sequencing of the 16S rDNA (Wallbanks et al., 1990). At first, this new genus contained four strains, *Carnobacterium divergens* comb. nov., *Carnobacterium piscicola*, *C. gallinarum* sp. nov. and *C. mobile* sp. nov. *C. piscicola* (Collins et al., 1987) and *Lactobacillus maltaromaticus* isolated from milk (producing a malty-like flavour and aroma) (Miller et al., 1974) are heterotypic synonyms (Mora et al., 2003). So, the authors validated the name *C. maltaromaticum* and described the physiological and biochemical properties of this new species.

* Corresponding author. Tel.: +33 (0) 3 83 59 58 84; fax: +33 (0) 3 83 59 57 71.
E-mail address: catherine.cailliez@ensaia.inpl-nancy.fr (C. Cailliez-Grimal).

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The type strain of *C. maltaromaticum* is DSM 20342^T (=ATCC 27865^T = CCUG 30142^T = CIP 103135^T = JCM 1154^T = LMG 6903^T = NRRL−B 14852^T) (Mora et al., 2003).

Lai and Manchester (2000) have revisited the current taxonomic classification of this genus using a numerical phenetic. Based on 92 unit characters, three cluster-groups were identified with the cluster group A equated with *C. maltaromaticum*. Currently the genus *Carnobacterium* contains ten species (Kim et al., 2009; Leisner et al., 2007) but only *C. maltaromaticum* and *C. divergens* have a potential interest in relation to dairy products (Laursen et al., 2005; Millière et al., 1994; Morea et al., 1999).

3. Identification tools

Both phenotypic and genotypic techniques have been utilized to deduce accurate phylogenetic information or to improve the quality of identification. Many typing methods and techniques, looking at phenotypic or genotypic level, have been developed (Vandamme et al., 1996). The use of one or a combination of different methods relies on four main characteristics: discriminatory levels (genus, species or intraspecies), reproducibility (at short and long term), ease of use and cost. Phenotypic methods are more time-consuming and are restricted to a few species; molecular methods require more expensive equipment but are more reproducible and applicable to a wider variety of species (Pot, 2008). In the case of *Carnobacterium* species, phenotypic or genotypic approaches were developed and applied on *C. maltaromaticum*, isolated from different food types (Table 1). These techniques could be applied for identification of *C. maltaromaticum* isolated in dairy products.

3.1. Phenotypic identification

To characterize *C. maltaromaticum* strains, the numerical taxonomic approach used traditional biochemical reactions, carbohydrate fermentation and inhibition tests (temperature, pH, salt, chemical preservatives, antibiotics, bacteriocins) (Lai and Manchester, 2000; Laursen et al., 2005). Simple identification keys must be used with caution. However, when a larger number of phenotypic tests were used and data evaluated by numerical taxonomy, the identification of *Carnobacterium* at species level was obtained with the same degree of confidence as with genotypic tools (Laursen et al., 2005).

The differentiation of *C. maltaromaticum* from *C. divergens* in meat and seafood has been obtained by the method of SDS-PAGE of whole-cell proteins (Laursen et al., 2005). It is possible to assign a protein profile to each known bacterial species by comparison with a database of normalized protein fingerprints derived from reference strains (Vandamme et al., 1996).

Since the development of chemometric methods to obtain biochemical information from hyperspectral data, Fourier Transform Infrared (FT-IR) spectroscopy has been used in a whole-cell analysis (Mariey et al., 2001). The FT-IR spectroscopy has allowed the differentiation of *C. maltaromaticum* taxon among 67 *Carnobacterium* strains. It is correlated with the results obtained by other methods, *i.e.* Pyrolysis mass spectrometry (PyMS) or DNA–DNA hybridization or 16S rDNA sequence comparisons (Manchester et al., 1995). The culture age has no major effect on the spectra obtained and the reproducibility is good over a six-month period, making FT-IR spectroscopy a rapid and reliable method for the identification of *Carnobacterium* species (Lai et al., 2004).

3.2. Genotypic identification

Carnobacteria have been identified at the genus level by nucleic acid hybridization by using 16S rDNA targeted genus specific probes (Nissen et al., 1994). Species-specific primers have been designed according to sequences of 16S rDNA (Barakat et al., 2000; Brooks et al., 1992; Nissen et al., 1994; Scarpellini et al., 2002) and used in multiplex PCR amplification (Macian et al., 2004; Pelle et al., 2005; Yost and Nattress, 2000). However, the PCR primers used were not specific enough to differentiate between *Carnobacterium* species. The intergenic spacer region (ISR) located between the 16S and the 23S genes was shown to be more adapted for discrimination of *C. maltaromaticum* at the species/strain level whereas 16S and 23S ribosomal genes, which are genetically close between the different species of a same genus, are more convenient for the genus level identification. *C. maltaromaticum* was successfully detected and identified using one of these PCR-derived techniques (Chenoll et al., 2003; Scarpellini et al., 2002).

Table 1
Methods used for the identification of *C. maltaromaticum*.

Methods	Isolates from	Reference
SDS-PAGE	Meat, seafoods	Laursen et al., 2005
Pyrolysis mass spectrometry	–[a]	Manchester et al., 1995
Fourier Transform Infrared Spectroscopy	–	Lai et al., 2004
16S or 23S rDNA	Meat, Poultry meat	Wallbanks et al., 1990, Brooks et al., 1992, Nissen et al., 1994, Barakat et al., 2000, Scarpellini et al., 2002
Multiplex PCR	Meat	Yost and Nattress 2000
Multiplex PCR	Various meat products	Macian et al., 2004
Multiplex PCR	Cold-smoked salmon	Pelle et al., 2005
Intergenic Spacer Region polymorphism (ISR)/Restriction Fragment Length polymorphism (RFLP)	–	Kabadjova et al., 2002; Chenoll et al., 2003; Rachman et al., 2004;
	Lake whitefish	Loch et al., 2008
Specific primers Pis A	Cold-smoked salmon	Pelle et al., 2005
	Lake whitefish	Loch et al., 2008
Amplified Fragment Length Polymorphism (AFLP)	Meat, seafoods	Laursen et al., 2005
Random Amplification of Polymorphic DNA (RAPD)	Mozzarella	Morea et al., 1999
	Atlantic cod	Seppola et al., 2006
	Refrigerated meat	Ercolini et al., 2009
Pulsed-Field Gel Electrophoresis (PFGE)	Cold-smoked fish	Duffes et al., 1999b
Peptidoglycan hydrolase	Cold-smoked fish	Duffes et al., 1999b

[a] not determined.

Restriction Fragment Length Polymorphism (RFLP) (Jensen and Stanton, 1993; Navarro et al., 1992) was applied on PCR-amplified 16S rDNA of 42 strains of eight *Carnobacterium* species (Kahadjova et al., 2002). Three PCR amplicons, designated small, medium and large ISR, were obtained with all *Carnobacterium* species tested. The large ISR sequence revealed the presence of two tRNA genes, which were separated by a spacer that varied from 24 to 38 bp in length depending on the *Carnobacterium* species. This variable region allowed the design of species-specific primers. The restriction patterns obtained by TaqI, HinfI and HindIII allowed a discrimination of *C. maltaromaticum* (Rachman et al., 2004).

Some *C. maltaromaticum* strains produce bactericiocins, e.g., piscicolin 126. As a consequence, this gene (*pisA*) can be used as a tool for this species identification, but it is restricted to bacteriocin-producing strains (Pelle et al., 2005). The *pisA* gene allowed the recent isolation of a new strain which is closely related to *C. maltaromaticum* involved in a freshwater fish (*Coregonus clupeaformis*) pseudokidney disease (Loch et al., 2008).

Amplified Fragment Length Polymorphism (AFLP) is based on the selective PCR amplification of restriction fragments from a total digest of genomic DNA (Vos et al., 1995). Laursen et al. (2005) applied this method to 59 *Carnobacterium* strains which allowed them to distinguish between two intraspecies groups of *C. divergens* strains but not *C. maltaromaticum* that appeared to be a monophyletic group.

Thanks to Random Amplification of Polymorphic DNA (RAPD), strains of *C. maltaromaticum* have been identified in various ecosystems (as discussed later by Ercolini et al., 2009; Morea et al., 1999). It is a discriminative identification method due to its high fingerprinting capacity. The oligonucleotide primers are arbitrarily designed, so it is not necessary to know the part of the genome involved in the fingerprinting. Moreover, its use of small amounts of DNA and is not very time-consuming (Gurtler and Mayall, 2001; Hadrys et al., 1992; Morea et al., 1999; Ruiz et al., 2008).

Pulsed-field gel electrophoresis (PFGE), a method developed for the separation of large DNA molecules, was applied to trace sources of microbial contamination (Dalgaard et al., 2003) and to characterize *Carnobacterium* strains (Dalgaard et al., 2003; Duffes et al., 1999b).

4. *C. maltaromaticum* in dairy products

4.1. Isolation and numeration of C. maltaromaticum

The isolation and identification of bacterial strains associated with food and dairy products appear to be the most restrictive and controversial steps in microbial taxonomy. Different culture media are available for the nonselective, semi-selective or selective recovery of this *Carnobacterium* genus (Hammes and Hertel, 2006). The Cresol Red Thallium Acetate Sucrose (CTAS) agar has been suggested for selective detection and isolation of carnobacteria (Baird et al., 1989; Holzapfel, 1992). This medium may allow the growth of some *Leuconostoc* and *Enterococcus*. Incubation is either at 30 °C for 24–48 h or at 25 °C for 3–4 days (Baird et al., 1989; Holzapfel, 1992). The elective EBRER medium (Millière et al., 1994) needed psychrotrophic conditions of incubation (7 °C for 10 d) to allow a good recovery of this genus. The Cresol Red Thallium Acetate Sucrose Inulin Agar (CTSI) has a limited use for automatic numeration due to its red colour, opacity, complexity and incubation time of 4 days (Wasney et al., 2001).

Specific isolation of *C. maltaromaticum* on a solid medium can be performed on the basis of its growth ability at an alkaline pH value (up to 9.6) and its antibiotic resistance pattern. Thus, a new medium, named CM (*C. maltaromaticum*), was designed. This selective medium is based on the TS–YE agar supplemented with vancomycin (3.5 mg L^{-1}), gentamicin (5.0 mg L^{-1}) and nalidixic acid (20 mg L^{-1}) with an alkaline pH value of 8.8. After incubation for 36–48 h at 25 °C, its high selectivity for *C. maltaromaticum* was demonstrated with multiple dairy strains (Edima et al., 2007). It was shown that this medium is suitable for pre-enrichment.

Three methods, flow cytometry, epifluorescent microscopy and *in situ* hybridization were used in order to enumerate *C. divergens* and *C. maltaromaticum* (Connil et al., 1998). These methods, once coupled together, allowed a rapid and specific enumeration of these bacteria in mixed cultures. At first, protein and lipids must be removed by the use of enzymes, detergent and centrifugation before appling these techniques to dairy products (Gunasekera et al., 2003).

DNA–DNA hybridization was also shown to be a powerful method for the detection and the quantification of *Carnobacterium* sp. in various food products. For instance, colony hybridizations with a specific DNA probe of the genus (CB1) were used to follow the presence of *Carnobacterium* (Larrouture-Thiveyrat et al., 2003).

A sensitive real-time PCR assay using Sybr Green I-based detection has been optimized to specifically quantify *Carnobacterium* sp. Using genus or species-specific primers (Rachman et al., 2004), a reliable and fast screening of *C. maltaromaticum* was reported (Cailliez-Grimal et al., 2005). Detection limits for real-time PCR were satisfactory ($\geq 10^4$ cfu g^{-1} of cheese) in relation to expected levels of this species in these products (10^5 to 10^8 cfu g^{-1} of cheese).

4.2. Presence of C. maltaromaticum in dairy products

C. maltaromaticum strains are widely found in food including a few dairy products (Table 2). It was first isolated from milk having developed a distinct malty or chocolate like flavour and aroma which correspond to the presence of aldehydes (3-methylbutanal, 2-methylbutanal, 2-methylpropanal) (Miller et al., 1974).

The presence of *C. maltaromaticum* in Brie cheeses, a variety of AOP (protected designation of origin) French surface mould-ripened soft cheese, was reported for the first time in 1994 (Millière and Lefebvre, 1994; Millière et al., 1994) and confirmed in 2007 (Cailliez-Grimal et al., 2007). Out of 30 French soft-ripened or red-smear cheeses made from cow's, ewe's or goat's milk (raw or pasteurized), 10 contained this species, and isolates from three cheeses exhibited anti-*Listeria* activity (Cailliez-Grimal et al., 2007). In cheeses tested, *C. maltaromaticum* constituted the main psychrotrophic LAB flora. Its growth was able to continue even at an alkaline pH value, reaching high levels (10^8–10^9 cfu g^{-1}) at the end of a further cold (4 °C) storage period (Cailliez-Grimal et al., 2007). It may appear as a potential ripening flora in soft cheese (Edima et al., 2008). This species was also found to be a citrate-fermenting member of the microflora involved in Mozzarella cheese fermentation and constitutes 70% of the curd (Morea et al., 1999).

C. maltaromaticum is a slow acidifying species compared to commercial starter LAB, such as *Lactococcus lactis* or *Streptococcus thermophilus*. However, the psychrotrophic *C. maltaromaticum* LMA 28 strain can sustain low pH values in co-culture with *Lc. lactis* DSMZ 20481 or *S. thermophilus* INRA 302 (Edima et al., 2008).

As a consequence of their slow acidifying activity, *Carnobacterium* strains cannot be used as a starter and can therefore be considered as NSLAB. Nevertheless, they could contribute to cheese ripening. From a cheese making technology point of view, some NSLAB strains showed appropriate technological characteristics for their use as adjunct cultures in soft and semihard cheeses (Briggiler-Marco et al., 2007). These NSLAB may positively contribute to the aroma development of soft cheeses. This production of aroma depends, among other things, on the catabolism of branched-chain amino acids, *i.e.* leucine, isoleucine

Table 2
Carnobacterium maltaromaticum in food products.

Products	Microbial counts	Number of isolates of C.m/total isolates[a]	Sensory impact	Reference
Dairy				
Skimmed milk		1	Malty aroma	Miller et al., 1974
Soft cheeses	10^6 cfu g^{-1}	10^6 cfu g^{-1}	No off-flavours	Millière and Lefebvre 1994
	10^8–10^9 cfu g^{-1}	33/36	No off-flavours	Millière et al., 1994
	10^6–10^7 cfu g^{-1}		No off-flavours	Cailliez-Grimal et al., 2007
Mozzarella	10^5–10^6 cfu g^{-1}	2/25	No off-flavours	Morea et al., 1999
Fish and shellfish				
MAP salmon[b]	10^6–10^8 cfu g^{-1}	21/28	No off-flavours	Emborg et al., 2002
Cold-smoked salmon	–	97/155	Malt culture medium	Leroi et al., 1998
	10^6–10^7 cfu g^{-1}	221/255	Slightly sour, sweet/nauseous (vacuum) Oxydized, bitter and fishy/malty (MAP)	Paludan-Muller et al., 1998
	–	22/160	No spoilage odour detected	Duffes et al., 1999a
Cooked and peeled	–	9/137	–	Jaffres et al., 2009
Shrimp	–	11/58	Sensory score acceptable with flavour of chlorine-like	Mejlholm et al., 2005
Brined shrimp	–	5/134	Fermented Fishy, sour Rancid, yellowish	Mejlholm et al., 2008
Meat				
MAP beef muscles	–	7/79	VOC Aldehydes, lactones, sulphur compounds[c]	Ercolini et al., 2009
MAP broiler	–	96/447	Not determined	Vihavainen et al., 2007
Marinated pork	–	Few	No spoilage odour detected	Schirmer et al., 2009

[a] C.m: *Carnabacterium maltoromaticum*.
[b] MAP: Modified atmosphere-packed.
[c] not use of sensory analysis but detection of VOC: volatile organic compounds.

and valine by the bacterial transaminases (Ardo, 2006; Marilley and Casey, 2004; Smit et al., 2004, 2005). From leucine, *C. maltaromaticum* produced α-ketoisocaproic acid, 3-methylbutanal and 3-methylbutanol, from isoleucine, 3-methylbutanoic acid, 2-methylbutanal and from valine, 2-methylbutanol, 2-methylpropanal and 2-methylpropanol. It has been suggested that these aldehydes convey the malty aroma that is the characteristic of *C. maltaromaticum* in milk (Larrouture-Thiveyrat and Montel, 2003; Laursen et al., 2006; Miller et al., 1974; Smit et al., 2005). The corresponding alcohols (3-methylbutanol, 2-methylbutanol and 2-methylpropanol) have an impact on the alcoholic and fruity odours (Thierry and Maillard, 2002). The branched-chain acids (2-methylpropanoic acid, 3-methylbutanoic acid, 2-methylbutanoic acid) are responsible for sweaty, rancid, faecal, putrid ester and rotten fruit like flavours (Marilley and Casey, 2004; Thierry and Maillard, 2002).

Not much is known about their metabolism during growth in cheese products, but this bacterium did not cause off-flavours and could possibly even play a beneficial role in texture and taste during cheese ripening (Millière and Lefebvre, 1994; Millière et al., 1994).

5. Preservation of food

The genus *Carnobacterium* is well known for its ability to produce bacteriocins (Leisner et al., 2007). Six bacteriocins, belonging to class IIa, IIc and one cyclic, have been described for different *C. maltaromaticum* strains. These bacteriocins are effective against spoilage microorganisms and inhibit the pathogenic bacteria *Listeria monocytogenes* (Table 3). The genera *Carnobacterium* and *Listeria* are both psychrotrophic and have the same behaviour towards pH and temperature variations (milk prematuration and end of ripening). The use of bacteriocin-producing *Carnobacterium* strains isolated from cheese or from other foods is very well documented. It could prevent the growth of *L. monocytogenes* during critical phases in a variety of refrigerated foods (Buchanan and Klawitter, 1992a, 1992b; Cailliez-Grimal et al., 2007; Herbin et al., 1997).

The effect of piscicolin 126, a bacteriocin produced by *C. piscicola* JG126, was investigated on the growth of *L. monocytogenes* and cheese starter bacteria in milk and camembert cheese (Wan et al., 1997). Its addition effectively inhibited the growth of *L. monocytogenes* in camembert cheese without affecting viable counts and acid production of starter cultures. The control of piscicolin 126 production in *C. maltaromaticum* UAL26 is an example of a regulated system of bacteriocin production that is effected by a specific growth temperature (25 °C), above which bacteriocin will not be produced at a detectable level in liquid media (Gursky et al., 2006).

Many studies have highlighted some biopreservation strategies for refrigerated foods such as cold-smoked salmon and ready-to-eat meat products (cooked chicken, minced meat) against the risk of *L. monocytogenes*. They used either bacteriocin produced by *Carnobacterium* species, including *C. maltaromaticum* strains, or the direct addition into food of a bacteriocin-producing *Carnobacterium* strain. The influence of different fermentation patterns on the production of a bacteriocin-like substance (BLS) from *C. maltaromaticum* Cp L103 strain using different culture broths has been investigated. This BLS was shown to have a bacteriostatic effect on *L. monocytogenes* on vacuum packaged salmon. However, as stated by the authors, the use of the single bacteriocin is not a sufficient safety factor against *L. monocytogenes* and other strategies such as a combination of two bacteriocins or the use of factors that increase their activity could be considered (Schöbitz et al., 2006). A lower storage temperature and the presence of bacteriocin-producing *C. maltaromaticum* have enhanced *L. monocytogenes* inhibition (Azuma et al., 2007; Brillet et al., 2004; Campos et al., 1997; Duffes et al., 1999a, 1999b; Yamazaki et al., 2003). Nevertheless, inactivation of bacteriocin by proteolytic enzymes, depending on the food, and the emergence of resistant strains (Schöbitz et al., 2003; Wan et al., 1997) have led to suggest the utilization of a bacteriocin negative *C. maltaromaticum* strain. This may be more suitable for the practical use of a biopreservative agent (Nilsson et al., 2004). Using such a strain, *L. monocytogenes* growth was reduced partly by glucose depletion (Buchanan and Bagi, 1997; Nilsson et al., 1999, 2005).

Table 3
Bacteriocins produced by *Carnobacterium maltaromaticum* strains.

Bacteriocins (class)	Strains	Bacteriocin activity against[c]	Molecular mass (AA)[b]	Location of gene (gene)	Reference
Carnobacteriocin BM1 (IIa)	LV17B		4524 (43)	Chromosome	Quadri et al., 1994
Cbn B1[a]	LV17B	*C. divergens*	4541 (43)	(*cbnBM1*)	
Piscicocin V1b[a]	V1	*E. faecalis*	4526 (44)		Bhugaloo-Vial et al., 1996
Carnocin CP51[a]	CP5	*Lb. curvatus*			Herbin et al., 1997
		Lb. plantarum			
		Ln. mesenteroides			
		L. innocua			
		L. monocytogenes			
		P. acidiloctici			
Carnobacteriocin B2 (IIa)	LV17B	*C. divergens*	4969 (48)	Plasmid	Quadri et al., 1994
A9b[a]	A9b	*L. monocytogenes*		(*cbnB2*)	Nilsson et al., 2002
Carnocin CP52[a]	CP5				Herbin et al., 1997
Piscicolin 126 (IIa)	JG126		4416 (44)	Chromosome	Jack et al., 1996
Piscicocin V1[a]	V1	*C. divergens*		(*pisA*)	Bhugaloo-Vial et al., 1996
Piscicolin 126[a]	UAL26	*Carnobacterium* sp.			Gursky et al., 2006
		E. faecalis			
		E. foecium			
		Lb. curvatus			
		Ln. dextranicum			
		Ln. mesenteroides			
		L. grayi			
		L. innocua			
		L. ivanovii			
		L. monacytognes			
		L. seeligeri			
		P. acidilactici			
		P. pentosaceus			
		S. thermophilus			
Carnobacteriocin A (IIc)	LV17A	*C. divergens*	5052 (53)	Plasmid	Worobo et al., 1994
Piscicolin 61[a]	LV61	*C. gallinarum*		(*cbnA*)	Holck et al., 1994
		C. maltaromaticum			
		E. faecalis			
Piscicocin CS526 (IIa)	CS526	*E. durans*	4430	ND	Yamazaki et al., 2005
		E. faecalis			
		E. faecium			
		E. hirae			
		Ln. mesenteroides			
		L. grayi			
		L. innocua			
		L. monocytogenes			
		P. pentosaceus			
Carnocyclin A	UAL307	*C. maltaromaticum*	5880–60	ND (*cclA*)	Martin-Visscher et al. 2008
		C. divergens			
		E. faecalis			
		E. foecium			
		Lc. lactis			
		Ln. mesenteroides			
		L. monocytogenes			
		P. acidilactici			
		S. aureus			

[a] Synonym.
[b] Numbers of amino acids.
[c] B: *Brachothrix*; C: *Carnobacterium*; E: *Enterococcus*; L: *Listeria*; Lb: *Lactobacillus*; Lc: *Lactococcus*; Ln: *Leuconostoc*; P: *Pediococcus*; S: *Staphylococcus*; ND: not determined.

6. Safety assessment

The use of the *C. maltaromaticum* species as a protective agent in various food products regarding food safety could be limited due to its ability to produce tyramine from tyrosine. The level of tyramine produced varies depending on the food and on the strains (Masson et al., 1996; Leisner et al., 2007). In soft cheeses artificially inoculated with *C. maltaromaticum* LMA 28, no tyramine and histamine were detected (Edima, 2007). Since 2005, one strain of *C. maltaromaticum* (CB1) has been classed as Generally Recognized as Safe (GRN 00159) for use in ready-to-eat meat products. *C. maltaromaticum* has never been involved in human infection and cannot be considered as an opportunistic pathogen; only one case of isolation of this species from human pus has been reported in the medical literature (Chmelar et al., 2002).

7. Potential interest in cheese technology

To conclude, what can the role of the *C. maltaromaticum* species be in cheese technology? This opportunistic lactic acid bacterium is present in a low proportion in raw milk. It can grow in milk without coagulation because of a low production of L (+)-lactic acid from lactose due to a weak β-galactosidase activity, with no competition from starter LAB. It sustains low pH value (4.9) at the end of draining of soft cheese. It encounters propitious conditions at the end of ripening, after a cold storage period. At this step of cheese manufacture, at low temperature (13–14 °C) and high pH value (7–8), *C. maltaromaticum* can take over from the traditional LAB (*i.e. Lc. lactis* and *S. thermophilus*) which have already been lysed (Chamba, 2008). In appropriate conditions, it also produces aromatic substances (with a malt flavour), which could allow

a diversification of the cheese panel, in order to satisfy consumer demands. No product defect is related to the presence of this species in AOP soft cheeses. Moreover, the ability of *C. maltaromaticum* to produce bacteriocins inhibiting the growth of well-known foodborne pathogens such as *Listeria* sp. should reinforce the interest for its use as a non-starter LAB. However, further knowledge about metabolic pathways involved in the biosynthesis of flavouring compounds, about the regulation of bacteriocin production and the mechanisms of action of these molecules appears to be still necessary in order to promote a wider use of *C. maltaromaticum* in the dairy industry.

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III. Biosynthèse et rôle du 3-méthylbutanal: voies métaboliques, enzymes et stratégies de contrôle dans des fromages

Biosynthesis and role of 3-methylbutanal in cheese: major metabolic pathways, enzymes involved and strategies for control

Muhammad Inam Afzal, Kenza-Amel Boulahya, Citlalli Celeste Gonzàlez-Ariceaga, Muriel Jacquot, Stéphane Delaunay and Catherine Cailliez-Grimal

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Abstract

Branched chain aldehyde, 3-methylbutanal is associated as a key flavour compound to many hard/semi-hard cheese varieties. The presence and impact of this flavour compound in various foods like bread, meat and certain beverages is recently documented, however its presence and consequences regarding cheese flavour were not clearly reported. This paper gives an overview of the role of 3-methylbutanal in cheese perspectives, along with the major metabolic pathways and key enzymes leading to its formation. Moreover, different strategies are highlighted for the control of this particular flavour compound in specific cheese types.

Keywords: Cheese, flavour formation, 3-methylbutanal, metabolic pathways, control strategies

1. Introduction

The presence of branched-chain volatile aldehydes has been reported to be perceived either as a malty/off-flavour or as nutty/chocolate-like aroma among various cheese types associated to both hard and soft categories. Special emphasis has been given to 3-methylbutanal, as its concentration or relative abundance always seemed to be higher as compared to 2-methybutanal and 2-methylpropanal due to the efficient breakdown of leucine by the cheese microbiota (Ayad et al., 2004b; Bosset and Gauch, 1993; Centeno et al., 2002; Deetae et al., 2007; Irigoyen et al., 2007; Morales et al., 2003; Tucker and Morgan, 1967).

Some decades ago, the identification, chemical nature of malty flavour and microbial defects in milk resulting from the use of malty flavour producing strain, *Streptococcus lactis* var. *maltigenes* were dealt with in various works (Jackson and Morgan, 1954; MacLeod and Morgan, 1955; Morgan et al., 1966; Morgan, 1970; Sheldon et al., 1971). Since then, the researchers gave attention to the possible consequences of the presence of these flavour compounds while characterising cheese flavour profiles and corresponding cheese microflora.

Recently, many studies have highlighted both the desirable and non desirable role of 3-methylbutanal with respect to various cheese types. It is assumed that the flavour perception might be related to numerous factors including cheese moisture, texture, milk type, microflora and more particularly, internal balance of various odorous compounds generated from various sources like proteins, fat and carbohydrates. However, to get a desired flavour from a cheese is yet a very complex and difficult task, as little imbalance between various odorous compounds could impair the final flavour quality (Wallace and Fox, 1997).

Bacteria such as lactic acid bacteria (LAB) belonging to genera *Lactococcus*, *Lactobacillus*, *Streptococcus*, *Carnobacterium* and *Enterococcus* are generally supposed to be involved in the formation of 3-methylbutanal in milk and cheese (Afzal et al., 2010; Afzal et al., 2012; Helinck et al., 2004; Miller et al., 1974; Smit et al., 2004; Ward et al., 1999). Moreover, some yeasts such as *Debaryomyces hansenii*, *Yarrowia lipolytica* and *Geotrichum candidum* when used as adjunct or starter in soft cheese manufacturing were also found to contribute the formation of 3-methylbutanal (Bintsis and Robinson, 2004; Boutrou and Guéguen, 2005; Sørensen et al., 2011).

Extensive studies have been carried out to investigate the possible biosynthetic pathways of 3-methylbutanal in bacteria. The intracellular biosynthesis of 3-methylbutanal from leucine catabolism generally takes place by the two possible metabolic pathways in LAB: either by a direct pathway using α-ketoacid decarboxylase enzyme (KADC) such as proven for *Lactobacillus delbrueckii* subsp. *lactis* CNRZ 207 by Helinck et al. (2004), or by an indirect pathway comprising α-ketoacid dehydrogenase enzyme (KADH) such as proven for *Lactobacillus helveticus* CNRZ 32 and *Enterococcus faecalis* 10C1 by Helinck et al. (2004) and Ward et al. (1999). Recently, it has been demonstrated that in *Carnobacterium maltaromaticum* both metabolic pathways were present and functional (Afzal et al., 2012).

Due to the importance of this flavour compound in particular cheese types, numerous strategies have been proposed to control its production. Different approaches have been pointed out using exogenous addition of substrate, microbial adjuncts possessing potential and complementary metabolic pathways, bacteriocin induced lysis, and modifying the environmental conditions by changing oxygen or potential redox.

Recently, Smit et al. (2009) documented the origin, presence and impact of branched-chain aldehydes such as 3-methylbutanal, 2-methylbutanal and 2-methylpropanal in food like

bread, meat and certain beverages. The aim of the present article is to clarify and highlight the role and significance of 3-methylbutanal related to particular cheese types and to summarize the current knowledge for its possible control in cheese products.

2. Key enzymes and metabolic pathways involved in the biosynthesis of 3-methylbutanal from leucine catabolism among LAB

The catabolism of leucine during cheese ripening is mainly initiated by the action of microbial aminotransferases, although chemical degradation (Strecker degradation) can also occur (Ardo, 2006; Smit et al., 2009; Yvon et al., 1997; Yvon and Rijnen, 2001). Among LAB, the deamination of glutamate to α-ketoglutarate (α-KG) catalyzed by glutamate dehydrogenase (GDH) is usually linked to a transamination route.

2.1. Aminotransferase and glutamate dehydrogenase

Aminotransferase activity (AT) was found to be present in a large group of cheese related LAB. Activities varied and diversity existed among the strains (Brandsma et al., 2008; Fernández de Palencia et al., 2006; Smit et al., 2004; Yvon et al., 1997). Yvon et al. (1997) purified and characterized the major aromatic aminotransferase (AraT) from *L. lactis* and demonstrated the role of this enzyme in the initiation of degradation of several amino acids including leucine, which was responsible for the synthesis of precursors of aroma compounds usually found active under cheese ripening conditions. Moreover, it was found to exhibit overlapping substrate specificities towards both branched chain and aromatic amino acids (Yvon et al., 1997; Yvon and Rijnen, 2001).

The role of both aromatic and branched chain aminotransferases (AraT/BcaT) was studied by Rijnen et al. (2003) in a cheese model and it was demonstrated that both BcaT and AraT were involved in the degradation of leucine. Leucine was converted into α-ketoisocaproate by aminotransferase enzyme and during this conversion the amino group of leucine was transferred to α-ketoglutarate resulted in the formation of glutamic acid.

To intensify cheese aroma, Tanous et al. (2002) pointed the importance of GDH activity as major criterion for the selection of flavour-producing LAB strains. Later on, a beneficial effect on aroma formation was observed by using a combination of GDH positive lactobacilli with *L. lactis* ssp. *cremoris* NCDO763 (Kieronczyk et al., 2004). Tanous et al. (2005) explored the

biosynthetic pathways for α-KG formation and its impact on cheese aroma development and it has been shown that the citrate-oxaloacetate pathway, that requires citrate permease (CitP), citrate lyase (CitL) and aspartate aminotransferase, was operative for *L. lactis* ssp. *diacetylactis* and hence stimulated the conversion of amino acids.

2.2. Major metabolic pathways for the biosynthesis of 3-methylbutanal

The transamination of leucine results in α-ketoisocaproate, which is the central metabolite in leucine catabolism (Smit et al., 2004), and gives rise to 3-methylbutanal either directly as a result of non-oxidative decarboxylation by α-ketoacid decarboxylase (KADC) or indirectly via an oxidative decarboxylation by the activity of α-ketoacid dehydrogenase (KADH) (Helinck et al., 2004; Larrouture-Thiveyrat and Montel, 2003). In the literature, the direct pathway for the biosynthesis of 3-methylbutanal was very well documented in LAB. On the contrary, the indirect pathway was not studied extensively (Table 1).

Table 1. Demonstration (*in vitro* and via gene product) of direct/indirect pathways for the biosynthesis of 3-methylbutanal in LAB.

Bacterial species	**Strain**	**Study model**	**Pathways KADC/KADH**	**Reference**
L. lactis subsp. *cremoris*	NIZO B1157	*In vitro*/via gene product	KADC	Ayad et al., 1999; Ayad et al., 2001; Smit et al., 2004; Smit et al., 2005
L. lactis	IFPL730	*In vitro*/via gene product	KADC	De la Plaza et al., 2004; Fernández de Palencia et al., 2006
L. delbrueckii subsp. *lactis*	CNRZ 207	*In vitro*	KADC	Helinck et al., 2004
P. freudenreichii subsp. *shermanii*	TL 34	*In vitro*	KADH	Thierry et al., 2002
L. helveticus	CNRZ 32	*In vitro*	KADH	Helinck et al., 2004
E. faecalis	10C1	Via gene product	KADH	Ward et al., 1999; Ward et al., 2000
L. casei	ATCC 334	Via gene product	KADH	Liu et al., 2008
C. maltaromaticum	LMA 28	*In vitro*/via gene product	KADC/KADH	Afzal et al., 2012

2.2.1. The direct pathway

The direct pathway appeared to be rare among LAB and KADC activity was found to be highly strain dependant. KADC activity has been reported in *L. lactis* (Smit et al., 2004), some *L. lactis* wild strains (Ayad et al., 1999; Ayad et al., 2001; De la Plaza et al., 2004; Fernández de

Palencia et al., 2006), *L. delbrueckii* (Helinck et al., 2004) and *C. maltaromaticum* (Afzal et al., 2012).

The gene encoding KADC enzyme (*kdcA*) was identified by N-terminal sequencing of the partially purified protein in *L. lactis* (De la Plaza et al., 2004) or by screening a mutant library in *L. lactis* (Smit et al., 2005). In *L. lactis*, the KADC activity would be of prime importance in order to control desired formation of 3-methylbutanal, as most of the aldehydes could be formed through this pathway (Smit et al., 2004).

2.2.2. The indirect pathway

The indirect pathway constituted the oxidative decarboxylation of α-ketoisocaproate to isovaleryl-CoA by the KADH enzyme complex along with several intermediate enzymes including phosphotransferase (PTA), acyl kinase (ACK), and aldehyde dehydrogenase for the biosynthesis of 3-methylbutanal. This pathway has been reported *in vitro* in *Propionibacterium freudenreichii* (Thierry et al., 2002), *L. helveticus* (Helinck et al., 2004), *C. maltaromaticum* (Afzal et al., 2012) and via the gene product in *E. faecalis* (Ward et al., 1999). The KADH enzyme activity was determined and found to be dependant of both NAD^+ and $NADP^+$ in *C. maltaromaticum* (Afzal et al., 2012).

The genes encoding KADH complex were found encoded in one operon (*ptb-buk-bkDABC*) in *E. faecalis* (Ward et al., 1999) and in *L. casei* (Liu et al., 2008). In *C. maltaromaticum* LMA 28 strain, these genes encoding KADH enzyme complex (*bkdA, bkdB, bkdC and bkdD*) have been identified by degenerate primer design (Afzal et al., 2012).

2.3. Regulation of 3-methylbutanal biosynthesis

The biosynthesis of 3-methylbutanal in bacteria depends on the functionality of intracellular pathways and is mainly regulated by redox environment (NAD^+/NADH, H^+ yield, presence/absence of oxygen). Indeed, high formation of 3-methylbutanal in *L. lactis* (Kieronczyk et al., 2006) and *Proteus vulgaris* (Deetae et al., 2011) has been attributed towards the presence of oxygen or stimulation of KADC enzyme activity.

3. Presence and role of 3-methylbutanal in various varieties of cheeses

An overview of previous studies on flavour characteristics of some cheeses revealed the presence and crucial role of 3-methylbutanal for the unique flavour development in these cheese types (Table 2).

The presence of Strecker aldehydes in hard cheddar cheese made from cow's pasteurized milk was reported to be responsible for a nutty/balanced flavour and was considered as desirable (Avsar et al., 2004; Hannon et al., 2006). However, on the contrary, Egyptian Ras and Manchego cheese usually made from either cow/buffalo/sheep's raw milk revealed an unclean/burnt flavour (Ayad et al., 2004a; Centeno et al., 2002), which was considered as non desirable. This unclean/burnt flavour was attributed to the high levels of aldehydes and alcohols and was related to the poor quality milk used for cheese manufacture. The flavour perception and desirability of aldehydes in Parmigiano Reggiano, Parmesan and Roncal cheese made from either cow/sheep's raw and pasteurized milk were not clearly reported (Barbieri et al., 1994; Bosset and Gauch, 1993; Irigoyen et al., 2007).

The perceived chocolate-like aroma after six weeks of cheese ripening in semi-hard Proosdij-type cheese made from cow's pasteurized milk using a mesophilic strain *L. lactis* ssp. *lactis* B851 with acidifying mesophilic and an adjunct thermophilic culture was attributed to the presence of high concentration of 3-methylbutanal (Ayad et al., 2003). A similar chocolate-like flavour was perceived as well in Gouda/Proosdij type cheese and was considered as desirable (Engels et al., 1997; Van Leuven et al., 2008). During the study of aroma development in reduced-fat semi-hard cheese using culture adjunct of *Lactobacillus paracasei* (CHCC 4256), 4 times higher concentration of aldehydes and alcohols were determined as compared to controls but flavour perception/desirability was not clearly mentioned (Thage et al., 2005). Some wild strains of *L. lactis* isolated from ewes' raw milk cheeses were reported to produce high levels of aldehydes and alcohols, which were considered as responsible for abnormal odours (Morales et al., 2003).

Aldehydes and alcohols were generally present and considered as potent odorants in soft cheeses. At low concentrations, they were perceived as a fruity flavor and considered desirable, while at high concentration, they resulted in rather off-flavour and highly non desirable (Sable and Cottenceau, 1999). The impact of various microorganisms on aromatic profiles of soft cheese has started to be elucidated (Bintsis and Robinson, 2004; Deetae et al., 2009; Irlinger et

al., 2012; Massouras et al., 2006). A richer pattern of aroma compounds namely aldehydes, alcohols and esters were achieved using *L. paracasei* subsp. *paracasei* and *Debaryomyces hansenii* as adjuncts in the manufacture of Feta-type cheese (Bintsis and Robinson, 2004). During the study of aromatic profile of Teleme cheese made from either sheep's or goat's milk or a combination of both, Massouras et al. (2006) found the highest level of volatile compounds in cheese made from sheep's milk.

Table 2. Presence and role of 3-methylbutanal in different cheese types.

Cheese type	Cheese variety	Milk type	Raw/pasteurized	Bacteria/yeasts	Flavour/perception	Desirability of flavour	Literature
Hard	Cheddar	Cow	Pasteurized	*L. lactis* ssp. *lactis*, *L. lactis* ssp. *cremoris*	Dark chocolate/malty/nutty	D	Avsar et al., 2004
	Cheddar	Cow	Pasteurized	*L. lactis* ssp. *lactis* 303, *L. lactis* ssp. *cremoris* 227 ± EMC powder	balanced	D	Hannon et al., 2006
	Egyptian Ras	Cow/buffalo	Raw	Without addition of starter cultures	unclean	ND	Ayad et al., 2004a
	Manchego	Sheep/ewes	Raw	*L. lactis* (BCV^+, BCV^- , CSC)	Burnt/toasted/unclean/nuts	ND	Centeno et al., 2002
	Parmigiano Reggiano	cow	Raw	Without addition of starter cultures instead natural whey culture added	NR	NR	Bosset and Gauch, 1993
	Parmesan	cow	Raw	–	NR	NR	Barbieri et al., 1994
	Roncal	Sheep/ewes	Pasteurized	*L. lactis* (CSC), *L. paracasei* (adjunct)	NR	NR	Irigoyen et al., 2007
Semi-hard	Proosdij type/gouda	Cow	Pasteurized	Mixed strain mesophilic starter culture Bos, Mixed strain thermophilic starter culture APS, *L. lactis* ssp. *lactis* B851	Chocolate-like	D	Ayad et al., 2003
	Proosdij type	Cow	Pasteurized	Mixed strain mesophilic starter culture Bos, Mixed strain thermophilic starter culture APS	Chocolate-like/nutty	D	Engels et al., 1997
	Gouda-type	Cow	Raw/pasteurized	*L. lactis* ssp. *lactis* biovar *diacetylactis*, *L. lactis* ssp. *cremoris*	Chocolate-like/nutty	D	Leuven et al., 2008
	Ewes' raw milk	Sheep/ewes	Pasteurized	*L. lactis* (CSC and wild strains)	Roasted hazel/nuts	ND	Morales et al., 2003
	Reduced-fat round-eyed	Cow	Pasteurized	DL-starter (CH-N11, Chr. Hansen A/S), *L. paracasei* ssp. *paracasei* CHCC 4256	NR	NR	Thage et al., 2005
Soft	Feta-type	Sheep/ewes	Pasteurized	*L. paracasei* ssp. *paracasei*, *Debaryomyces hansenii*	NR	NR	Bintsis and Robinson, 2004
	Teleme	Sheep/ewes	Pasteurized	*L. lactis* ssp. *lactis*, *L. lactis* ssp. *cremoris*, *L. delbrueckii* ssp. *bulgaricus*, *Streptococcus thermophilus*	NR	NR	Massouras et al., 2006
	Camembert-type cheese model	Cow	Pasteurized	*Proteus vulgaris* 1M10, *Debaryomyces hansenii* 304	Fruity	NR	Deetae et al ., 2009
	Smear soft cheese model	Cow	Pasteurized	*L. lactis* ssp. *lactis*, model community (7 bacteria, 4 yeast), ± *Psychrobacter celer* 91, ± *Hafnia alvei* 2920	Fruity	NR	Irlinger et al., 2012

BCV^+ branched chain volatile compounds producing strains; BCV^- branched chain volatile compounds not producing strains; CSC commercial starter cultures; D desirable; ND non desirable; NR not reported

4. Strategies for control of 3-methylbutanal concentration in cheese

The significance and impact of 3-methylbutanal in cheese has attracted considerable attention for obtaining desired formation in specific cheese types. A number of strategies have been followed during these recent years to control the formation of 3-methylbutanal in cheese using cheese models at laboratory scale or *in vitro* investigations (Table 3). In this respect, a balanced flavour may be obtained by accelerating cheddar cheese ripening and proteolysis by the addition of free amino acids and enzyme modified cheese powder (EMC), however both strategies seemed to be expensive and not practical at industrial scale use (Hannon et al., 2006; Wallace and Fox, 1997). Most of the volatile compounds generated through amino acid catabolism, and defined the final aroma characteristics of particular cheese. To obtain desired flavour formation, the use of particular isolates with either potential enzyme activities (Ayad et al., 2003; Garde et al., 2007; Thage et al., 2005; Whetstine et al., 2006) or enzymes complementing metabolic pathways (Amárita et al., 2006; Ayad et al., 2001) seemed to be the most promising approach. Another interesting strategy for the enhancement of cheese aroma and more particularly, the aldehydes, could be the use of bacteriocin producing strains to induce lysis of the bacteriocin sensitive adjunct cultures and as a consequence, to promote the release of intracellular enzymes and their accessibility towards corresponding substrates (Fernández de Palencia et al., 2004; Martínez-Cuesta et al., 2002; Martínez-Cuesta et al., 2006). Many studies have started to investigate the influence of various parameters like oxygen or redox potential on the flavour forming pathways, for the control of desired formation of aldehydes/alcohols in cheese (Afzal et al., 2012; Deetae et al., 2011; Kieronczyk et al., 2006). Indeed, the presence of oxygen/oxidizing agent was found responsible for the increased formation of 3-methylbutanal (Deetae et al., 2011; Kieronczyk et al., 2006). Until now, a chocolate-like flavour due to 3-methylbutanal could not be perceived in soft cheese, instead main emphasis has been given to obtain a well balanced flavour.

Table 3. Proposed strategies for control of 3-methylbutanal concentration in cheese.

Proposed strategies	Cheese variety/model used	Type	Bacteria used	Resulted flavour	Literature
Addition of free amino acids at intermediate level	Cheddar	Hard	*L. lactis* ssp. *cremoris* 223	clean/mature	Wallace and Fox, 1997
Addition of enzyme-modified cheese powder for flavour/ripening acceleration	Cheddar	Hard	*L. lactis* ssp. *lactis* 303, *L. lactis* ssp. *cremoris* 227 + EMC powder	Balanced	Hannon et al., 2006
Addition of culture adjuncts	Cheddar	Hard	*L. lactis* 850, *L. lactis* ATCC 29146 (adjunct)	Nutty/chocolate-like	Whetstine et al., 2006
	Hispànico	Hard	*L. lactis* ssp. *lactis* INIA 639, *L. lactis* ssp. *lactis* INIA 437, *L. helveticus* LH 92	Intensed	Garde et al., 2007
	Proosdij	Semi-hard	Mixed strain mesophilic starter culture Bos, Mixed strain thermophilic starter culture APS, *L. lactis* ssp. *lactis* B851 (adjunct)	Nutty/chocolate-like	Ayad et al., 2003
	Reduced-fat round-eyed	Semi-hard	DL-starter (CH-N11, Chr. Hansen A/S), *L. paracasei* ssp. *paracasei* CHCC 4256 (adjunct)	Aromatic/sweet	Thage et al., 2005
Strains possessing enzymes complementing metabolic pathways	Gouda/cheddar	Hard	*L. lactis* ssp. *cremoris* SK110, *L. lactis* ssp. *cremoris* NIZO B1157	Chocolate-like	Ayad et al., 2001
	Milk	-	*L. lactis* IFPL730, *L. lactis* IFPL326	Ripened cheese	Amarita et al., 2006
Bacteriocin induced lysis	*In vitro*	-	*L. lactis* ssp. *lactis* IFLP359, *L. lactis* IFLP105 (lacticin 3147 producer)	Intensed	Martinez Cuesta et al., 2002
	In vitro	-	*L. lactis* ssp. *lactis* IFLP359, *L. lactis* IFLP730, *L. lactis* IFLP105 (lacticin 3147 producer)	Intensed	Martinez Cuesta et al., 2006
	Cheese model	-	*L. lactis* ssp. *lactis* IFLP3593, *L. lactis* IFLP730	Intensed	De la Plaza et al., 2004
Environmental modifications by static and shaking conditions	*In vitro*	-	*P. vulgaris* 1M10	Off flavour	Deetae et al., 2011
Oxidizing or reducing agents	*In vitro*	-	*L. lactis* ssp. *cremoris* NCDO 763, *L. lactis* ssp. *lactis* NCDO 1867	Off flavour	Kieronczyk et al., 2006

5. Conclusions

The branched chain aldehyde, 3-methylbutanal is associated as a key flavour compound to many hard/semi-hard cheese varieties, while, it is considered as potent aromatic compound in soft cheese. This flavour compound arises from leucine catabolism either by the direct pathway or indirect pathway or from both depending upon the functionality of these pathways in cheese related microorganisms. In some of the hard/semi-hard cheese varieties, the presence and role of 3-methylbutanal is regarded as chocolate-like and highly desirable. Many strategies have been proposed for the control of this flavour compound. The combination of knowledge of flavour forming pathways, control strategies, final flavour perception could lead to a better control of this specific flavour formation before considering its use in industrial applications.

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2ème Chapitre

Résultats

I. Impact de l'utilisation d'une flore lactique psychrotrophe (*Carnobacterium maltaromaticum*) sur l'écologie d'un fromage

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Carnobacterium maltaromaticum: Impact and interaction as a barrier species against the undesired flora of soft cheese

Muhammad Inam Afzal, Emilie Lhomme, Nehal Kamel Ali, Sophie Payot, Claire Gaiani, Frédéric Borges, Anne-Marie Revol-Junelles, Stéphane Delaunay and Catherine Cailliez-Grimal

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Chapitre II : Résultats

I. Impact de l'utilisation d'une flore lactique psychrotrophe (*Carnobacterium maltaromaticum*) sur l'écologie d'un fromage

I.1 Introduction

En technologie fromagère, les bactéries lactiques interviennent tout au long des nombreux processus de transformation du lait en fromage. Elles proviennent généralement des levains lactiques commerciaux mais sont aussi présentes dans le lait cru et dans l'environnement laitier, celles-ci sont alors appelées bactéries lactiques opportunistes.

Dans de nombreux fromages français d'Appellation d'Origine Protégée (AOP), en fin d'affinage, une flore lactique opportuniste a été mise en évidence : *Carnobacterium* qui, après stockage au froid ne provoque pas de déclassement des produits. Ce genre contient dix espèces isolées de diverses niches écologiques. Psychrotrophe et acidotolérante, l'espèce *maltaromaticum* peut constituer la flore lactique dominante de certains produits lactiques réfrigérés.

Les intérêts majeurs de l'utilisation de *C. maltaromaticum* dans les produits laitiers pourraient être basés sur quatre facteurs. i) cette espèce est capable de se maintenir dans le lait sans concurrence avec les bactéries starters, ii) elle synthétise des composés aromatiques, comme le 3-méthylbutanal, iii) elle peut produire des bactériocines à activité anti-*Listeria* iv) elle n'a jamais été impliquée dans des maladies humaines directement liées à la consommation de produits laitiers la contenant (Afzal et al., 2010).

Certaines souches de *C. maltaromaticum* sont déjà utilisées dans les produits alimentaires comme la viande et les fruits de mer en tant que cultures bio-protectrices contre *L. monocytogenes* (Brillet et al., 2005; Duffes et al., 1999; Matamoros et al., 2009; Vescovo et al., 2006).

Des études portant sur ce genre bactérien ont été menées au laboratoire à partir d'une collection comprenant trente trois souches lactiques appartenant au genre *Carnobacterium*, *Lactobacillus*, *Lactococcus* et *Leuconostoc*. Une présélection de six souches a été réalisée sur la base de leurs aptitudes à se développer dans le lait et sur des analyses sensorielles de caillés lactiques (Edima, 2007). Une analyse sensorielle des fromages dont le lait de fabrication a été

ensemencé avec ces différentes souches de *C. maltaromaticum* a permis de retenir la souche *C. maltaromaticum* LMA 28, comme ayant des potentialités aromatiques et texturantes intéressantes.

L'objectif de ce travail est, dans un premier temps, de conforter l'analyse sensorielle par l'étude des différences entre la souche retenue et les autres souches de *C. maltaromaticum* du laboratoire en comparant leurs aptitudes technologiques telles que leur capacité d'acidification du lait, leur tolérance à l'acidité, leur production de composés maltés ainsi que leur activité inhibitrice.

Dans un second temps, la souche *C. maltaromaticum* LMA 28 ayant été sélectionnée, il s'agira d'évaluer son impact sur la dynamique de diverses communautés microbiennes au sein d'un fromage à pâte molle.

Les résultats obtenus sont présentés sous la forme d'une publication soumise à Food Microbiology : ***"Carnobacterium maltaromaticum*: Impact and interaction as a barrier species against the undesired flora of soft cheese"**.

I.2 *Carnobacterium maltaromaticum*: Impact and interaction as a barrier species against the undesired flora of soft cheese

Abstract

Carnobacterium maltaromaticum is a lactic acid bacterium isolated from atypical soft cheese. The objective of this work was to study its potential positive impact when used in cheese technology. Phenotypic and genotypic characterization of six strains of *C. maltaromaticum* showed that they belong to different phylogenetic groups. Although these strains lacked the ability to coagulate milk quickly, they were acidotolerant. They did not affect the coagulation capacity of starter lactic acid bacteria, *Lactococcus lactis* and *Streptococcus thermophilus*, used in dairy industry. The study of the impact of *C. maltaromaticum* LMA 28 on the bacterial flora of the cheese revealed that its presence caused the decrease in the concentration of *Psychrobacter* sp., which might be responsible for accelerating the aging phenomena of cheese. An experimental plan was built to elucidate the inhibition of *Psychrobacter* sp. and *Listeria monocytogenes* and possible interaction between various factors (cell concentration, NaCl, pH and incubation time). Experiments showed that the cellular concentration of *C. maltaromaticum* LMA 28 was the main factor involved in the inhibition of *Psychrobacter* sp. and *L. monocytogenes* in the conditions tested.

Keywords: *Carnobacterium maltaromaticum*, soft cheese, bacterial interaction, *Psychrobacter*, *Listeria monocytogenes*

1. Introduction

Cheeses host a complex ecosystem characterized by a succession of different microbial groups occurring during the milk fermentation, ripening or storage. Lactic acid bacteria (LAB) are usually involved throughout the numerous processes, converting milk into cheese. The microorganisms generally play a positive role during the cheese maturation process, as they provide specific cheese flavors (Irlinger and Mounier, 2009). They generally come from commercial starter cultures but are also present in raw milk and in the dairy environment and are thus called opportunistic LAB (Fleet, 1999). The presence of an opportunistic psychrotrophic LAB named *Carnobacterium maltaromaticum* has been demonstrated in numerous French

cheeses (Appellation d'Origine Protégée, AOP) at the end of ripening and cold storage, without affecting the final product quality (Cailliez-Grimal et al., 2007; Millière et al., 1994).

Many *Carnobacterium* strains have been used previously as protective cultures against pathogenic bacteria (for instance *L. monocytogenes*) in fish and meat products (Brillet et al., 2005; Duffes et al., 1999; Matamoros et al., 2009; Vescovo et al., 2006). The bacteriocin-producing ability of *Carnobacterium* strains was found to play a key role in controlling spoilage and pathogenic bacteria in food and in *in vitro* model systems (dos Reis et al., 2011; Martin-Visscher et al., 2011; Bernardi et al., 2011), while external factors, including pH, salt and storage temperatures, were also shown to have a significant impact (Hwang, 2009; Leroi et al., 2012).

The identification, isolation, ecology and technological aspects of *C. maltaromaticum* in cheese were recently documented (Afzal et al., 2010). The objective of this study was to characterize several *C. maltaromaticum* strains isolated from soft cheese by comparing their technological aptitude, acid tolerance and inhibitory activities. Moreover, its impact on the dynamics of microflora during soft cheese manufacture was studied by a molecular identification method (TTGE) and an experimental plan (Doehlert matrix).

2. Material and methods

2.1. Bacterial strains, media and growth conditions

Strains used in this study, along with culture media and growth conditions, are presented in Table 1. Strains were grown in trypticase soy broth (Biokar, Beauvais, France) supplemented with yeast extract (TSB-YE), except for *Escherichia coli*, which was grown in Lysogeny broth (LB) (Biokar, Beauvais, France) and *Psychrobacter* sp. grown in TSB-YE containing 8 % NaCl (Table 1). For selective enumeration, PALCAM agar (Biokar, Beauvais, France) was used for *L. monocytogenes* CIP 82110, MCM (Edima et al., 2007) was used for *C. maltaromaticum* LMA 28 and TSB-YE with NaCl (8 %) for *Psychrobacter* sp.

Table 1. Bacterial strains used in this study.

Bacterial species	Strain designation[a]	Growth medium	Incubation temperature (°C)
Carnobacterium maltaromaticum	LMA 5	TSB-YE	30
C. maltaromaticum	LMA 7	TSB-YE	30
C. maltaromaticum	LMA 14	TSB-YE	30
C. maltaromaticum	LMA 23	TSB-YE	30
C. maltaromaticum	LMA 28	TSB-YE	30
C. maltaromaticum	LMA 32	TSB-YE	30
C. divergens	DSM 20623^T	TSB-YE	30
C. gallinarum	DSM 4847^T	TSB-YE	25
C. mobile	DSL 4848^T	TSB-YE	30
C. viridans	CIP 107728	TSB-YE	25
Listeria grayi	CIP 6818^T	TSB-YE	30
L. innocua	CIP 12511	TSB-YE	30
L. innocua	CIP 107775	TSB-YE	30
L. ivanovii	CIP 12510	TSB-YE	30
L. ivanovii	CIP 7842	TSB-YE	30
L. monocytogenes	CIP 82110^T	TSB-YE	37
L. monocytogenes	CIP 7831	TSB-YE	37
Enterococcus faecalis	ATCC 19433	TSB-YE	37
E. faecalis	CIP 78117^T	TSB-YE	37
E. faecium	CIP 106742	TSB-YE	37
Escherichia coli	XL-1Blue	LB	37
Lactococcus lactis	DSM 20481	TSB-YE	30
Streptococcus thermophilus	INRA 302	TSB-YE	37
Psychrobacter sp.	LMA 1	TSB-YE + NaCl (8 %)	30

[a] ATCC: American Type Culture Collection, Mannassas, USA; CIP: Collection de l'Institut Pasteur, Paris, France; DSM: Deutsche Sammlung von Mikro-Organismen und Zellkulturen, Göttingen, Germany; INRA: Institut National de la Recherche Agronomique; LMA: Laboratoire de Microbiologie Alimentaire, ENSAIA-INPL, Nancy, France; SLCC: Special *Listeria* Culture Collection, University of Wurzburg, Germany; T: Type.

2.2. Identification

2.2.1. Determination of carbon assimilation profiles

Carbohydrate fermentation patterns were determined using API 50 CH test strips (BioMérieux, Marcy l'Etoile, France), according to the manufacturer's instructions.

2.2.2. Molecular typing using pulsed-field gel electrophoresis (PFGE)

Carnobacterium strains were inoculated in 10 ml of TSB-YE broth and grown at 30 °C overnight. The culture (1 ml) was inoculated in 20 mL of TSB-YE broth and incubated at 30 °C until the bacterial suspension absorbance reached 0.4 to 0.8 (exponential phase). Pulsed-Field

Gel Electrophoresis (PFGE) of all *Carnobacterium* strains was performed with the enzyme *SmaI* as described by Haenni *et al.* (2010).

2.2.3. Antibacterial assays and primers used for the detection of genes encoding bacteriocins

Antibacterial activity of six strains of *C. maltaromaticum* was determined against *Carnobacterium*, *Listeria and Enterococcus* species by the agar well diffusion method (Mathieu et al., 1993) (Table 1). Primers (Eurogenetec, Herstal, Belgium) (Table 2) were used for the detection of genes encoding different bacteriocins in DNA of *C. maltaromaticum* strains

Table 2. Oligonucleotides used in this study.

Target	Target gene	Primer	Sequences (5'- 3')	Reference
Carnobacteriocin BM1 (IIa)	cbnBM1	cbnBM1-for cbnBM1-rev	GCT ATC TCT TAT GGC AAT GGT G TAG AAG CCC ATC CAC CGA TA	Quadri et al., 1994
Carnobacteriocin B2 (IIa)	cbnB2	cbnB2-for cbnB2-rev	TGA ATA GCG TAA AAG AAT TAA ACG TG TTA CGG TCT CCT ACC AAT GGA	Quadri et al., 1994
Piscicolin Pi126 (IIa)	pisA	pisA-for pisA-rev	CGG CTC CAC CTG TAG TCA A TGG CGT TTC CTG TAA TAA AAA TG	Jack et al., 1996
Carnobacteriocin CbnA (IIc)	cbnA	cbnA-for cbnA-rev	TGG TGG AGA CCA AAT GTC AG AAC CAG CCT AAA GGA CCT GAA	Worobo et al., 1994
Divergicin DvnA (IIc)	dvnA	dvnA-for dvnA-rev	GGG GCA ACA TTT TTC TCA AC ACC TCC TGC TAT TGC ACC AC	Worobo et al., 1995
Divergicin Dvn750 (IIc)	dvn750	dvn750-for dvn750-rev	CAG AAC AAT TTC TTC CCT TGG TTT ATT CCA GCC CAC ACT CC	Holck et al., 1996
Carnocyclin CcnA (circular)	ccnA	ccnA-for ccnA-rev	GCA TAT GGT ATC GCA CAA GG GCA ATT GCT GCT TTA ACT GCT	Martin-Visscher et
Total bacterial community	V3 region of 16S rDNA	HDA1-GC HDA2	CGCCCGGGGCGCGCCCCGGGCGGGGCGGGGCAC GGGGGGACTCCTACGGGAGGCAGCAGT GTATTACCGCGGCTGCTGGCA	Ogier et al., 2002
		2HDA1-EcoR1 2HDA2-EcoR1	CCGGAATTCGACTCCTACGGGAGGCAGCAGT CCGGAATTCGTATTACCGCGGCTGCTGGCA	Serhan et al., 2009

2.2.4. Isolation of Psychrobacter sp.

Cheese sample (5 g) was suspended in 50 mL of citrate buffer (trisodium citrate, 2 % w/v and NaCl, 8.5 g/L) and homogenized in stomacher (Interscience, St. Nom-la-gatehouse, France). A volume of 1.5 mL was then inoculated into three different selective media: brilliant green bile broth, lactose broth (BLBVB Biokar, Beauvais, France) and TSB-YE + NaCl (8 %). These media were incubated at 4 ^{o}C and 30 ^{o}C during 48 and 24 h respectively. After enrichment,

bacteria were isolated on TSA-YE. Gram staining, oxidase and catalase tests were performed on each isolated colony to confirm bacterial species.

2.3. Technological aptitude

2.3.1. Milk acidification

Bacterial cultures were inoculated in 150 mL of semi-skimmed milk supplemented with 1 g/L of yeast extract at the initial population of 10^8 cfu/mL. The cultures were incubated in a thermostatic water bath at 30 ^oC to monitor acidification using an automatic multimeter (Consort D230, Neuilly-sur-Seine, France). The kinetic parameters, maximum acidification rate (V_m) and time corresponding to V_m (T_m) were determined by the method described by Spinnler and Corrieu (1989).

2.3.2. Milk coagulation

The bacterial strains were subcultured twice in TSB-YE at their optimum growth temperatures during 16 h. The cell pellets were obtained as described above. Milk coagulation times were obtained by the use of a rheometer (Stress Tech, Rheologica Instruments AB, Sweden). The cell pellets were used to inoculate 18 mL (placed in a C25 cup) of semi-skimmed UHT milk supplemented with yeast extract (1 g/L) in a tank thermostatically controlled by a water bath at 30 ^oC. The geometry used to monitor milk coagulation is a paddle system (four blades placed at right angles to each other) specially designed by Rheologica to follow milk coagulation. The shear rate was fixed at 100 s^{-1} and remained constant throughout the analysis. Data are collected automatically every 20 seconds and all runs are carried out at least in duplicate.

2.3.3. Acid tolerance

The strains were subcultured twice in TSB-YE at 30 ^oC for 16 h. These precultures were used to inoculate TSB-YE and semi-skimmed UHT milk at 1:10 ratio and incubated at 30 ^oC. Cultures were stopped in exponential growth phase (OD_{660nm} = 0.4). Volume of 1 mL of the suspension was used to inoculate 9 mL of TSB-YE or milk to a specified value of pH. The pH range selected varied from 6.5 to 3, adjusted using HCl or lactic acid in aseptic conditions. After

a contact time of 4 h at 30 °C, spreadings and numerations were carried out by inclusion method in Petri dishes using 1 mL of sample and 12 mL of TSA-YE.

2.3.4. Analysis of flavor compounds by headspace gas chromatography

Flavor compounds produced by the different strains were investigated in semi-skimmed (UHT) milk and in different reaction mixtures. Strains (5 mL) were inoculated in TSB-YE culture medium at optimum growth temperatures. Cells in late exponential phase were harvested by centrifugation (5000 x g, 4 °C, 10 min) and resuspended in 5 mL of semi-skimmed UHT milk. These suspensions were used to inoculate 45 mL semi-skimmed UHT milk incubated at 30 °C and 4 °C for 24 h. Samples were stored at – 25 °C until analysis by headspace gas chromatography (Afzal et al., 2012).

2.3.5. Cheese manufacture

Among the six laboratory strains tested, only *C. maltaromaticum* LMA 28 was selected to study its impact on microbial community and final quality of soft cheese. Soft cheeses were manufactured in the laboratory (Edima et al., 2007). Milk was inoculated with different initial populations (10^1, 10^4 and 10^7 cfu/mL named 2A, 3B and 4C) of *C. maltaromaticum* LMA 28 and without *C. maltaromaticum* LMA 28 (control named 1T). After ripening for 10 days at 10 °C, the cheeses were stored at 4 °C for 42 days. Cheese samples were taken at different time intervals during 42 days and frozen at – 20 °C.

2.3.6. Sensory Analysis

Two experiments were carried out. In the first experiment, the panel was composed of seven people. Milk samples inoculated with *C. maltaromaticum* strains were tested for the presence of aroma and its intensity was numerically expressed in a scale from one (low) to five (high).

In the case of the second experiment, the contribution of *Carnobacterium* to aroma development of soft cheeses was assessed during the ripening period by comparing the aroma of cheeses made with or without adjunct cultures by a triangle aroma test (AFNOR, 1995) as described by Milesi *et al.* (2007). Samples (5 g) were placed in sealed glass containers and maintained in an oven at 30 °C for 30 min, after which they were provided to the panel. The

same containers were re-equilibrated in the oven and reused twice. Three samples were supplied to each panelist; 2 of the samples had been taken from the same cheese, whereas the third was different. The samples were identified with random 3-digit codes. The panel, composed of 12 untrained members, was asked to find the sample that differed and to comment briefly on the differences. The panelists evaluated cheese duplicates but they did not replicate measurements with the same cheeses.

2.3.7. Statistical Analysis

Data from chemical composition, microbiological counts, FAA, and organic acid and volatile compound assays were analyzed by one-way ANOVA with a 95 % confidence level. All analyses were made in duplicate. All statistical analyses were performed using the SPSS 10.0 software (SPSS Inc., Chicago, IL).

2.4. Nucleic acid extraction

2.4.1. Samples from isolate colonies or broth culture

The bacterial DNA was extracted as previously described (Serhan et al., 2009). gDNA was extracted with DNA stool kit (Qiagen, France) and plasmid DNA using NucleoSpin Plasmid kit (Macherey Nagel, France) according to manufacturer recommendations.

2.4.2. Samples of cheeses

Cheese sample (5 g) was suspended in 50 mL of citrate buffer and homogenized using a stomacher (Interscience, France). For each sample, 40 mg of pronase (Sigma) and 100 µL of β-mercaptoethanol were added and placed at 37 ^{o}C overnight. The cells were washed twice using ultrapure water after centrifugation (5000 rpm, 10 min and 4 ^{o}C). DNA was extracted as previously described (Serhan et al., 2009).

2.4.3. PCR amplification of the V3 region of 16S rDNA and analysis of PCR products by TTGE

DNAs extracted from cheeses were subjected to universal PCR targeting the eubacterial 16S rRNA gene. The V3 region of the gene was amplified using the primers HDA1-GC and HDA2 (Ogier et al., 2002) for the TTGE analysis, or the universal primers 2HDA1-EcoR1 and 2HDA2-EcoR1 (Serhan et al., 2009) for the cloning experiments. The PCR products obtained

from amplification of the V3 region of 16S rRNA were analyzed by Temporal Temperature Gel Electrophoresis TTGE (Dcode universal mutation detection system, Bio-Rad Laboratories, USA) as described by Serhan *et al.* (2009) (Table 2).

2.4.4. Classical PCR protocols

The amplification was carried out in a thermal cycler iCyclerTM (Bio-Rad Laboratories, Hercules, CA, USA). For bacteriocin gene detection, the thermal program for amplification was 95 °C for 5 min, followed by 35 cycles of 95 °C for 1 min, 53 °C for 30 s, 72 °C for 30 s with a final step for 5 min. For TTGE, the amplification program was 94 °C for 4 min, followed by 30 cycles of 94 °C for 30 s, 58 °C for 30 s, 68 °C for 1 min with a final step for 7 min.

2.4.5. Cloning and sequencing for bacterial specie identification

To identify the bacterial species present in cheese, the amplified 16S rDNA were inserted into a cloning vector, plasmid pUC19, containing the gene for ampicillin resistance as described by Serhan *et al.* (2009). The constructions were sequenced (GATC, Germany) and sequences were used to identify bacterial species using BLAST analysis on NCBI (http://blast.ncbi.nlm.nih.gov/Blast.cgi).

2.5. Experimental design and data analysis for investigation of bacterial interactions in milk

In order to study the bacterial interactions in co-cultures and to investigate the optimum culture conditions for the inhibition of *Psychrobacter* sp. LMA 1 and *L. monocytogenes* CIP 82110 in semi skimmed UHT milk, a Doehlert experimental design (Doehlert, 1970) was carried out. Four variable factors (X) of different ranges were: cellular concentration of *C. maltaromaticum* LMA 28 (X_1 = 0 - 10^8 cfu/mL; 5 levels); NaCl (X_2 = 0 - 9 g/L; 7 levels); pH (X_3 = 5.5 - 8.5; 7 levels) and duration of incubation (X_4 = 24 - 72 h; 3 levels) (Table 3).

Table 3. Experimental plan (Doehlert matrix) using four variable factors (X) (*Carnobacterium* cells concentration, NaCl, pH and duration) to check the growth reponse (R expressed as $Log_{10}N$) of *Psychrobacter* sp. and *L. monocytogenes* CIP 82110 in semi skimmed UHT milk.

	FACTORS			
Experiment No.	***C. maltaromaticum* LMA 28 (log_{10})**	**NaCl (g/L)**	**pH**	**Duration (h)**
1	8.0	4.50	7.00	48
2	0.0	4.50	7.00	48
3	6.0	9.00	7.00	48
4	2.0	0.00	7.00	48
5	6.0	0.00	7.00	48
6	2.0	9.00	7.00	48
7	6.0	6.00	8.50	48
8	2.0	3.00	5.50	48
9	6.0	3.00	5.50	48
10	4.0	7.50	5.50	48
11	2.0	6.00	8.50	48
12	4.0	1.50	8.50	48
13	6.0	6.00	7.38	72
14	2.0	3.00	6.62	24
15	6.0	3.00	6.62	24
16	4.0	7.50	6.62	24
17	4.0	4.50	8.13	24
18	2.0	6.00	7.38	72
19	4.0	1.50	7.38	72
20	4.0	4.50	5.87	72
21	4.0	4.50	7.00	48
22	4.0	4.50	7.00	48
23	4.0	4.50	7.00	48
24	4.0	4.50	7.00	48

Each experiment was started with the initial population of *Psychrobacter* sp. LMA 1 and/or *L. monocytogenese* CIP 82110 at about 10^3 cfu/mL and incubated at 25 ^oC for respective duration. Selective enumerations were carried out after incubation for *C. maltaromaticum* LMA 28, *Psychrobacter* sp. LMA 1 and *L. monocytogenes* CIP 82110 using selective media, MCM (Edima et al., 2007), TSA-YE + NaCl 8 % and PALCAM. The factorial growth response (R) of either *Psychrobacter* sp. LMA 1 and/or *L. monocytogenes* CIP 82110 expressed as $Log_{10}N$ can be predicted in all experimental regions according to the following equations

$$R\ (Log_{10}N) = \beta_0 + \beta_1X_1 + \beta_2X_2 + \beta_3X_3 + \beta_4X_4 + \beta_{11}X_1^2 + \beta_{22}X_2^2 + \beta_{33}X_3^2 + \beta_{44}X_4^2 + \beta_{12}X_1X_2 + \beta_{13}X_1X_3 + \beta_{14}X_1X_4 + \beta_{23}X_2X_3 + \beta_{24}X_2X_{24} + \beta_{34}X_3X_4$$

Where β_0 is the constant term; β_1 determines the influence of *C. maltaromaticum*; β_2 the influence of NaCl; β_3 the influence of pH; β_4 the influence of duration; β_{12} the interaction effect between *C. maltaromaticum* and NaCl; β_{13} the interaction effect between *C. maltaromaticum* and pH; β_{14} the interaction effect between *C. maltaromaticum* and duration; β_{23} the interaction effect between NaCl and pH; β_{24} the interaction effect between NaCl and duration; β_{34} the interaction effect between pH and duration and β_{11}, β_{22}, β_{33} and β_{44} are "shape" parameters. Data analysis, ANOVA and polynomial regressions were performed using the NEMROD-W software (LPRAI, Marseille, France).

3. Results

3.1. Identification of the strains and comparison of the technology ability

3.1.1. Biochemical and genotypic identifications

The differentiation of the 6 strains of *C. maltaromaticum*, isolated from soft cheeses was performed using biochemical and genotypic tests. A comparison of their biochemical characteristics showed weak differences in their fermentation pattern (data not shown). All isolates were consistent in their ability to produce acid from β-gentiobiose, saccharose, N-acetyl glucosamine, amygdaline, arbutine, esculine, salicine, cellobiose, D-mannose, D-fructose, D-glucose and ribose. All were able to used lactose and hydrolyzed products of lactose (glucose and galactose). Like other carnobacteria (Cailliez-Grimal et al., 2007), isolates did not produce acid from erythritol, D-arabinose, L-xylose, adonitol, rhamnose, dulcitol, inuline, melezitose, D-raffinose, xylitol, D-lyxose, D-fucose, D-arabitol and L-arabitol.

In the conditions tested, three strains (LMA 7, 14 and 32), among the *C. maltaromaticum* strains tested, exhibited no antibacterial activity against the target strains tested (data not shown). The three other strains (LMA 5, 23, 28) were active against all the *Carnobacterium* and *Listeria* sp. (data not shown). Moreover, *C. maltaromaticum* LMA 5 and 28 were found to possess inhibitory activities against the three strains of *Enterococcus* (data not shown). DNA extracted from all the strains was tested with six pairs of bacteriocin primers. An amplicon was obtained with BM1 primers for all the strains and nonewith the other primers (pil6, CbnA, Dvn750, CcnA). Only DNA extracted from *C. maltaromaticum* LMA 7 and 14 did not give an amplicon with the B2 primers (data not shown).

The diversity of the *C. maltaromaticum* isolates was examined by the PFGE method. The restriction patterns observed for the isolates were diverse and generated between 12 and 21 bands (Figure 1). The strains displayed a unique band pattern with seven or more than seven differences indicative of a genetic diversity. So each strain was declared non-associated to each other according to the criteria of Tenover *et al.* (1995).

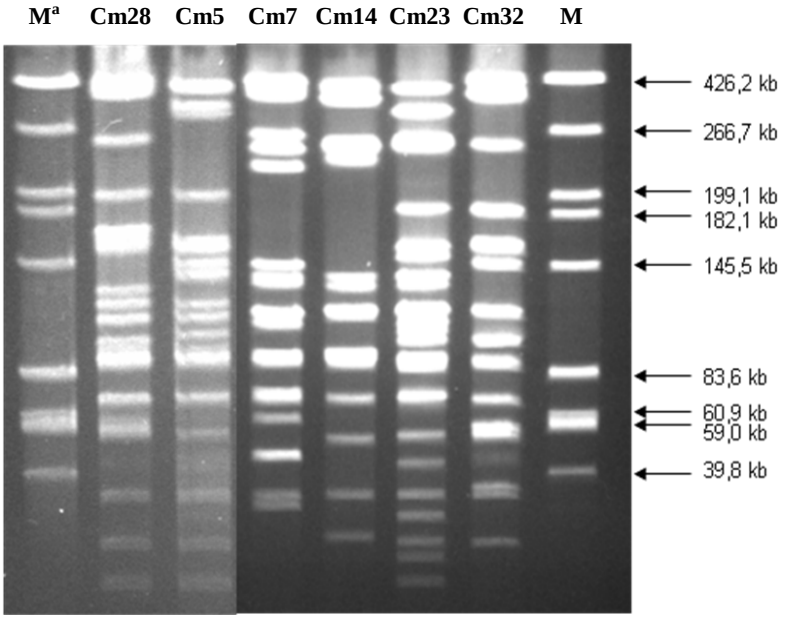

Figure 1. Pulsed-field gel electrophoretic profiles of gDNA of *C. maltaromaticum* strains digested by *Smal*.
[a] Molecular weight marker from gDNA of *Streptococcus agalactiae* A 909 digested by *SmaI*.
C. maltaromaticum LMA 28; *C. maltaromaticum* LMA 5; *C. maltaromaticum* LMA 7; *C. maltaromaticum* LMA 14; *C. maltaromaticum* LMA 23; *C. maltaromaticum* LMA 32.

3.2. Technological aptitude

Among the six strains of *C. maltaromaticum* cultured in milk supplemented with yeast extract (1 g/L), four strains (*C. maltaromaticum* LMA 5, 14, 23 and 28) showed weak and similar acidification kinetics of milk with a V_m of 0.1 pH/h, T_m of 20 ± 1 h and a final pH of 5.4 compared to *L. lactis* and *S. thermophilus*, starter LABs used in dairy industry (Table 4). In the presence of HCl and lactic acid, the weak acidifying strains, *C. maltaromaticum* LMA 14, 23 and 28 conserved a survival rate of 100 % at lower pH in milk compared to non acidifying strains, *C. maltaromaticum* LMA 7 and 32.

In the conditions tested, all the strains were able to produce 3-methylbutanal in milk cultured at 4 °C and at 30 °C after 24 h of incubation (Table 4). Milk bottles tasted and smelled

significantly differently ($P < 0.05$) for each *Carnobacterium* species after 48 hours. The taste panel could clearly distinguish the milk malty flavor in bottles inoculated with *C. maltaromaticum* LMA 28 and lactic and aromatic flavor with LMA 14. The other strains did not exhibit particular flavors. The strain *C. maltaromaticum* LMA 28 was tested for cheese assay.

Table 4. Acidification kinetic parameters, bacterial cells viability in milk at pH 4, detection of 3-methylbutanal and sensory analysis of milk at 30 °C during 24 h of strains tested.

Strains	**Acidification kinetics parameters**			**% Bacterial viability in milk at pH 4**		**3-methylbutanal (µM) (SE ≤ 0.5)**	**Sensory analysis 1(weak) to 5 (high)**
	V_m (pH/h)[a] (SE ≤ 0.01)	T_m (h) SE ≤ 0.1	Final pH SE ≤ 0.1	HCl	Lactic acid		
C. maltaromaticum LMA 5	0.11	21.0	5.4	22	70	70.0	2
C. maltaromaticum LMA 7	ND	ND	6.2	10	35	65.0	2
C. maltaromaticum LMA 14	0.10	19.0	5.4	60	100	50.0	3
C. maltaromaticum LMA 23	0.11	19.0	5.4	100	100	50.0	3
C. maltaromaticum LMA 28	0.11	18.0	5.4	80	100	65.0	4
C. maltaromaticum LMA 32	ND	ND	5.9	8	40	20.0	2
S. thermophilus INRA 302	0.57	7.0	4.3	ND	ND	0.0	ND
L. lactis DSM 20481	0.46	12.0	4.4	100	100	70.0	ND

V_m: Maximum acidification rate; T_m: Corresponding time to V_m; ND: Non detected
[a] Results are expressed as the mean of three independent experiments with the standard error (SE).

The milk coagulation time of *C. maltaromaticum* LMA 28 was significantly different from to the one of starter bacteria (Table 5). However, the coagulation times of *L. lactis* and *S. thermophilus* were not significantly different when used in single or in combination with *C. maltaromaticum* LMA 28 indicating that the presence of *C. maltaromaticum* LMA 28 has no influence on the coagulation capacity or velocity of these starters (Table 5).

Table 5. Coagulation time of semi-skimmed milk in presence of different LAB at 30 °C.

Strains	Coagulation time[a] (h)
C. maltaromaticum LMA 28	7.93 ± 1.69
L. lactis DSM 20481	1.8 ± 0.87
S. thermophilus INRA 302	2.36 ± 1.26
L. lactis DSM 20481 + *C. maltaromaticum* LMA 28	2.25 ± 0.21
S. thermophilus INRA 302 + *C. maltaromaticum* LMA 28	3.26 ± 1.36
S. thermophilus INRA 302 + *L. lactis* DSM 20481	1.76 ± 0.25
S. thermophilus INRA 302 + *L. lactis* DSM 20481+ *C. maltaromaticum* LMA 28	1.93 ± 0.23

[a] Values are mean ± standard deviation of three independent experiments.

3.2.4. Sensorial impact of C. maltaromaticum LMA 28 on cheeses

After 20 and 34 days of ripening, cheeses tasted and smelled significantly differently ($P < 0.05$) to the reference without *C. maltaromaticum* LMA *28*. The taste panel clearly distinguished the malty and butyric flavor in cheeses inoculated with *C. maltaromaticum* LMA 28.

3.3. Impact of C. maltaromaticum LMA 28 on bacterial flora of soft cheese

The electrophoretic profiles of bacterial populations corresponding to cheese samples after 25 days of ripening (Figure 2) show that cheese 1T, non-inoculated with *C. maltaromaticum* LMA 28 generated 6 bands and cheeses inoculated with different initial levels of *C. maltaromaticum* LMA 28 generated 5 bands. The comparison of DNA profiles from cheese with those obtained from DNA extracted from bacterial cultures (*C. maltaromaticum*, *L. lactis* DSM 20481 and *S. thermophilus* INRA 302) revealed a co-migration of some bands. Specifically, the band from the pure culture of *L. lactis* DSM 20481 co-migrated with band profiles of four cheeses (band b) suggesting that this species was present in these cheeses. In addition, the band corresponding to *C. maltaromaticum* LMA 28 (band c) co-migrated with band profiles of cheeses 3B and 4C. However, TTGE technique has a detection limit of 10^4 cfu/g and below this threshold, the bacterial species are not detected (Ogier et al., 2002). The additional band (band d) present in cheese non-inoculated with *C. maltaromaticum* LMA 28 was absent in the profiles of other cheeses. A similar analysis was performed on cheeses after 30 and 42 days of ripening and similar profiles were observed (data not shown).

The band d, only present in control cheese, was selected for cloning and sequencing and the sequence was compared by performing blastn alignments. This corresponded to *Psychrobacter* sp. with 100 % identity (closest accession number FN433052.1)

After enrichment using different selective media (BLBVB, Lasseur and TSB-YE + NaCl 8 %) and isolation at 4 ^{o}C and 30 ^{o}C on TSA-YE agar, Gram staining, catalase and oxidase tests were performed. Gram-negative colonies with positive catalase and oxidase tests were isolated to obtain pure cultures. The colonies obtained after 10 days at 4 ^{o}C or 48 h at 30 ^{o}C were round, mucous, whitish with regular edges. The V3 regions of the 16S rRNA of these colonies were amplified by PCR with primers HDA1-GC and HDA 2. The resulting fragments were then sequenced (GATC, Germany) and corresponded to *Psychrobacter* sp.

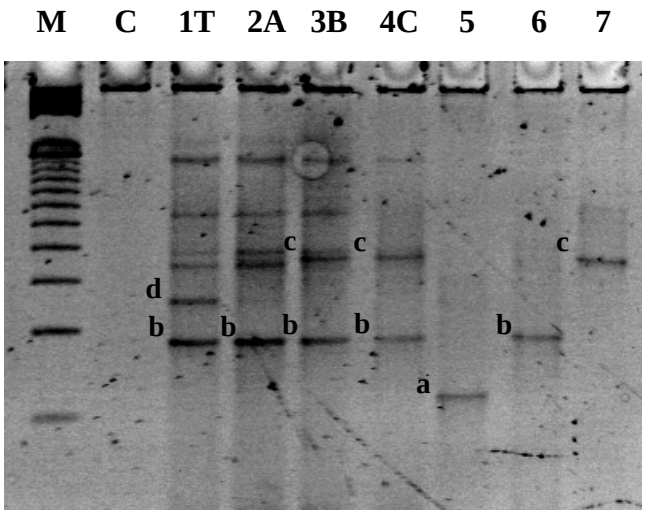

Figure 2. TTGE of PCR amplification products with primers a) HDA1-GC, b) HDA2. M: marker; C: control; 1T, 2A, 3B, 4C: PCR products of DNA extracted from cheeses 1T, 2A, 3B and 4C; 5: PCR product of gDNA of *S. thermophilus* INRA 302 (band a); 6: PCR product of gDNA of *L. lactis* DSM 20481 (band b); 7: PCR product of gDNA of *C. maltaromaticum* 28 (band c).

3.4. Interactions of C. maltaromaticum LMA 28 with Psychrobacter sp. LMA 1 and L. monocytogenes CIP 82110

To study the impact of *C. maltaromaticum* LMA 28 cells concentration on the two target strains *Psychrobacter* sp. LMA 1, isolated previoulsly and *L. monocytogenes* CIP 82110, sensitive to the bacteriocins CbnBM1 and CbnB2, and to search for the optimum conditions of

their inhibition in milk (NaCl, pH and duration), a Doehlert experimental design was used and polynomial equations were obtained. Table 6 shows the coefficient estimates and the determination of model coefficients that were fitted to the experimental data.

The coefficient terms of polynomial equations for *C. maltaromaticum* LMA 28 were the least ($\beta 1$ = - 1.532; - 1.547) predicted that the cellular concentration of *C. maltaromaticum* LMA 28 influenced the response by decreasing *Psychrobacter* sp. LMA 1 and *L. monocytogenes* CIP 82110 counts cells number. However, NaCl, pH and duration had less influence toward the response of *Psychrobacter* sp. LMA 1 and *L. monocytogenes* CIP 82110, but the interactions between them had an influence represented by negative coefficient values. Therefore, the increase in the cellular concentration of *C. maltaromaticum* LMA 28 improved the inhibition of both *Psychrobacter* sp LMA 1 and *L. monocytogenes* CIP 82110 in milk.

Thus, keeping the pH and NaCl constant, the maximum inhibitory growth responses of *Psychrobacter* sp. LMA 1 could be predicted by an increase in the concentration of *C. maltaromaticum* LMA 28 and decrease in the duration of incubation (Figure 3a). Moreover, keeping the NaCl and duration constant, the maximum inhibitory growth responses of *L. monocytogenes* CIP 82110 could be predicted by an increase in the concentration of *C. maltaromaticum* LMA 28 and decrease in pH (Figure 3b).

Maximum inhibition for *Psychrobacter* sp. LMA 1 was obtained at 10^6 cfu/mL cellular concentration of *C. maltaromaticum* LMA 28, 4 g/L of NaCl, and 6.6 of pH during 27 h respectively. Maximum inhibition for *L. monocytogenes* CIP 82110 was obtained at 10^5 cfu/mL cellular concentration of *C. maltaromaticum* LMA 28, 5.6 g/L of NaCl, and 5.6 of pH during 42 h respectively.

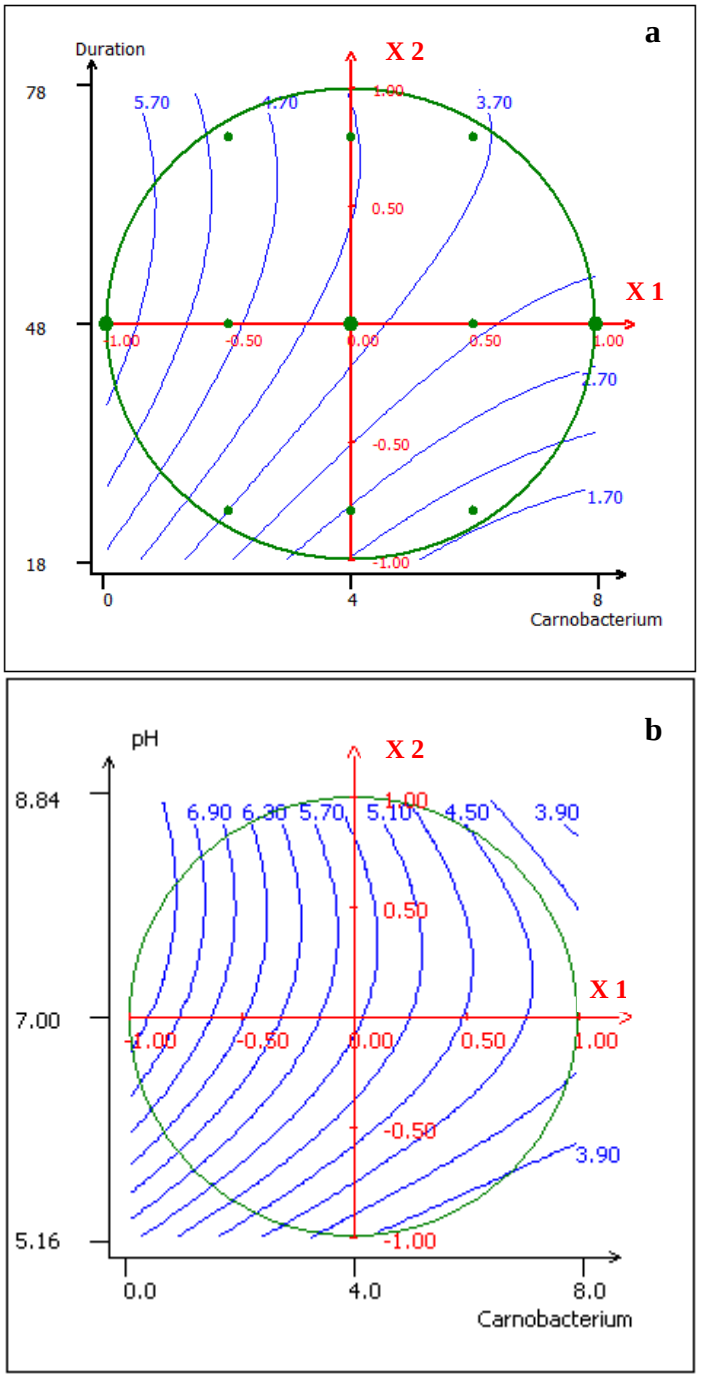

Figure 3. 3D graphical presentation for the optimum inhibitory response (R) in milk of a) *Psychrobacter* sp. LMA 1 in function of cellular concentration of *C. maltaromaticum* LMA 28 (log_{10}) and duration where, X1 axis = cellular concentration of *C. maltaromaticum* LMA 28 and X2 = duration at fixed values of NaCl and pH (4.5 g/L, 7, centre of the domain) and of b) *L. monocytogenes* CIP 82110 in function of cellular concentration of *C. maltaromaticum* LMA 28 (log_{10}) and pH where, X1 axis = cellular concentration of *C. maltaromaticum* LMA 28 and X2 = pH at fixed values of NaCl and duration (4.5 g/L, 48 h, centre of the domain).

4. Discussion

The LABs comprise a wide variety of species which themselves include highly diverse strains. The carbon assimilation profiles of six strains of *C. maltaromaticum* isolated from the same habitat showed only few differences between these strains. All strains were found to possess a gene encoding c*bnBM1* and only *C. maltaromaticum* LMA 7 and LMA 14 does not possess the gene encoding *cbnB2*. Although all strains of *C. maltaromaticum* have at least one gene encoding a bacteriocin, none of the target bacteria tested was inhibited by *C. maltaromaticum* LMA 7 and LMA 14, suggesting that the gene was not expressed in the culture conditions tested. The PFGE analysis showed that all the six strains of *C. maltaromaticum* were different from each other according to the criteria of Tenover (Tenover et al., 1995), showing a diversity of this species in the same biotope.

In cheese industry, two species played a major role in the transformation of milk to cheese i.e. *L. lactis* and *S. thermophilus,* which acidify and coagulate milk quickly. Our results revealed that *C. maltaromaticum* has no function during the acidification and coagulation stage of milk. Moreover, despite an optimal growth at alkaline pH, the weak acidifying strains of *C. maltaromaticum* are able to withstand low pH between 3 and 4.5 during acidification of milk. The TTGE profiles of cheeses inoculated with a different initial population of *C. maltaromaticum* LMA 28 showed that this species was present after 42 days of ripening regardless of its initial concentration.Thus, *C. maltaromaticum* was found compatible with starter LAB and its presence did not alter the ability of starter LAB to quickly transform milk into lactic gel and is able to survive during the maturation of cheese. In presence of *C. maltaromaticum* LMA 28, the band corresponding to the genus *Psychrobacter* present in cheese after 25 days does not appear after 35 days in cheese inoculated with an initial population of 10^1 cfu/mL of *C. maltaromaticum* LMA 28 and in other cheeses inoculated with an initial population of 10^4 and 10^7 cfu/mL of *C. maltaromaticum* LMA 28. These bacteria can be able to grow at low temperatures and tolerate high percentage of NaCl between 1 to 6 %. Different species of *Psychrobacter* have been isolated from various ecosystems; cold or hot, slightly or heavily salted, frozen sea, fish, refrigerated meat and from clinical samples. In case of cheese, this bacterium could arise either from raw milk or environmental contamination. It belongs to the order *Pseudomonadales*, well known in food microbiology as spoilage flora of dairy products

that exhibit high proteolytic activities. It could play a role in accelerating the aging process of cheese. To reduce this process, *C. maltaromaticum* could be use as ripening flora

During cheese manufacture, milk samples were inoculated with *L. lactis, S. thermophilus* and different quantities of *C. maltaromaticum* along with ripening flora *Geotrichum candidum* and *Penicillium camemberti*. From the results of TTGE profiles, post-environmental contaminations were evident. Indeed 5 to 6 species might be present as 5 to 6 bands were observed (Figures 2 and 3). Furthermore, it should be considered with this method that a band might be a representative of different species if these species co-migrated. According to the literature, when there was more than 97 % homology between the sequences of 16S rRNA, the sequences can be considered as arising from identical species (Stackebrandt et al., 2002). The identification of species present on the TTGE gel by sequencing and BLAST analysis has helped to highlight the band 'c' corresponded to *L. lactis*, found consistent as it was used as starter LAB in cheese manufacture.

The study on the inhibition of the two target strains *Psychrobacter* sp. LMA 1 and *L. monocytogenes*, in milk predicted that the cellular concentration of *C. maltaromaticum* the most inhibiting factor resulted in high inhibitory growth response of *Psychrobacter* sp. and *L. monocytogenes* in milk. However, the combination of other variables had also an impact. The exact mechanism of inhibition is still unknown and deserves further investigations. It could be related to competition effects for substrates/nutrients, food environmental changes like pH, oxygen or redox, and to the release of antimicrobial compounds targeting competing cells (Leroy and De Vuyst, 2001). Various predictive models have been adapted to study the interactions of spoilage bacteria in growth media and food systems including mixed cultures (Gimenez and Dalgaard, 2004; Malakar et al., 1999), in mono and co-cultures (Antwi et al., 2007; Antwi et al., 2008; Charlier et al., 2009; Cornu et al., 2011). Therefore, the modeling approach will be an interesting tool to predict the factors responsible for the inhibition of *Psychrobacter* sp. and *L. monocytogenes* in growth media and food systems.

5. Conclusion and perspectives

The use of one strain of *C. maltaromaticum* in cheese technology and its impact on the microflora of soft cheese has been demonstrated in this study. *C. maltaromaticum* cannot be used as starter in cheese industry, as it lacked the necessary acidifying capacity compared to other

currently used starter cultures (for instance *S. thermophilus* and *L. lactis*) but it has no interference on these starters.

In soft cheese, its presence caused the decrease in the concentration of *Psychrobacter* sp. which might be responsible for accelerating the aging phenomena of cheese. Moreover, the cellular concentration of *C. maltaromaticum* LMA 28 is the main factor involved in the inhibition of *Psychrobacter* sp. and *L. monocytogenes* CIP 82110 in our experimental conditions. However, the exact mechanism of inhibition is still unknown and deserves further investigations. In addition, *C. maltaromaticum* LMA 28 produced malty/chocolate like aroma due to 3-methylbutanal from the catabolism of leucine (Afzal et al., 2012). However, it is essential to study its impact on proteolysis and lipolysis during cheese ripening. Being psychrotrophic and alcalinophil, having antibacterial activity and an ability to increase flavor, this species could play a major role as cheese ripening flora.

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I.3 Contributions de l'article

Bien que les profils d'assimilation des sucres et des spectres antibactériens soient assez semblables, des différences ont été révélées par typage moléculaire (PFGE). La caractérisation génotypique des six souches de *C. maltaromaticum* isolées de fromages à pâte molle a permis de montrer qu'elles ne sont pas phylogénétiquement identiques.

La cinétique d'acidification du lait par ces souches de *C. maltaromaticum* était faible par rapport à celle observée avec *L. lactis* et *S. thermophilus*. Cependant, ces souches conservent un taux de survie de 100 % à des valeurs de pH de 4 dans le lait.

L'analyse sensorielle de laits inoculés avec six souches de *C. maltaromaticum* a permis de sélectionner *C. maltaromaticum* LMA 28 pour la fabrication de fromage sur la base de son profil aromatique, de texture et de sa croissance dans le lait.

En industrie fromagère, deux espèces jouent un rôle majeur lors de la transformation du lait en fromage, *L. lactis* et *S. thermophilus*. En effet, par leurs propriétés acidifiantes fortes et rapides, elles sont utilisées comme levain en fabrication fromagère. Ainsi, au début du process de transformation, *S. thermophilus* acidifie rapidement le lait à 39 °C, puis, *L. lactis* lui succède lorsque la température diminue.

La présence de *C. maltaromaticum* LMA 28 en co-cultures avec *L. lactis* ou *S. thermophilus* lors de la coagulation du lait s'est avérée n'avoir aucune influence significative sur la capacité de coagulation ces starters. Ces résultats montrent que *C. maltaromaticum* n'a aucune fonction lors de cette première étape d'acidification du lait. De plus, malgré une croissance optimale à des pH alcalins, elle est capable de résister à des valeurs de pH faibles, compris entre 3 et 4,5, lors de l'acidification du lait par la production d'acide lactique à partir du glucose par les bactéries permettant la coagulation du lait.

Ainsi, *C. maltaromaticum* est compatible avec les starters d'autant plus que sa présence n'altère en rien la capacité de ces dernières à transformer rapidement le lait en gel lactique. Par conséquent, *C. maltaromaticum* pourrait survivre dans le milieu si elle était utilisée comme levain lactique, puis réussir à s'implanter après la phase d'acidification afin de persister lors de la phase d'affinage.

L'étude de l'impact de *C. maltaromaticum* LMA 28 sur la microflore du fromage à pâte molle a révélé que sa présence était responsable de la diminution de la concentration de *Psychrobacter*. D'après la littérature (Bowman, 2006), le genre *Psychrobacter* inclut les

bactéries à Gram négatif, coccobacilles, aérobies, non-mobiles, osmotolérantes et strictement aérobies. Ces bactéries ont une croissance à de basses températures et tolèrent des pourcentages de NaCl compris entre 1 et 8 %. Les différentes espèces de *Psychrobacter* ont été isolées d'écosystèmes variés, froids ou chauds, peu ou fortement salés, allant de la mer glacée aux poissons et viandes réfrigérés, et aux échantillons cliniques. Dans le cas des fromages analysés, ce genre pourrait provenir soit de lait cru, soit d'une contamination environnementale. Ce genre peut résister aux conditions d'affinage et au froid. Il appartient à l'ordre des *Pseudomonadales* bien connu en microbiologie alimentaire comme flore d'altération des produits laitiers ayant un fort pouvoir protéolytique. Il pourrait jouer un rôle dans l'accélération du phénomène de vieillissement des fromages. D'après les résultats obtenus, *C. maltaromaticum* diminuerait la concentration en *Psychrobacter*, limitant ainsi les problèmes de vieillissements prématurés. Les mécanismes entrant en jeu sont à déterminer.

L'étude des interactions entre *Psychrobacter* sp. et *L. monocytogenes* par rapport aux quatre facteurs (population initiale de *C. maltaromaticum*, concentration en NaCl, pH et durée d'incubation) en utilisant un plan d'expériences a permis de révéler que la concentration cellulaire initiale de *C. maltaromaticum* LMA 28 est le facteur principal impliqué dans l'inhibition de *Psychrobacter* sp. et de *L. monocytogenes* CIP 82110 dans nos conditions expérimentales.

II. Identification des voies de biosynthèse du 3-méthylbutanal à partir du catabolisme de la leucine chez *Carnobacterium maltaromaticum* LMA 28

Identification of metabolic pathways involved in the biosynthesis of flavor compound 3-methylbutanal from leucine catabolism by *Carnobacterium maltaromaticum* LMA 28

Muhammad Inam Afzal, Stéphane Delaunay, Cédric Paris, Frédéric Borges, Anne-Marie Revol-Junelles and Catherine Cailliez-Grimal

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II. Identification de la voie de biosynthèse du 3-méthylbutanal à partir du catabolisme de la leucine chez *Carnobacterium maltaromaticum* LMA 28

II.1 Introduction

Il est généralement admis que la formation des composés aromatiques pendant le processus de fermentation dans les produits laitiers est principalement liée aux bactéries lactiques, levures et moisissures, équipées d'un grand nombre d'enzymes intracellulaires. Les principaux composés odorants proviennent de la glycolyse, la protéolyse et la lipolyse au cours de l'affinage du fromage. La caséine est dégradée en acides aminés (AA), qui peuvent être convertis en composés aromatiques suivant plusieurs voies métaboliques. Parmi les aldéhydes à chaîne branchée, le 3-méthylbutanal ayant une flaveur maltée ou chocolatée est un composé aromatique trouvé dans de nombreux fromages. Parmi les bactéries lactiques, les genres *Lactococcus, Lactobacillus, Streptococcus, Enterococcus* et *Carnobacterium* sont généralement impliqués dans la formation de 3-méthylbutanal dans le lait et le fromage (Afzal et al., 2010; Helinck et al., 2004; Miller et al., 1974; Smit et al., 2004; Ward et al., 1999). Certaines levures telles que *D. hansenii, Y. lipolytica* et *G. candidum,* utilisées comme ajout ou starter dans des fromages à pâte molle contribuent également à la formation de 3-méthylbutanal (Brintsis et Robinson, 2004; Boutrou et Guéguen, 2005; Sørensen et al., 2011).

Dans la littérature, la présence de 3-méthylbutanal a été signalée et perçue soit comme une saveur maltée soit comme un défaut dans des fromages à pâte dure ou à pâte molle. La biosynthèse intracellulaire de 3-méthylbutanal à partir du catabolisme de la leucine a généralement lieu selon deux voies métaboliques possibles: soit par une voie directe en utilisant l'α-cétoacide décarboxylase (KADC) (*L. delbrueckii* subsp. *lactis* CNRZ 207) (Helinck et al., 2004), ou par une voie indirecte comprenant une α-cétoacide déshydrogénase (KADH) (*L. helveticus* CNRZ 32, *E. faecalis* 10C1) (Helinck et al., 2004; Ward et al., 1999).

Nous avons étudié les voies métaboliques responsables de la biosynthèse de 3-méthylbutanal à partir du catabolisme de la leucine chez *C. maltaromaticum* LMA 28, une souche isolée du fromage à pâte molle et sélectionnée précédemment (Résultats, chapitre II, partie I) sur la base de ses potentialités aromatiques dans le lait. Deux souches de contrôle ont également été incluses dans l'étude présentée ici, *L. delbrueckii* subsp. *lactis* CNRZ 207 présentant une activité KADC et une absence d'activité KADH (KADC+, KADH-), et *L.*

helveticus CNRZ 32, ayant seulement une activité KADH (KADH +, KADC-) (Helinck et al., 2004).

Des cellules non croissantes des différentes souches bactériennes ont été incubées en présence et en absence de méta-arsénite de sodium, inhibiteur spécifique du complexe enzymatique KADH afin d'étudier l'impact de ce dernier sur la biosynthèse de 3-méthylbutanal chez *C. maltaromaticum* LMA 28. Des dosages enzymatiques ont été également réalisés. La présence de gènes codant pour diverses enzymes a été recherchée en utilisant des amorces dégénérées.

Les résultats obtenus sont présentés sous la forme d'une publication publiée dans International Journal of Food Microbiology (2012, 157, 332-339) : **"Identification of metabolic pathways involved in the biosynthesis of flavor compound 3-methylbutanal from leucine catabolism by *Carnobacterium maltaromaticum* LMA 28"**.

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Identification of metabolic pathways involved in the biosynthesis of flavor compound 3-methylbutanal from leucine catabolism by *Carnobacterium maltaromaticum* LMA 28

Muhammad Inam Afzal [a], Stéphane Delaunay [b], Cédric Paris [a], Frédéric Borges [a], Anne-Marie Revol-Junelles [a], Catherine Cailliez-Grimal [a,*]

[a] Laboratoire d'ingénierie des biomolécules, Université de Lorraine, 2 avenue de la Forêt de Haye B.P. 172, 54505 Vandoeuvre-lès-Nancy, France
[b] Laboratoire réactions et génie des procédés, CNRS, Université de Lorraine, 2 avenue de la Forêt de Haye B.P. 172, 54505 Vandoeuvre-lès-Nancy, France

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ABSTRACT

Carnobacterium maltaromaticum strains are widely found in food including fish, meat and some dairy products. Producing a malty/chocolate like aroma due to 3-methylbutanal from the catabolism of leucine is a general characteristic of this species. In this study, we investigated metabolic routes responsible for the biosynthesis of this flavor compound from the catabolism of leucine in *C. maltaromaticum* LMA 28, a strain isolated from mold ripened soft cheese. Depending on the lactic acid bacterium, leucine can be converted into 3-methylbutanal following two possible metabolic pathways: either directly by α-ketoacid decarboxylase (KADC) pathway or indirectly by α-ketoacid dehydrogenase (KADH) pathway. Both KADC (41.0 ± 3.0 nmol/mg protein/min) and KADH (1.43 ± 0.62 nmol/mg protein/min) activities were detected and determined in vitro in *C. maltaromaticum* LMA 28. *C. maltaromaticum* LMA 28 slightly reduced the production of 3-methylbutanal from leucine in the presence of a specific inhibitor of KADH enzyme complex, i.e. sodium meta-arsenite, suggesting that both pathways were involved in vivo in leucine catabolism. Moreover the presence of genes encoding aminotransferase, glutamate dehydrogenase, α-ketoacid decarboxylase, α-ketoacid dehydrogenase and aldehyde dehydrogenase was confirmed. *C. maltaromaticum* is then the first lactic acid bacterium in which presence of both metabolic routes responsible for the biosynthesis of 3-methylbutanal from leucine catabolism was confirmed in vitro and in vivo as well.

1. Introduction

Flavor formation in dairy products is mainly related to lactic acid bacteria (LAB), yeasts and molds, which are equipped with a large number of key intracellular enzymes responsible for its formation during the fermentation process in specific food product. Casein, the main constituent of milk protein is degraded into amino acids (AAs), which can be converted into flavor compounds following several metabolic routes during ripening of cheese. These involved enzymatic process carried out by intracellular enzymes both from starter and non starter LAB (Klein et al., 2001; Smit et al., 2004; Thierry et al., 2002). During the past decade extensive studies have been conducted on the generation of flavor compounds in milk and in cheese (Amárita et al., 2006; Asteri et al., 2009; Avsar et al., 2004; Ayad et al., 2001; Bourdat-Deschamps et al., 2004; Brandsma et al., 2008; Deetae et al., 2009; Delgado et al., 2010; Garde et al., 2006; Kieronczyk et al., 2003; Rijnen et al., 2003; Tanous et al., 2005; Whetstine et al., 2006; Ziadi et al., 2010). Among them a branched aldehyde i.e. 3-methylbutanal having malty or chocolate note was found to be a potent aroma compound in many food products, both fermented and non-fermented, resulting from the degradation of leucine (Afoakwa et al., 2009; Smit et al., 2009). The formation of 3-methylbutanal in high amounts has been reported as off-flavors in raw milk (Molimard and Spinnler, 1996; Morgan, 1976), despite the fact, it is also recognized as a key aroma compound in many cheeses (Avsar et al., 2004; Ayad et al., 2003; Barbieri et al., 1994; Bosset and Gauch, 1993; Whetstine et al., 2006), suggesting that good control of flavor production by the starter or non starter cultures could be essential for a well balanced flavor (Kieronczyk et al., 2006; Smit et al., 2004).

The first step in the biosynthesis pathway of this flavor compound is the transamination of leucine yielding α-ketoisocaproic acid as demonstrated by the fact that a ketoacid is required to accept the amino group from leucine degradation (Kieronczyk et al., 2006; Smit et al., 2004). This ketoacid then rises to 3-methylbutanal via two catabolic routes either direct or indirect (Helinck et al., 2004; Thierry et al., 2002) (Fig. 1). The direct pathway is a non-oxidative decarboxylation by α-ketoacid decarboxylase (KADC). The indirect pathway, an oxidative decarboxylation via α-ketoacid dehydrogenase complex (KADH), generates isovaleryl-CoA which is transformed into isovaleric acid by acylkinase (ACK) and phosphotransacylase (PTA) and then to 3-methylbutanal via aldehyde dehydrogenase (AldDH) (Beck et al., 2004).

* Corresponding author.
E-mail address: Catherine.Cailliez@ensaia.inpl-nancy.fr (C. Cailliez-Grimal).

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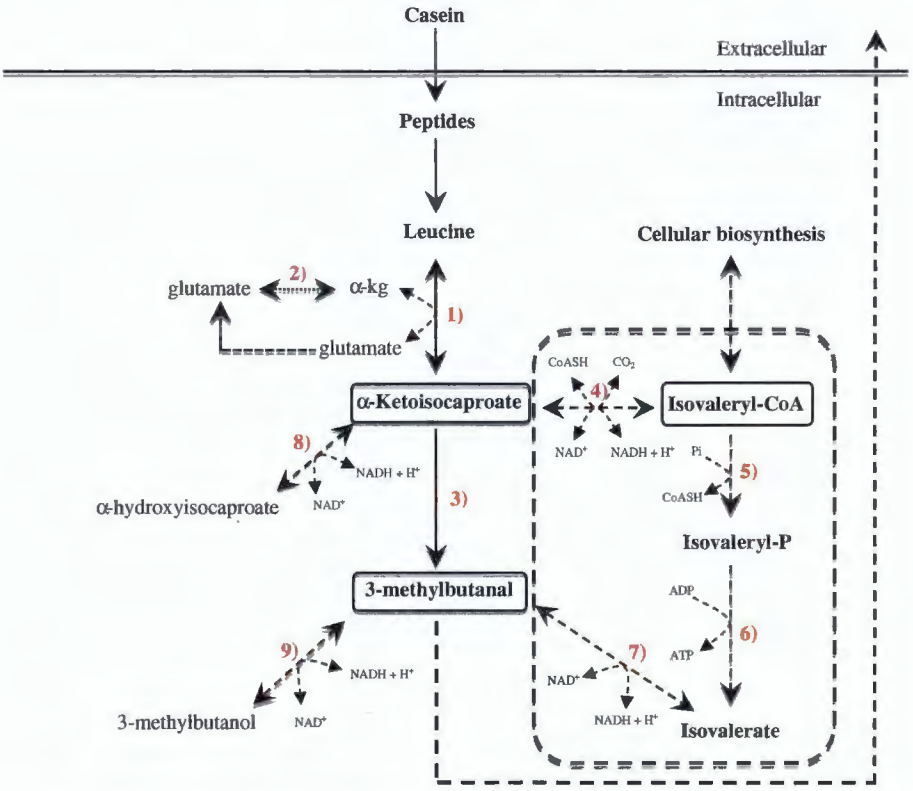

Fig. 1. Intracellular possible metabolic pathways for the biosynthesis of 3-methylbutanal from leucine catabolism by lactic acid bacteria (LAB). Enzymes of the direct pathway (in the solid line): 1) AT, aminotransferase, 2) GDH, glutamate dehydrogenase, and 3) KADC, α-ketoacid decarboxylase. Enzymes of indirect pathway (in the dashed lines): 4) KADH, α-ketoacid dehydrogenase, 5) PTA, phosphotransferase, 6) ACK, acylkinase, and 7) AldDH, aldehyde dehydrogenase. Other enzymes include 8) HADH, α-hydroxyacid dehydrogenase and 9) AlcDH, alcohol dehydrogenase.

KADH comprises 3 catalytic components including α-ketoacid dehydrogenase (subunits E1-α and E1-β), dihydrolipolyltransacylase (E2) and lipoamide dehydrogenase (E3). E1 catalyzes both decarboxylation of α-ketoacid and oxidative transfer of acyl group to the lipolyl moiety of E2 using thiamine pyrophosphate (TPP) and Mg^+ as cofactors. E2 catalyzes the acyl group transfer to CoA generating an acyl-CoA. Finally the resulting dihydrolipoamide is oxidized back to the disulphide form by the E3 component at the expense of NAD^+ as cofactor resulting in acyl-CoA formation (Liu et al., 2008; Reed, 1974). α-Hydroxyacid dehydrogenase (HADH) activity, results in the production of α-hydroxyisocaproic acid, which according to literature does not contribute to any flavor compound (Smit et al., 2004).

The leucine catabolism by *Carnobacterium* has previously been studied in *Carnobacterium piscicola* 545, a strain of meat origin (Larrouture-Thiveyrat and Montel, 2003). It was proposed that leucine catabolism was increased with the addition of glucose and α-ketoisocaproic acid under shaking conditions. *Carnobacteria* are complex facultative anaerobes, heterofermentative microorganisms that can be able to grow at low temperatures under different environmental conditions (both aerobic and anaerobic with increased CO_2 concentrations) and can produce lactic acid, ethanol and CO_2 from pyruvate under reducing conditions (anaerobically), whereas oxidizing conditions (aerobic) favor the pyruvate metabolization through the TCA cycle generating additional NADH, H^+ and ATP.

The presence of *C. maltaromaticum* in Brie cheeses, a variety of AOP (protected designation of origin) French surface mold-ripened soft cheese, was reported for the first time in 1994 (Milliere et al., 1994) and confirmed in 2007 (Cailliez-Grimal et al., 2007). This species constituted the main psychrotrophic LAB that are able to grow even at alkaline pH value reaching high levels (10^8–10^9 cfu/mL). Producing the malty or chocolate-like compound, 3-methylbutanal, is a general characteristic of this species (Afzal et al., 2010; Miller et al., 1974). However the metabolic routes involved in the biosynthesis of 3-methylbutanal are not yet identified. This work focuses on the identification of the metabolic routes involved in leucine catabolism for the biosynthesis of this potent flavor compound, 3-methylbutanal in *C. maltaromaticum* LMA 28.

2. Material and methods

2.1. Bacterial strains and growth conditions

Bacterial strains used in this study were purchased from different collections (Table 1) and were maintained in trypcase-soy, broth (TSB; Biomérieux, Craponne, France) supplemented with 0.6% yeast extract (YE, Biokar Diagnostics, Beauvais, France) at −25 °C in 20% (v/v) glycerol. All strains were subcultured in TSB-YE and incubated at their optimal growth temperatures (Table 1). *Carnobacterium maltaromaticum* LMA 28, which previously showed good growth patterns in milk and in TSB-YE (Edima et al., 2008), with the reference strain, *C. maltaromaticum* DSM 20730^T (Collins et al., 1987; Hiu et al.,

Table 1
Bacterial strains used in this study.

Bacterial species	Strain designation[a]	Incubation temperature (°C)
Carnobacterium maltaromaticum	LMA 28	30
C. maltaromaticum	DSM 20730^T	30
Lactococcus lactis	DSMZ 20481	30
Lactobacillus helveticus	CNRZ 32	44
Lactobacillus delbrueckii subsp. *lactis*	CNRZ 207	44

[a] DSM: Deutsche Sammlung von Micro-Organismen und Zellkulturen, Gottingen, Allemagne; LMA: Laboratoire de Microbiologie Alimentaire, ENSAIA-INPL, Nancy, France; CNRZ: Centre National de la Recherche Zootechnique, Jouy-en-Josas, France; T: Type.

1984) were selected to study in vitro leucine catabolic pathways. Two control strains were selected, which were previously well studied for AAs catabolism (Helinck et al., 2004). *Lactobacillus delbrueckii* subsp. *lactis* CNRZ 207 was found to exhibit KADC activity and to lack KADH activity ($KADC^+$, $KADH^-$), while *Lactobacillus helveticus* CNRZ 32, exhibits KADH activity and lacks KADC activity ($KADH^+$, $KADC^-$) (Helinck et al., 2004). One commercial starter strain, *Lactococcus lactis* DSM 20481 was also included. All chemicals were purchased from Sigma-Aldrich (St. Louis, MO, USA).

2.2. Preparation of cell suspensions and cell free extracts

Strains were grown as described above. Cells in the late exponential phase were harvested by centrifugation (10,000 ×g, 15 min, 4 °C), washed with saline solution (NaCl 8.5 g/L), and then washed with 100 mM Tris–HCl buffer (pH 7.0). Cell pellets were then suspended in the same buffer to an optical density of 200 at 600 nm. Aliquots of cell suspensions were stored at −80 °C. Cell free extracts (CFE) were obtained by sonication of cell suspensions (40 kHz, 8 cycles of 30 s and 60 s space out). All steps were performed at 0 to 4 °C. Cellular debris was removed by centrifugation (15,000 ×g, 20 min, 4 °C) and the supernatants were kept on ice until enzyme assays. Protein contents of CFE were determined by BCA protein assay reagent kit (Sigma-Aldrich, St. Louis, MO, USA). Triplicate measurements were performed.

2.3. Production of 3-methylbutanal by the strains as determined by headspace gas chromatography

Metabolite production was assessed in *C. maltaromaticum* LMA 28, *C. maltaromaticum* DSM 20730^T along with *L. delbrueckii* subsp. *lactis* CNRZ 207, *L. helveticus* CNRZ 32 and *L. lactis* DSM 20481 by headspace gas chromatography (Smit et al., 2004). Cell suspensions (OD_{600} of 200) were incubated in reaction mixtures containing leucine 3 mM, α-ketoglutarate (α-KG) 10 mM (pH adjusted to 5.6 by adding NaOH), pyridoxal 5 phosphate (PLP) 0.05 mM, glucose 0.5% in Tris–HCl buffer 100 mM (pH 5.6) with and without the addition of sodium meta-arsenite ($NaAsO_2$) 10 mM, a specific inhibitor of KADH enzyme complex (Webb, 1966). Controlled samples containing cell suspensions and glucose without the addition of substrate and cofactors were also incubated. Incubations were done at 37 °C for up to 48 h. Samples (1 mL) were taken at different intervals and stored in hermetic 10 mL vials at −25 °C until analysis by headspace gas chromatography. A 1.0 ml headspace sample was injected splitless on the column after 2 min of incubation at 90 °C. The chromatograph used (PR 2100, Périchrom, Saulx-lès-Carthusian, France) was equipped with an injector with headspace (Headspace HT 300A, Périchrom) and with a flame ionization detector (FID). The volatile compounds were separated using a capillary column (Périchrom) of 30 m × 0.25 mm × 0.2 μm and proportioned by integrator WINILAB III (Périchrom). The carrier gas was nitrogen under a constant flow of 2.0 mL/min. The temperature of the detector was maintained at 190 °C. The vials were pressurized during 30 s and the injection lasts 1 s. The adjustment of the quantity of product by the valve of open escape or "Split" is 5:5. The oven temperature was initially kept at 45 °C for 1 min, and then it was increased by 5 °C/min up to 190 °C and then kept at 190 °C for 12 min. Standard curves were carried out with solution of 3-methylbutanal with concentrations varying between 1 and 5000 μM.

2.4. Enzyme assays

2.4.1. α-Ketoacid decarboxylase activity

α-Ketoacid decarboxylase activity was determined according to method described by Fernández De Palencia et al. (2006). Aldehyde dehydrogenase was used as a coupling enzyme. The basic reaction mixture consisted of 30 mM α-ketoisocaproic acid, 50 mM magnesium chloride, 1.5 mM thiamine pyrophosphate, 0.25 mM NAD^+, and 100 mM Tris–HCl buffer (pH 7.0), with or without 2.5 U of aldehyde dehydrogenase using different volumes of CFE. Activities were determined by measuring reduction rate of NAD^+ at 340 nm for 15 min at 30 °C. Specific activities were expressed as nmol of NAD^+ reduced/mg protein/min.

2.4.2. α-Ketoacid dehydrogenase activity

α-Ketoacid dehydrogenase activity was determined according to the method described by Tajima et al. (2004). The basic reaction mixture consisted of 7.5 mM α-ketoisocaproic acid, 7.5 mM CoASH (coenzyme A trilithium salt), 1 mM thiamine pyrophosphate, 35 mM magnesium chloride, 2.5 mM NAD^+, and 50 mM Tris–HCl buffer (pH 7.5). KADH activity started by the addition of CFE. Incubations were done at 37 °C for 1 h. Several samples were taken after 0, 10, 20, 30 and 60 min incubation. The reaction was stopped by lowering the pH (2 to 3) by adding 50 μl of 65% perchloric acid. The samples were then frozen at −20 °C. Before analysis, the samples were thawed and centrifuged at 10,000 rpm, (Eppendorf, 5804 R, Hamburg, Germany) for 10 min at 4 °C. α-Ketoisocaproic acid and isovaleryl-CoA concentrations in the supernatants were determined by liquid chromatography coupled with mass spectrometry (LC–MS) (see below). Assays were confirmed with and without addition of 20 mM $NaAsO_2$ using *L. delbrueckii* subsp. *lactis* CNRZ 207 ($KADC^+$, $KADH^-$) and *L. helveticus* CNRZ 32 ($KADH^+$, $KADC^-$). Specific activities were expressed as nmol of isovaleryl-CoA formed/mg protein/min.

2.4.3. Aminotransferase activity

Aminotransferase activity was determined as described by Smit et al. (2004) by incubating different volumes (10 to 200 μL) of CFE with leucine for 1 h at 30 °C. The reaction mixture contained 100 mM potassium phosphate buffer (pH 7.5), 0.05 mM pyridoxal-5-phosphate, 20 mM leucine and 10 mM α-KG. After incubation, the reaction was stopped by lowering the pH (as previously). The samples were then frozen at −20 °C. Before analysis, the samples were thawed and centrifuged at 10,000 rpm (Eppendorf 5804 R, Hamburg, Germany) for 15 min at 4 °C. Leucine and α-ketoisocaproic acid concentrations in the supernatants were determined by LC–MS. The specific activities were expressed as nmol of α-ketoisocaproic acid formed/mg protein/min.

2.4.4. Glutamate dehydrogenase activity

Glutamate dehydrogenase activity was determined according to the method described by Amárita et al. (2006). The reaction mixture contained 10 mM L-glutamate (Sigma-Aldrich), 1 mM NAD^+ or $NADP^+$, 40 mM Tris–HCl buffer (pH 8.8) and CFE. Glutamate dependent reduction of NAD^+ or $NADP^+$ was measured by absorbance at 340 nm for 15 min at 37 °C. Specific activities were expressed as nmol/mg protein/min.

2.4.5. Aldehyde dehydrogenase activity

Aldehyde dehydrogenase activity was determined by incubating different volumes of CFE in a reaction mixture containing 150 mM 3-methylbutanal, 0.25 mM NAD^+, and 100 mM Tris HCl (pH 7.0) at 30 °C. Activities were determined by measuring reduction rate of NAD^+ measured by absorbance at 340 nm for 15 min at 30 °C. Specific activities were expressed as nmol of NAD^+ reduced/mg protein/min.

2.5. Liquid chromatography–mass spectrometry

The LC–MS system comprised a binary solvent delivery pump and a linear ion trap mass spectrometer (LTQ-MS, ThermoFinnigan, San Jose, CA, USA). LTQ was equipped with an atmospheric pressure ionization interface operating in ESI (electrospray ionization) mode. Leucine was detected in ESI positive mode, while all other catabolites (α-ketoisocaproic acid, α-hydroxyisocaproic acid, isovaleryl-CoA, isovaleric acid) were detected in ESI negative mode. Data were processed using Xcalibur 2.0. The operational parameters of the

mass spectrometer were optimized by infusing (at 5 μL/min) the analytes in mobile phase at a flow rate of 0.2 mL/min. The spray voltage was 4.0 kV and the temperature of the heated capillary was set at 300 °C. The flow rate of sheath gas, auxillary gas and sweap gas were set (in arbitrary units/min) to 35, 10 and 10 respectively. Capillary voltage was +11 V, tube lens was +45 V, split lens was 54 V, and the front lens was 5.75 V. LC parameters comprised the mobile phase; acetate buffer pH 4.70 (A) and acetonitrile (B) at the flow rate of 0.2 mL/min through C18-amide (model Alltima HP, Alltech^R) column of dimensions 150×2.1 mm (5 μm). The monitoring of all catabolites was realized through MS detection via SRM (single reaction monitoring) and SIM (single ion monitoring) as follows: leucine (SRM = 132 to 86), HICA (SRM = 131 to 85), KICA (SRM = 129 to 85), isovaleric acid (SIM = 101), and isovaleryl-CoA (SIM = 850), respectively. SRM and SIM monitoring gave a very good specificity especially in complex mixture and allowed to realize a robust external quantification of each compound (100 to 1000 μM).

2.6. Degenerate primer design for *bcaT*, *gdh*, *kdcA*, *bkdA*, *bkdB*, *bkdC*, *bkdD*, and *adhE* and detection by PCR

Complete LAB genome sequences for particular enzymes were obtained from the NCBI microbial genome database (National Centre for Biotechnology Information (USA) http://www.ncbi.nlm.nih.gov/) as described by Liu et al. (2008). Homologous sequences from each enzyme family derived from BLAST were collected, and the redundant sequences were removed. Orthologous relationships between the various homologs were determined on the basis of phylogeny and synteny. Primers were designed using conserved regions of gene sequences found in different LAB strains (Table 2). The *bcaT* genes were found in the genome of lactococcal and streptococcal strains (Liu et al., 2008). The *gdh* genes were only found in the genomes of *Lactobacillus plantarum* WCFS1, *Streptococcus thermophilus* CNRZ 1066, *S. thermophilus* LMG 18311 and *S. thermophilus* LMD9. The α-ketoacid decarboxylase (*kdcA*) gene from *L. lactis* B1157 was well characterized (Smit et al., 2005), which was found to share partial homology with *kdcA* gene in the genome of *L. lactis* subsp. *lactis* IL1403. The α-ketoacid dehydrogenase an enzyme complex consisting of four subunits E1-α, E1-β, E2 and E3 (*bkdA*, *bkdB*, *bkdC*, *bkdD* genes) was well characterized in *Enterococcus faecalis* (Ward et al., 1999). Only *Lactobacillus casei* genome was found to contain similar orthologous operon. Bifunctional aldehyde/alcohol dehydrogenase (*adhE*) with aldehyde dehydrogenase (AldDH) catalytic domains were found in the genome of *L. lactis* subsp. *cremoris* SK11, *L. lactis* subsp. *lactis* IL1403, *L. lactis* subsp. *cremoris* MG1363, *L. sakei* subsp. *sakei* 23K, *L. casei* ATCC 334, *L. brevis* ATCC 367 and *L. acidophilus* NCFM. However, experimental evidence of AldDH from LAB is lacking (Liu et al., 2008).

The genes of specified enzymes were amplified in *C. maltaromaticum* LMA 28 by polymerase chain reaction. Bacterial DNA was extracted according to the method described by Leblond et al. (1996). PCR reaction consisted of 38.4 μL of ultrapure water; 5 μL of TE buffer (10×); 0.4 μL of dNTP at 0.25 mM; 2.5 μL of each primer at 10 μM; 0.25 μM of Taq polymerase (Q-biogene) at 5 U/μL in a total volume of 50 μL. Amplifications of PCR were done using thermocycler (Biorad, USA) using denaturing step at 94 °C for 5 min, followed by 35 cycles including denaturing at 94 °C, annealing at 43 °C for 2 min, and extension/elongation at 68 °C for 1 min. The aliquots 20 μL of amplified products were subjected to electrophoresis in agarose gels (Agarose DNA grade, Euromedex, France) at different concentrations depending upon the size of amplified fragment. The gels were stained with SYBR Safe (Invitrogen) and visualized under UV light at 350 nm. The smart ladder SF (Biorad, New England) was used as size marker (100–1000 bp). Amplified fragments were than purified by PCR gel cleanup protocol and sequenced (GATC-biotech, Germany) for respective gene.

3. Results

3.1. Effect of sodium meta-arsenite on growth and metabolite production from leucine by *C. maltaromaticum* LMA 28

Leucine catabolism was studied by incubating resting cells of strains with 10 mM of $NaAsO_2$, a specific inhibitor of KADH in the reaction mixture (Fig. 2). Arsenite binds to the SH group of lipoamide and makes KADH enzyme complex unstable. The effect of $NaAsO_2$ (10 mM) on the viability of bacterial cells and 3-methylbutanal production was checked after 48 h of incubation. The cell viability was not affected by the presence of $NaAsO_2$. The production of 3-methylbutanal was slightly reduced in *C. maltaromaticum* (40–60%) suggesting that only a proportion of 3-methylbutanal could be synthesized via KADH pathway. The metabolite production was completely stopped in *L. helveticus* CNRZ 32 (KADH positive) which is in accordance with the previous study (Helinck et al., 2004). On the contrary $NaAsO_2$ did not affect the formation of 3-methylbutanal in *L. lactis* DSM 20481 and *L. delbrueckii* subsp. *lactis* (KADC positive) as both strains strictly used KADC pathway. The results obtained lead to the hypothesis that both pathways i.e. KADC and KADH might be present and functional in *C. maltaromaticum* LMA 28.

Table 2
Degenerate primers used for *bcaT*, *gdh*, *kdcA*, *bkdA*, *bkdB*, *bkdC*, *bkdD* and *adhE* genes with the respective size of the amplified DNA fragments obtained.

Gene	Protein	Function	Sequences	PCR product	Size of amplified fragment (bp) obtained
bcaT	BcAT	Branched-chain aminotransferase (EC 2.6.1.42)	for 5′ ATT ATG GNC AAC AAK VTT TTG AAG GHT TAA AAG C 3′ rev 5′ TAA CTT CTT CAD CCA MGD CGN TTA AAG AAA C 3′	I	500
gdh	GDH	Glutamate dehydrogenase (EC 1.4.1.2)	for 5′ GAT GAT GAA GTT ATG CGC TT 3′ rev 5′ GAC ATA GTC GAA ATC TTT ACT 3′	II	733
kdcA	KdcA	Ketoacid decarboxylase (EC 4.1.1.72)	for 5′ AAW TGG RTY GGW AAT RCH AAT GA 3′ rev 5′ TTG HAR KGA WCC ATC MCC AAT 3′	III	1187
bkdA	KADH E1-α	Ketoacid dehydrogenase (EC 1.2.4.4)	for 5′ TSA AAA YAA TGG ATA CGC WAT 3′ rev 5′ TCA ATY AAD GTT GGR CCD T 3′	IV	188
bkdB	KADH E1-β	Ketoacid dehydrogenase (EC 1.2.4.4)	for 5′ TYY TYG GDG AAG ATG THG G 3′ rev 5′ ADN CCT TTT GCR TCR TAW GG 3′	V	398
bkdC	KADH E2	Ketoacid dehydrogenase (EC 2.3.1.168)	for 5′ GGH GAT ACH RTY AAW GAA GA 3′ rev 5′ ACR TAD GGB AAG AAT GTT A 3′	VI	757
bkdD	KADH E3	Ketoacid dehydrogenase (EC 1.8.1.4)	for 5′ CHG GAG GHT ATG TAG CNG C 3′ rev 5′ TCT CCR ATT GCA TAR ATR T 3′	VII	923
adhE	AldDH/AlcDH	Aldehyde dehydrogenase (EC 1.2.1.10)	for 5′ ACT GGT GGY YCA GSN ATG GT 3′ rev 5′ ACH GCA AAT GGA GTY ACT TC 3′	VIII	1297

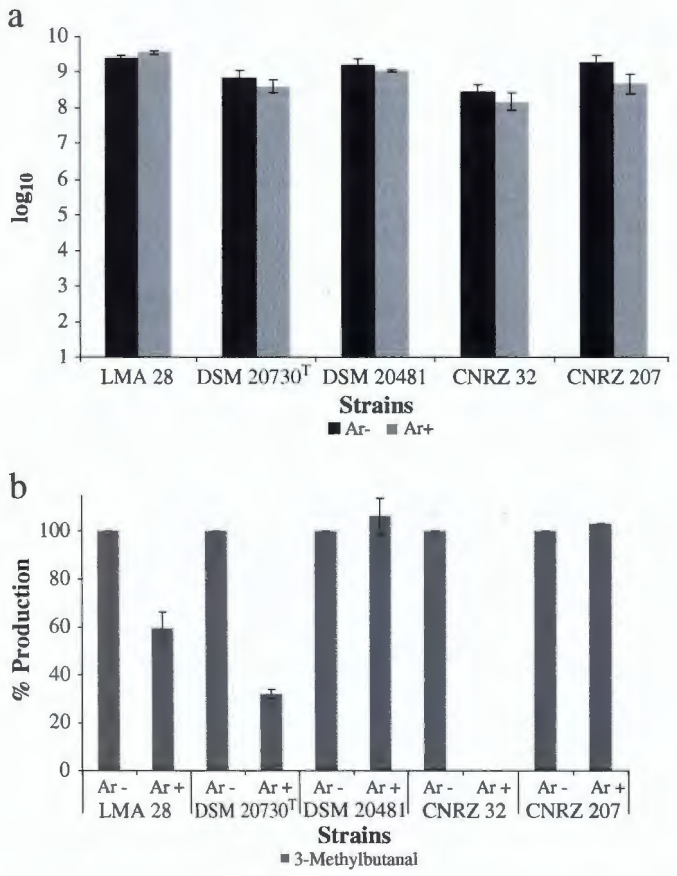

Fig. 2. a) Effect of $NaAsO_2$ (10 mM) on the bacterial cells' growth and b) on the production of 3-methylbutanal from leucine catabolism in a reaction medium containing leucine, α-KG, PLP in the absence (Ar−) or presence (Ar+) of $NaAsO_2$ by the strains studied after 48 h: LMA 28: *Carnobacterium maltaromaticum*; DSMZ 20730^T: *C. maltaromaticum*; DSMZ 20481: *Lactococcus lactis*; CNRZ 32: *Lactobacillus helveticus*; and CNRZ 207: *Lb. delbrueckii* subsp. *lactis*.

3.2. Detection of bcaT, gdh, kdcA, bkdA, bkdB, bkdC, bkdD, and adhE genes using degenerate primers in genomic DNA of C. maltaromaticum LMA 28

In order to detect the genes encoding enzymes involved in leucine catabolism in *C. maltaromaticum* LMA 28, degenerate primers were designed (Table 2). Eight PCR products were obtained with genomic DNA (gDNA) of *C. maltaromaticum* LMA 28 as expected (Table 2). Amplified fragments were sequenced and compared by performing blast analysis for closely related enzymes (Table 3). All sequenced PCR products were found highly homologous, showing 98–100% similarities, with the corresponding genes of respective enzymes in the genome of *C. maltaromaticum* ATCC 35586. The homologous proteins for PCR product III was annotated as indole-3-pyruvate decarboxylase in the genome of *C. maltaromaticum* ATCC 35586, whereas, for PCR product VIII, it was found to be annotated as iron-containing alcohol dehydrogenase. Four PCR products IV, V, VI and VII were found to share 100% similarity with the components of *C. maltaromaticum* ATCC 35586 annotated as pyruvate dehydrogenase, while, they shared 27 to 80% similarity with branched chain α-ketoacid dehydrogenase component of *E. faecalis* v583, *L. casei* BL23 and *Bacillus subtilis* str. 168 (Table 3).

3.3. Leucine catabolic pathways in C. maltaromaticum LMA 28

In order to identify the metabolic pathways used by *C. maltaromaticum* LMA 28 for the biosynthesis of 3-methylbutanal, KADC and KADH activities were determined. Both enzyme activities were detected in *C. maltaromaticum* (Table 4). Both *C. maltaromaticum* strains, LMA 28 and DSM 20730^T, possess a KADC activity of 41 ± 3 and 38 ± 15 nmol/mg protein/min respectively. KADH activity was found to be dependent on both NAD^+ and $NADP^+$. NAD^+ dependent KADH activity was detected as 1.43 ± 0.62 and 2.70 ± 0.50 nmol/mg protein/min in both strains, while $NADP^+$ dependent KADH activity was found to be 0.57 ± 0.15 and 0.75 ± 0.23 nmol/mg protein/min respectively. The measured enzyme activities supported the previous hypothesis that both pathways were present and functional in *C. maltaromaticum*. Validity of enzyme assays was confirmed using the reference strains. No KADC activity was detected in *L. helveticus* CNRZ 32 and no KADH activity was determined in both *L. lactis* DSMZ 20481 and *L. delbrueckii* subsp. *lactis* CNRZ 207.

In addition to the identification of KADC and KADH activities, aminotransferase activity (AT) which is a key enzyme directly implicated in leucine catabolism was also determined along with glutamate dehydrogenase (GDH) and aldehyde dehydrogenase (AldDH) (Table 4). All studied strains possessed AT activity, which is the first step in AA catabolism. Slight $NADP^+$ dependent GDH activities were detected in both *C. maltaromaticum* strains suggesting that this species could be able to catabolize leucine without the addition of exogenous α-ketoglutarate in the medium. All studied strains possessed AldDH activity, although activities varied between strains and were found at a very low level in *C. maltaromaticum* LMA 28. A slight HADH activity was also detected in both *C. maltaromaticum* strains (data not shown).

4. Discussion

This study is the first report on the identification of metabolic routes involved in the catabolism of leucine in *C. maltaromaticum*. To date, only the influence of various parameters including pH, temperature, oxygen, glucose and α-ketoisocaproic acid concentration was investigated on the production of 3 methylbutanal, 3 methylbutanol and isovaleric acid in *C. maltaromaticum* previously *piscicola* 545, a strain of meat origin. It was proposed that leucine catabolism was increased by the addition of glucose and α-ketoisocaproic acid under shaking conditions (Larrouture-Thiveyrat and Montel, 2003).

Previous studies revealed that enzymes involved in flavor formation are highly strain and species dependent and either direct (KADC) or indirect (KADH) pathways may be present (Helinck et al., 2004; Thierry et al., 2002). Contrary to direct pathway, indirect pathway was not extensively studied. The KADC pathway, which is constituted by a single step biotransformation of α-ketoacids deriving from branched chain amino acids (BCAAs) to aldehydes has been reported in a number of dairy related microorganisms including *L. delbrueckii* subsp. *lactis* (Helinck et al., 2004), *L. lactis* var. *maltigenes* (Morgan, 1976; Tucker and Morgan, 1967) and *Staphylococcus xylosus* (Beck et al., 2002). However, KADC activity was determined only in a strain of *L. lactis* and *Corynebacterium ammoniagenes* ranging from 3 to 29 nmol/mg protein/min (Smit et al., 2004) while strains of *C. maltaromaticum* showed high KADC activity (38 to 41 nmol/mg protein/min). According to Smit et al. (2004), KADC is the rate controlling step in aldehyde formation. It is interesting to note that aldehyde resulting from α-ketoacid decarboxylation can be further reduced to alcohol by alcohol dehydrogenase or oxidized to carboxylic acid by an aldehyde dehydrogenase depending upon the intracellular redox environment. Genes encoding enzymes were well characterized in *L. lactis* (De La Plaza et al., 2004; Smit et al., 2005; Yvon et al., 1997).

The multistep KADH pathway, leading to the formation of aldehyde from α-ketoacids via multienzyme complex KADH was reported in *L. helveticus* (Helinck et al., 2004), *Propionibacteria* (Thierry et al., 2002), non dairy *E. faecalis* (Ward et al., 1999), *B. subtilis* (Lowe et al., 1983; Wang et al., 1993) and extensively studied in *E. faecalis* and *B. subtilis*. *E. faecalis* was found to possess pyruvate dehydrogenase (PDH) in addition to KADH complex (Ward et al., 1999), while in *B. subtilis*, only PDH catalyzed the decarboxylation of α-ketoacids derived from leucine (Lowe et al., 1983). To our knowledge, KADH

Table 3
Similarity among PCR products amplified in gDNA of *C. maltaromaticum* LMA 28 with closely related enzymes.

PCR product	Product size (bp)	Gene	Homologous protein	Source	Protein ID	% identity	Reference
I	500	*bcaT*	Branched chain amino acid aminotransferase	*C. maltaromaticum* ATCC 35586	ATCC_539	98	Leisner et al. (2012)
				Carnobacterium sp. 17-4	AEB31100	76	Voget et al. (2011)
				Lactococcus lactis IL1403	ADQ55723.1	68	Passerini et al. (2010)
				Lactococcus lactis KF 147	ADA65032	68	Siezen et al. (2008)
II	733	*gdh*	Glutamate dehydrogenase	*C. maltaromaticum* ATCC 35586	ATCC_1754	100	Leisner et al. (2012)
				Carnobacterium sp. 17-4	YP_004374626.1	73	Voget et al. (2011)
				Streptococcus suis P1/7	CAR44587.1	64	Holden et al. (2009)
III	1187	*kdcA*	α-Ketoacid decarboxylase	*C. maltaromaticum* ATCC 35586	ATCC_2688[a]	99	Leisner et al. (2012)
				Lactococcus lactis B1157	AAS49166	51	Smit et al. (2005)
				Lactococcus lactis IL1403	AAK05402	51	Bolotin et al. (2001)
IV	188	*bkdA*	Branched chain α-ketoacid dehydrogenase (E1-α)	*Bacillus subtilis* subsp. *subtilis* str. 168	NP_390285.1	52	Barbe et al. (2009)
				Enterococcus faecalis V583	NP_815368.1	48	Wecker et al. (2009)
				Lactobacillus casei BL23	YP_001987607.1	44	Maze et al. (2010)
			Pyruvate dehydrogenase	*C. maltaromaticum* ATCC 35586	ATCC_1757	100	Leisner et al. (2012)
				Carnobacterium sp. 17-4	YP_004374627.1	82	Voget et al. (2011)
				Enterococcus faecalis V583	NP_815074.1	79	Wecker et al. (2009)
				Lactobacillus casei BL23	YP_001987475.1	75	Maze et al. (2010)
				Bacillus subtilis subsp. *subtilis* str. 168	NP_389341.1	75	Barbe et al. (2009)
V	398	*bkdB*	Branched chain α-ketoacid dehydrogenase (E1-β)	*Bacillus subtilis* subsp. *subtilis* str. 168	NP_390284.1	53	Barbe et al. (2009)
				Lactobacillus casei BL23	YP_001987606.1	50	Maze et al. (2010)
				Enterococcus faecalis V583	NP_815367.1	47	Wecker et al. (2009)
			Pyruvate dehydrogenase	*C. maltaromaticum* ATCC 35586	ATCC_1758	100	Leisner et al. (2012)
				Enterococcus faecalis V583	NP_815075.1	88	Wecker et al. (2009)
				Carnobacterium sp. 17-4	YP_004374628.1	85	Voget et al. (2011)
				Lactobacillus casei BL23	YP_001987476.1	83	Maze et al. (2010)
				Bacillus subtilis subsp. *subtilis* str. 168	NP_389342.1	79	Barbe et al. (2009)
VI	757	*bkdC*	Branched chain α-ketoacid dehydrogenase (E2)	*Bacillus subtilis* subsp. *subtilis* str. 168	NP_389343.1	80	Barbe et al. (2009)
				Lactobacillus casei BL23	YP_001987605.1	56	Maze et al. (2010)
				Enterococcus faecalis V583	NP_815366.1	27	Wecker et al. (2009)
			Pyruvate dehydrogenase (E2)	*C. maltaromaticum* ATCC 35586	ATCC_1759	100	Leisner et al. (2012)
				Carnobacterium sp. 17-4	YP_004374629.1	84	Voget et al. (2011)
				Enterococcus faecalis V583	NP_815076.1	80	Wecker et al. (2009)
				Lactobacillus casei BL23	YP_001987477.1	71	Maze et al. (2010)
VII	923	*bkdD*	Branched chain α-ketoacid dehydrogenase (E3)	*Enterococcus faecalis* V583	NP_815369.1	49	Wecker et al. (2009)
			Pyruvate dehydrogenase (E3)	*C. maltaromaticum* ATCC 35586	ATCC_1760	100	Leisner et al. (2012)
				Carnobacterium sp. 17-4	YP_004374630.1	86	Voget et al. (2011)
				Enterococcus faecalis V583	NP_815077.1	79	Wecker et al. (2009)
				Lactobacillus casei BL23	YP_001987478.1	71	Maze et al. (2010)
				Bacillus subtilis subsp. *subtilis* str. 168	NP_389344.1	63	Barbe et al. (2009)
VIII	1297	*adhE*	Bifunctional alcohol-aldehyde dehydrogenase	*C. maltaromaticum* ATCC 35586	ATCC_1561[b]	100	Leisner et al. (2012)
				Carnobacterium sp. 17-4	YP_004374408.1	81	Voget et al. (2011)
				Lactobacillus sakei subsp. *sakei* 23 K	YP_394992.1	68	Chaillou et al. (2005)
				Lactococcus lactis subsp. *cremoris* SK11	YP_812009.1	44	Makarova et al. (2006)

[a] ATCC_2688 was found to be annotated as indole-3-pyruvate decarboxylase (*ipdC*).
[b] ATCC_1561 was found to be annotated as iron-containing alcohol dehydrogenase (*adh*).

activity was never determined in dairy related microorganisms. The slight NAD^+ dependent KADH activity that was detected in *C. maltaromaticum* (1.43 to 2.70 nmol/mg protein/min) is then the first report of a KADH activity in dairy related microorganisms.

Surprisingly, both KADC and KADH activities were detected in *C. maltaromaticum*, and in the absence of KADH activity ($NaAsO_2$ addition) the 3-methylbutanal production was reduced but still present. These two observations lead to the conclusion that both pathways are functional in this bacterium. It was previously shown that in *L. lactis*, oxidative conditions favored the conversion of α-ketoisocaproic acid into 3-methylbutanal through KADC activity. This increase was attributed to a direct stimulation of the enzyme activity by the oxidative conditions or by an accumulation of α-ketoisocaproic acid, which could not be transformed into α-hydroxyisocaproic acid in such conditions (Kieronczyk et al., 2006). This confirmed that the production of 3-methylbutanal might be affected by the presence of oxygen i.e.

Table 4
Enzyme activities including α-ketoacid decarboxylase (KADC), α-ketoacid dehydrogenase (KADH), aminotransferase (AT), glutamate dehydrogenase (GDH) and aldehyde dehydrogenase (AldDH), determined by spectrophotometer and LC–MS.

Bacterial species	Strains	KADC	KADH	AT	GDH ($NADP^+$)	AldDH (NAD^+)
C. maltaromaticum	LMA 28	41.0 ± 3.0	1.43 ± 0.62 (NAD^+)[a] 0.57 ± 0.15 ($NADP^+$)[a]	21.05 ± 0.07	2.35 ± 0.26	0.30 ± 0.11
C. maltaromaticum	DSM 20730^T	38.0 ± 5.0	2.70 ± 0.50 (NAD^+)[a] 0.75 ± 0.23 ($NADP^+$)[a]	31.25 ± 5.30	2.19 ± 0.43	0.47 ± 0.09
L. lactis	DSM 20481	195 ± 9	ND	30.25 ± 8.13	ND	0.81 ± 0.05
L. helveticus	CNRZ 32	ND	2.93 ± 0.19 ($NADP^+$)[b]	5.87 ± 1.51	ND	0.61 ± 0.15
L. delbrueckii subsp. *lactis*	CNRZ 207	12 ± 3	ND	16.57 ± 3.86	ND	3.35 ± 0.26

Values are means ± standard deviation of three independent experiments. Values are expressed in nmol/mg protein/min. ND: not detected.
[a] KADH activities from *C. maltaromaticum* LMA 28 and DSM 20730^T were found to be dependent on both NAD^+ and $NADP^+$.
[b] KADH activity from *L. helveticus* CNRZ 32 was found to be dependent on only $NADP^+$.

the extracellular redox potential (E_h). The repartition between the direct and the indirect pathway in *C. maltaromaticum* could be influenced by the E_h. The functionality of both pathways could then be an advantage in order to catabolize BCAAs under various redox conditions in different food products.

In recent years, emphasis has started to give in selecting bacterial strains with potential enzyme activities, efficient in degrading amino acids to produce aromatic compounds (Brandsma et al., 2008; Ziadi et al., 2010). Such strains could allow diversification as well as enhancement of cheese aroma development (Amárita et al., 2006; Brandsma et al., 2008; Fernández De Palencia et al., 2006; Kieronczyk et al., 2006; Smit et al., 2004; Williams et al., 2002). However, on the contrary, accelerated AA catabolism due to unsuitable adjunct of bacterial strains can sometimes generate non-desired flavor compounds (Urbach, 1995). Aminotransferase activity was found in a large number of lactococcal strains and other dairy related lactic flora ranging from 10 to 150 nmol/mg protein/min (Amárita et al., 2006; Smit et al., 2004). In the present study high AT activity was also found in *L. lactis* (30 nmol/mg protein/min) along with *C. maltaromaticum* LMA 28 (21 nmol/mg protein/min) which is consistent with the above findings. Previous studies revealed the importance of microflora possessing GDH activities generating α-KG, hence efficiently catabolizing AA without the addition of exogenous α-KG. In the literature NADPH, H^+ dependent GDH activity was detected in some wild lactococcal and some *Lactobacillus* strains ranging from 1.2 to 20 nmol/mg protein/min (Amárita et al., 2006; Fernández De Palencia et al., 2006). Slight GDH activities were detected in *C. maltaromaticum* suggesting that it could also efficiently catabolize AA without the addition of α-KG in the reaction medium. In this study, GDH activity was not detected in both lactobacilli strains (*L. delbrueckii* subsp. *lactis* CNRZ 207 and *L. helveticus* CNRZ 32), which is in accordance with the previous study (Helinck et al., 2004).

This study also revealed the presence of genes encoding *bcaT*, *kdcA*, *gdh*, *bkdA*, *bkdB*, *bkdC*, *bkdD* and *adhE* in *C. maltaromaticum* LMA 28. These genes were previously well characterized in *L. lactis*, *E. faecalis and B. subtilis* (De La Plaza et al., 2004; Smit et al., 2005; Yvon et al., 1997; Wang et al., 1993; Ward et al., 1999). Amplified gene sequences for particular enzymes were found highly homologous compared to genes amplified previously in LAB genomes. Recently, the genome sequence of *C. maltaromaticum* ATCC 35586 isolated from diseased salmon was determined by whole-genome shutgun approach and automatic annotation was performed by using RAST annotation system (Leisner et al., 2012). The genes from *C. maltaromaticum* LMA 28, isolated in this study, appeared to be identical or very similar to those from *C. maltaromaticum* ATCC 35586. However, the genome sequence of *C. maltaromaticum* ATCC 35586 was found to encode potential virulence genes concerning to extracellular matrix and invasion, capsule and cell wall, iron acquisition, metabolism and transcriptional regulators (Leisner et al., 2012). Before the use of *C. maltaromaticum* LMA 28 in cheese technology, expression of genes encoding virulence factors will have to be determined to verify this specific point. Moreover, the application of *C. maltaromaticum* species in cheese technology regarding food safety could be limited due to its ability to produce tyramine from tyrosine. In soft cheeses artificially inoculated with *C. maltaromaticum* LMA 28, no tyramine and histamine were detected (Edima et al., 2007).

5. Conclusion and perspectives

The presence of genes encoding enzymes involved in the catabolic pathways of leucine and the in vitro activities of these enzymes have been demonstrated in this study. Moreover, in vivo biosynthesis of 3-methylbutanal was shown to be possible using both KADC and KADH pathways. To our knowledge, this is the first time that the coexistence of both pathways is demonstrated in a LAB. Such a study, demonstrating the presence of pathways of leucine catabolism at the genetical, enzymatical levels and the consequences on the production of 3-methylbutanal was never conducted in a single LAB before.

Previous studies mentioned the importance of lactobacilli, especially *L. delbrueckii* subsp. *lactis* in flavor development as they have the enzymatic potential to produce potent and varied aroma compounds from AAs. Since both pathways for leucine catabolism are functional in *C. maltaromaticum* LMA 28, it can play a major role in cheese aroma development under certain conditions of pH, temperature and oxygen or E_h environment.

Recently it has been demonstrated that, a good control of E_h in a milk product like cheese or yogurt could allow a diversification in flavor (Martin et al., 2011). Various factors including mixing, agitation and selection of species of NSLAB capable to grow under oxidizing conditions must be taken into account during ripening as it could enhance the formation of 3-methylbutanal, which if too pronounced may result in an off-flavor development. So therefore, further studies including effect of oxygen and potential redox under controlled conditions would lead us to better understand these enzymatic conversions in *C. maltaromaticum* LMA 28 in order to optimize the flavor generation capacity.

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II.3 Contributions de l'article

Deux voies métaboliques de biosynthèse intracellulaire de 3-méthylbutanal à partir du catabolisme de la leucine sont connues: soit une voie directe impliquant une α-cétoacide décarboxylase (KADC) soit une voie indirecte empruntant une α-cétoacide déshydrogénase (KADH).

Dans notre étude, l'addition de méta-arsénite de sodium, inhibiteur du complexe KADH, réduit légèrement la production de 3-méthylbutanal au cours de la croissance cellulaire, sans affecter la viabilité de *C. maltaromaticum* LMA 28. Ceci indique que seulement une proportion de 3-méthylbutanal pourrait être synthétisée par la voie de la KADH. Cela nous conduit à l'hypothèse que les deux voies (KADC et KADH) pourraient être présentes et fonctionnelles chez *C. maltaromaticum* LMA 28.

Le catabolisme des acides aminés commence par l'élimination du groupe amine par les aminotransférases. Cette enzyme a été détectée chez *C. maltaromaticum* LMA 28 à un niveau comparable à celui de *L. lactis*. Cette enzyme utilise l'α-KG comme accepteur du groupe amine afin de synthétiser le glutamate. L'activité GDH, même faible chez *C. maltaromaticum* LMA 28 lui permet de regénérer l'α-KG, un des facteurs limitant pour la réaction catalysée par l'aminotransférase. Ce composé peut alors être transformé suivant deux voies grâce à l'enzyme KADC et/ou au complexe enzymatique KADH. Ces deux activités enzymatiques ont été détectées.

Dans nos conditions expérimentales, l'activité KADC (voie directe) chez *C. maltaromaticum* LMA 28, 5 fois plus faible que chez *L. lactis* et 2 fois plus forte que chez *L. delbrueckii* subsp. *lactis* lui permet de produire le 3-méthylbutanal. Cette activité enzymatique est rarement détectée chez les bactéries lactiques d'origine laitière.

L'activité KADH (voie indirecte) est plus couramment retrouvée chez ces LAB. Elle permet la conversion de l'α-ketoisocaproate en isovaleryl-CoA. Ce complexe enzymatique est composé de quatre sous unités. Chez *C. maltaromaticum* LMA 28, cette activité KADH comparable à celle de *L. helveticus* s'est avérée être dépendante du NAD^+ et du $NADP^+$.

L'isovaleryl-CoA est ensuite converti en isovalerate par la phosphotransférase et l'acylkinase dont les activités enzymatiques n'ont pas été mises en évidence.

La dernière étape de cette voie est la conversion de l'isovalerate en 3-méthylbutanal par l'aldéhyde deshydrogénase. Cette activité, rarement recherchée chez les LAB, a été détectée chez *C. maltaromaticum* LMA 28.

La mesure de ces activités confirme donc la présence et la fonctionnalité de ces deux voies chez *C. maltaromaticum* LMA 28.

Les séquences partielles des gènes (*bcaT, gdh, kdcA, adhE*) codant pour les enzymes dont les activités ont été détectées se sont avérées être hautement homologues aux gènes du génome de *C. maltaromaticum* ATCC 35586. Pour les gènes du complexe KADH, la comparaison est plus difficile car l'annotation des homologues est définie comme des complexes pyruvate ou acétoïne déshydrogénase.

Ce travail nous a permis de démontrer pour la première fois chez une bactérie lactique la coexistence des deux voies fonctionnelles de biosynthèse du 3-méthylbutanal. *Carnobacterium maltaromaticum* pourrait donc jouer un rôle majeur dans le développement des arômes du fromage dans certaines conditions de pH, température, d'environnement en oxygène ou en potentiel redox. Notre deuxième objectif a donc été d'étudier comment différents facteurs, en particulier l'oxygène ou le potentiel redox, peuvent influencer ces voies de biosynthèse chez *C. maltaromaticum* LMA 28.

III. Effet de l'oxygène sur la biosynthèse du 3-méthylbutanal chez *Carnobacterium maltaromaticum* LMA 28

Effect of oxygen on the biosynthesis of flavour compound 3-methylbutanal from leucine catabolism during batch fermentation in *Carnobacterium maltaromaticum* LMA 28

Muhammad Inam Afzal, Kenza-Amel Boulahya, Cédric Paris, Stéphane Delaunay and Catherine Cailliez-Grimal

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III. Effet de l'oxygène sur la biosynthèse du 3-méthylbutanal chez *Carnobacterium maltaromaticum* LMA 28

III.1 Introduction

Dans la littérature, il a été démontré que la fonctionnalité des voies métaboliques impliquées dans la biosynthèse de 3-méthylbutanal et 3-méthylbutanol pouvait être fortement influencée par différent facteurs, comme le pH, la température, le sel et l'environnement en oxygène ou le potentiel redox (De la Place et al., 2004; Kieronczyk et al., 2006; Deetae et al., 2011). Kieronczyk et al. (2006) ont observé une augmentation de la production de 3-méthylbutanal et 3-méthylbutanol chez *L. lactis* sous un état d'oxydation élevé. Des résultats similaires ont été obtenus chez *Proteus vulgaris* par Deetae et al. (2011) dans des conditions aérobies. Cependant, les voies de biosynthèse du 3-méthylbutanal sont encore peu connues chez ces bactéries.

Après avoir démontré la coexistence et le fonctionnement des deux voies métaboliques chez *C. maltaromaticum* LMA 28 (Afzal et al., 2012), nous nous sommes intéressés à la façon dont les biotransformations métaboliques dans cette bactérie pouvaient être influencées par la modification de l'environnement en oxygène pendant une culture en mode discontinu, sous environnement contrôlé, en bioréacteur.

Carnobacterium maltaromaticum LMA 28 a été cultivée dans un bioréacteur avec 5 L de volume utile, sur un milieu synthétique (MCGC) supplémenté en extrait de levure (1 g/L), à pH 6,8 sous trois différentes conditions d'oxygénation (0, 50 et 90 % de la saturation en air). Ce milieu a été initialement développé pour la croissance de *Corynebacterium glutamicum*. Nous avons fait le choix d'utiliser ce milieu afin de pouvoir suivre plus facilement l'évolution des concentrations en substrats et en produits.

Durant les cultures, nous avons déterminé la viabilité cellulaire, les concentrations en glucose résiduel, lactate, 3-méthylbutanal et 3-méthylbutanol. De plus, les activités enzymatiques (KADC et KADH) ont été mesurées lorsque les taux de production de 3-méthylbutanal et de 3-méthylbutanol étaient maximaux afin d'estimer l'influence de l'oxygène sur l'utilisation de l'une et/ou l'autre des deux voies de biosynthèse du 3-méthylbutanal existante chez *C. maltaromaticum* LMA 28.

Les résultats obtenus sont présentés sous la forme d'une publication publiée dans Journal of Dairy Science (2012, 96, 352-359) : **"Effect of oxygen on the biosynthesis of flavor compound 3-methylbutanal from leucine catabolism during batch culture in *Carnobacterium maltaromaticum* LMA 28"**.

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Effect of oxygen on the biosynthesis of flavor compound 3-methylbutanal from leucine catabolism during batch culture in *Carnobacterium maltaromaticum* LMA 28

M. I. Afzal,* K.-A. Boulahya,†‡ C. Paris,* S. Delaunay,†‡ and C. Cailliez-Grimal*[1]
*Laboratoire d'ingénierie des biomolécules, Université de Lorraine, 2 avenue de la Forêt de Haye B.P. 172, Vandoeuvre-lès-Nancy F-54505, France
†Laboratoire réactions et génie des procédés, Centre National de la Recherche Scientifique (CNRS), UPR 3349, Vandoeuvre-lès-Nancy F-54505, France
‡Laboratoire réactions et génie des procédés, Université de Lorraine, UPR 3349, Vandoeuvre-lès-Nancy, F-54505 France

ABSTRACT

In this study, we demonstrated the effect of different dissolved oxygen concentrations (DOC) on cell growth and intracellular biosynthesis of 3-methylbutanal from leucine catabolism in *Carnobacterium maltaromaticum* LMA 28 during batch culture. The maximum specific growth rate was obtained in culture when DOC was controlled at 50% of air saturation. The specific consumption rates of glucose and specific production rates of lactate were higher at a DOC at 50 or 90% of air saturation. *Carnobacterium maltaromaticum* LMA 28 produced high quantities of 3-methylbutanal and 3-methylbutanol during culture with DOC maintained at 90%, suggesting that oxygen had a significant effect of the formation of these flavor compounds. This high formation of flavor compounds in an oxygen-rich environment was attributed to the simultaneous activation and stimulation of both α-ketoacid decarboxylase (KADC) and α-ketoacid dehydrogenase (KADH) pathways. Thus, intracellular biosynthesis of 3-methylbutanal can be controlled by modifying the DOC of the culture or food product during fermentation.
Key words: *Carnobacterium maltaromaticum*, flavor formation, L-leucine, metabolic pathway

INTRODUCTION

Carnobacterium maltaromaticum belongs to the lactic acid bacteria (**LAB**) group, and strains of this species have been reported in a variety of foods, including a few dairy products (Leisner et al., 2007; Afzal et al., 2010). Its presence in Brie, a protected designation of origin French surface mold-ripened soft cheese, was first reported in 1994 (Millière et al., 1994) and confirmed in 2007 (Cailliez-Grimal et al., 2007). Works published in the past decade have shown that this species could be considered a nonstarter LAB (**NSLAB**), because it constitutes the main psychrotrophic LAB, is able to grow even at alkaline pH value, and reaches high concentrations (10^8 to 10^9 cfu/mL) during cheese ripening (Cailliez-Grimal et al., 2007). Producing the malty or chocolate-like compound 3-methylbutanal from the catabolism of leucine is a general characteristic of this species (Miller et al., 1974; Leisner et al., 2007; Afzal et al., 2010). In the literature, it is reported that leucine can be converted into 3-methylbutanal by 1 of 2 metabolic pathways. A direct pathway implicating α-ketoacid decarboxylase (**KADC**) enzyme or an indirect one with α-ketoacid dehydrogenase (**KADH**) enzyme complex might be present in microorganisms, particularly those of the genera *Lactococcus*, *Lactobacillus*, *Streptococcus*, *Enterococcus*, and *Staphylococcus* (Yvon and Rijnen, 2001; Helinck et al., 2004; Smit et al., 2004; Figure 1).

Recently, we demonstrated the co-existence of both metabolic routes in *C. maltaromaticum* LMA 28 (Afzal et al., 2012). The functionality of these pathways is highly influenced by various factors including pH, temperature, salt, and oxygen or redox environment (de la Plaza et al., 2004; Kieronczyk et al., 2006). Indeed, the presence of oxygen or high oxidation state results in increased production of 3-methylbutanal and 3-methylbutanol in *Lactococcus lactis* and *Proteus vulgaris*, respectively (Kieronczyk et al., 2006; Deetae et al., 2011). At the same time, enzyme activities involved in amino acid catabolism could be strongly controlled by the generation of oxidizing (NAD^+) and reducing agents (NADH, H^+) (Bourel et al., 2003; Pham et al., 2008). Anaerobic microorganisms convert pyruvate, resulting from glycolysis, into lactate through the activity of lactate dehydrogenase (**LDH**), generating NAD^+ and ATP in the absence of oxygen. The activity of LDH has been shown to be dependent on the redox environment, exhibiting high specific activity in reducing conditions in *Escherichia coli* (Riondet et al.,

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[1]Corresponding author: catherine.cailliez@ensaia.inpl-nancy.fr

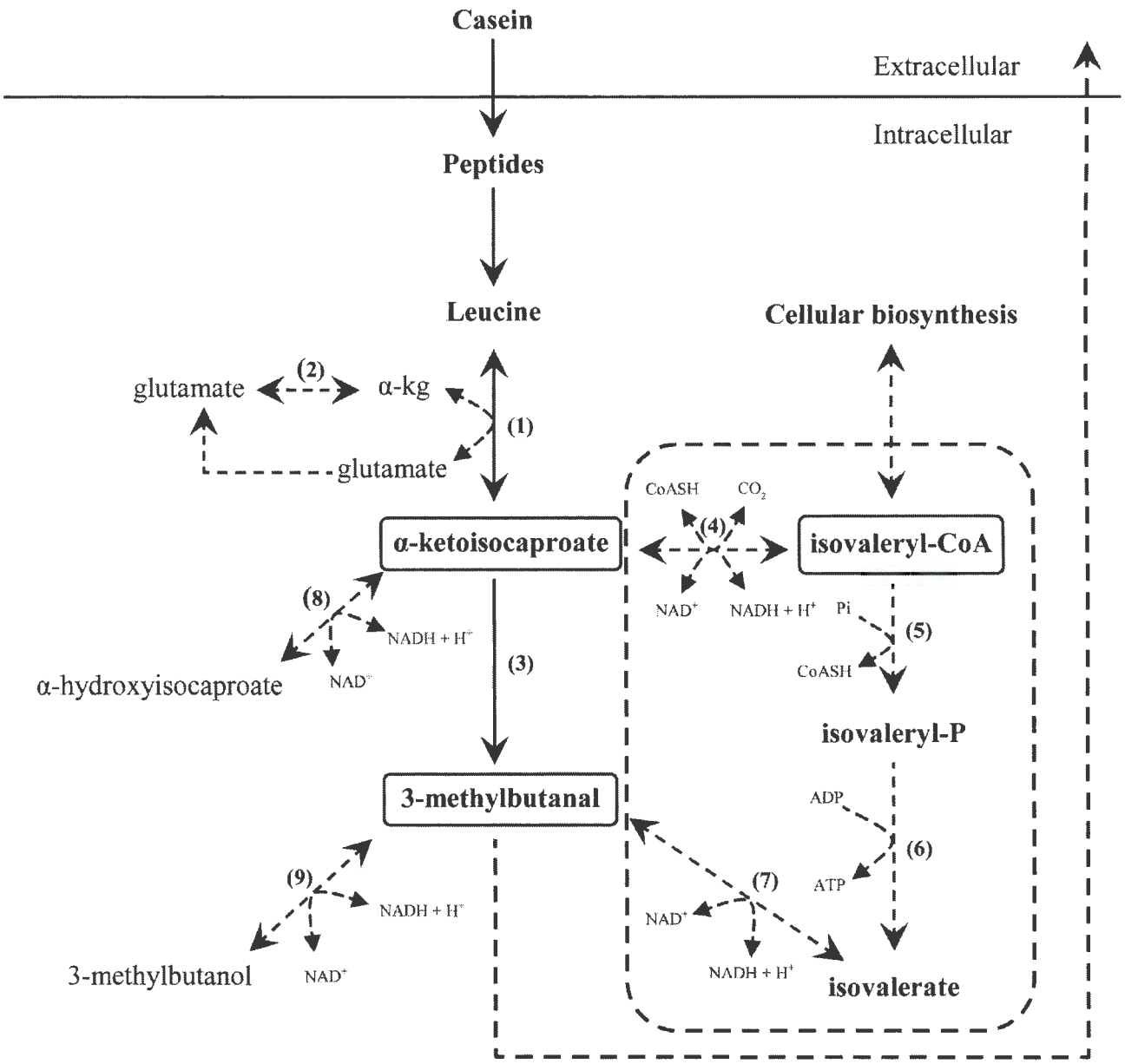

Figure 1. Intracellular metabolic pathways for the biosynthesis of 3-methylbutanal from catabolism of leucine by *Carnobacterium maltaromaticum* LMA 28 (Afzal et al., 2012). Enzymes of direct pathway (solid lines): (1) aminotransferase (AT), (2) glutamate dehydrogenase (GDH), (3) α-ketoacid decarboxylase (KADC); enzymes of indirect pathway (dashed lines): (4) α-ketoacid dehydrogenase (KADH), (5) phosphotransferase (PTA), (6) acylkinase (ACK), (7) aldehyde dehydrogenase (AldDH); other enzymes: (8) α-hydroxyacid dehydrogenase (HADH), (9) alcohol dehydrogenase (AlcDH).

2000). However, in aerobic microorganisms, pyruvate formation from glycolysis generates more NADH, H^+, and ATP in the presence of high dissolved oxygen concentration (**DOC**; Ardö, 2006; Pham et al., 2008). The presence of oxygen in culture medium, and consequently the redox environment, may have a dramatic effect on the control of amino acid catabolism. Previously, the increased production of 3-methylbutanal from leucine in *Lactococcus lactis* NCDO1867 under oxidative conditions was thought to be stimulated either by the increase in KADC activity or by substrate availability (Kieronczyk et al., 2006).

Carnobacteria are complex facultative anaerobic microorganisms, able to grow under low or high DOC, and able to produce lactic acid, ethanol, and CO_2 from pyruvate. Therefore, the intracellular balance of NAD^+

and NADH, H^+ could play a major role in controlling the biosynthetic pathways of 3-methylbutanal. Because *C. maltaromaticum* LMA 28 possesses both metabolic pathways, our objective was to study how different DOC states in a chemically defined culture medium influence these pathways involved in the biosynthesis of the flavor compound 3-methylbutanal.

MATERIALS AND METHODS

Strain and Medium Composition

The bacterial strain studied, *C. maltaromaticum* LMA 28, was obtained from the culture collection of the Laboratoire de Microbiologie Alimentaire (Université de Lorraine, France). It was isolated from a mold-ripened soft cheese (Millière et al., 1994), and the strain was cultivated in a flask on a synthetic minimal medium called MCGC (Von der Osten et al., 1989), in which citrate was replaced by deferoxamine (Delaunay et al., 1999). The MCGC medium consisted of glucose (40 g/L), $Na_2HPO_4 \cdot 12H_2O$ (45.5 g/L), KH_2PO_4 (3.6 g/L), NaCl (1.2 g/L), $(NH_4)_2SO_4$ (4.8 g/L), $MgSO_4 \cdot 7H_2O$ (0.4 g/L), $FeSO_4 \cdot 7H_2O$ (40 mg/L), $FeCl_3$ (4 mg/L), $ZnSO_4 \cdot 7H_2O$ (1 mg/L), $CuCl_2 \cdot 2H_2O$ (0.4 mg/L), $MnSO_4 \cdot H_2O$ (4 mg/L), $(NH_4)_6Mo_7O_{24} \cdot 4H_2O$ (0.2 mg/L), $Na_2B_4O_7 \cdot 10H_2O$ (0.4 mg/L), $CaCl_2$ (84 mg/L), biotin (2 mg/L), thiamine (20 mg/L), deferoxamine (3 mg/L), and glycine betaine (2 g/L). This medium was modified by the supplementation of yeast extract (1 g/L) and its pH was adjusted to 6.8. The bacterial strain was grown under aerobic conditions at 30°C and 200 rpm in 500-mL flasks for 40 h. All chemicals were purchased from Sigma-Aldrich (St. Louis, MO).

Batch Culture in Bioreactor Under Different DOC. *Carnobacterium maltaromaticum* LMA 28 was cultured in a 5-L bioreactor (Applikon, Schiedam, the Netherlands) with a 1.5-L working volume. The MCGC medium for batch culture in the stirred bioreactor was the same as described earlier but modified by supplementation with leucine (6.5 g/L). The volume of preculture used to inoculate the bioreactor was determined by aiming for an initial cellular concentration of approximately 10^5 cfu/mL. The pH was regulated to 6.8 by the addition of NaOH (12 *N*) or HCl (2 *N*) solution. The DOC was controlled by agitation, gas flow, and composition of the gas (air or N_2). The gas flow varied from 60 to 100 L/h. To study the influence of oxygen on the biosynthesis of 3-methylbutanal from leucine catabolism by *C. maltaromaticum* LMA 28, cultures were carried out under different DOC: in the absence of oxygen (0%) or in the presence of oxygen (50 and 90%) for 55 h. The absence of oxygen in the culture medium (0%) was achieved by continuous sparging of N_2. Experiments were performed in duplicate, and samples were collected throughout culture to determine viable cell counts and concentrations of glucose, lactate, 3-methylbutanal, and 3-methylbutanol.

Viable Cell Number. Serial dilutions of samples were carried out in tryptone salt solution. A volume of 100 μL was spread on agar plates (trypticase soy agar supplemented with yeast extract) with the help of a spiral plater (AES, Combourg, France). Agar plates were incubated at 30°C, and bacterial counts ($\log_{10}$ cfu/mL) were recorded after 48 h.

Determination of Glucose and Lactate. After centrifugation of culture samples (12,000 × *g* for 5 min at 4°C), the concentrations of glucose and lactate were determined enzymatically (glucose, Elitech, Paris, France; lactate, bioMérieux, Marcy l'Etoile, France).

Determination of 3-Methylbutanal and 3-Methylbutanol by Headspace Gas Chromatography. During culture of *C. maltaromaticum* LMA 28 under different DOC, samples (1 mL) were collected at different intervals and stored immediately in hermetic 10-mL vials at −25°C until analysis by headspace gas chromatography. A 1.0-mL headspace sample was injected (splitless) on the column after 2 min of incubation at 90°C. The chromatograph (PR 2100, Périchrom, Saulx-lès-Carthusian, France) was equipped with an injector with headspace (Headspace HT 300A, Périchrom) and a flame-ionization detector (Périchrom). The volatile compounds were separated using a capillary column of 30 m × 0.25 mm × 0.2 μm and proportioned by integrator WINILAB III (Périchrom). The carrier gas was nitrogen at a constant flow of 2 mL/min. The temperature of the detector was maintained at 190°C. The vials were pressurized for 30 s and injection lasted 1 s. The adjustment of the quantity of product by the valve of open escape or "split" was 5:5. The oven temperature was initially kept at 45°C for 1 min, increased by 5°C/min to 190°C, and then maintained at 190°C for 12 min. Standard curves were carried out with solutions of 3-methylbutanal and 3-methylbutanol with concentrations varying between 1 and 5,000 μ*M*.

Determination of KADC and KADH Enzyme Activities. One liter of MCGC medium was taken from each culture at the maximum production rate of 3-methylbutanal and 3-methylbutanol in sterile conditions. Cell-free extracts and protein contents, along with determination of KADC and KADH, were measured as described previously (Afzal et al., 2012).

RESULTS AND DISCUSSION

Bacterial Growth

The growth of *C. maltaromaticum* LMA 28 during batch culture was influenced by the presence of oxygen

(Figure 2). The viable cell concentration reached 6 $\log_{10}$ cfu/mL with a maximum specific growth rate of 0.02 h^{-1} in the absence of oxygen (Figure 2A), whereas the maximum viable cell concentration reached 7 $\log_{10}$ cfu/mL in the presence of oxygen, with maximum specific growth rates of 0.06 and 0.035 h^{-1} after 5 h of culture at a DOC of 50 and 90% of air saturation, respectively (Figure 2B and 2C).

Our results are consistent with previous findings showing that many LAB grow aerobically at a specific growth rate higher than that determined in the anaerobic state. For instance, *Leuconostoc mesenteroides* ssp. *mesenteroides* and *Brochothrix thermosphacta* ATCC 11509 showed high specific growth rates (0.66 and 0.50 h^{-1}) under aerobic conditions compared with those achieved in the anaerobic states (0.45 and 0.20 h^{-1}, respectively; Blickstad and Molin, 1984; Borch and Molin, 1989; Plihon et al., 1995).

The slightly different growth response of *C. maltaromaticum* LMA 28 during culture in the presence of oxygen might be due to its facultative anaerobic nature, consuming a substantial proportion of oxygen during the aerobic state, with increased growth yield and specific growth rates, as shown previously for *Leuconostoc* and *Carnobacterium* strains (Borch and Molin, 1989; Plihon et al., 1995).

Influence of DOC on Glucose Consumption and Lactic Acid Production

In the absence of oxygen, very slight consumption of glucose was noticed during the first 5 h of culture, with a specific consumption rate of 0.01 g/$\log_{10}$ cfu per hour (Figure 3A). Moreover, no lactate was detected in the culture medium. The bacterial growth previously determined in this culture condition was thus probably due to the use of yeast extract instead of glucose as a carbon source. The maximum specific consumption rates of glucose were 0.03 and 0.04 g/$\log_{10}$ cfu per hour with DOC of 50 and 90%, respectively. In both culture conditions, 33 g/L glucose remained in the medium at the end of the cultures, indicating that only a small amount of glucose was consumed (Figures 3B and 3C). The production of lactate was 1.8 and 0.9 g/L, with a specific production rate less than 0.01 g/$\log_{10}$ cfu per hour in the presence of 50 or 90% air saturation, respectively (Figures 4A and 4B).

The production of lactate was mainly due to LDH, which remained active under the anaerobic environment, converting pyruvate into lactate. The acidifying activity of *C. maltaromaticum* LMA 28 was previously reported to be weak compared with that of commercial starter LAB (Edima et al., 2008). However, the capacity of *Carnobacterium* species to produce some lactic acid in the presence of oxygen was previously demonstrated. Various strains were shown to produce 0.21 to 0.28 mol of lactic acid per 1 mol of glucose during aerobic batch culture in yeast-peptone-thiamine growth medium (Borch and Molin, 1989).

Effect of DOC on Biosynthesis of 3-Methylbutanal and 3-Methylbutanol

The production of 3-methylbutanal and 3-methylbutanol was similar in the absence of oxygen and in presence of 50% air saturation, whereas a dramatic increase in the concentration of these 2 metabolites was determined with 90% air saturation. The maximum concentrations of 3-methylbutanal and 3-methylbutanol were, respectively, 190 and 320 μM in presence of 90% air saturation, whereas they were 120 to 130 and 100 μM in the absence of oxygen and with 50% air saturation, respectively (Figures 5 and 6). This finding suggests that DOC in the culture medium might have a significant effect on the biosynthesis of 3-methylbutanal and 3-methylbutanol in *C. maltaromaticum* LMA 28. Regarding the weak concentrations of 3-methylbutanal and 3-methylbutanol produced (0.5 mM), no significant decrease in leucine concentration was determined (data not shown). This also suggests that leucine was catabolized only through 3-methylbutanal and 3-methylbutanol producing pathways. These results are in agreement with previous studies showing that such a change in the production of metabolites could be attributed to the modification of redox potential (E_h) of the culture medium by incorporation of oxygen or addition of oxidizing (NAD^+) and reducing agents (NADH, H^+) (Kieronczyk et al., 2006; Deetae et al., 2011). However, no significant difference in the production of metabolites from leucine catabolism was observed in the presence or absence of oxygen by *Carnobacterium piscicola* strain 545 (Larrouture-Thiveyrat and Montel, 2003).

The biosynthesis of the flavor compounds 3-methylbutanal and 3-methylbutanol from leucine catabolism may arise via 2 metabolic routes: the direct (KADC) or the indirect (KADH) pathway, or both (Thierry et al., 2002; Helinck et al., 2004; Afzal et al., 2012). To determine the biosynthetic route responsible for synthesis of 3-methylbutanal and 3-methylbutanol in the absence or presence of oxygen, activities of KADC and KADH were determined during culture with 90% air saturation and during culture without oxygen. These determinations were performed when the specific production rates of 3-methylbutanal and 3-methylbutanol were maximal (Figures 5 and 6). The activity of KADC was 3 times higher with 90% air saturation (3.95 $\pm$ 1.48 nmol/mg per minute) than without oxygen (1.35 $\pm$ 0.49 nmol/mg per minute), suggesting that KADC

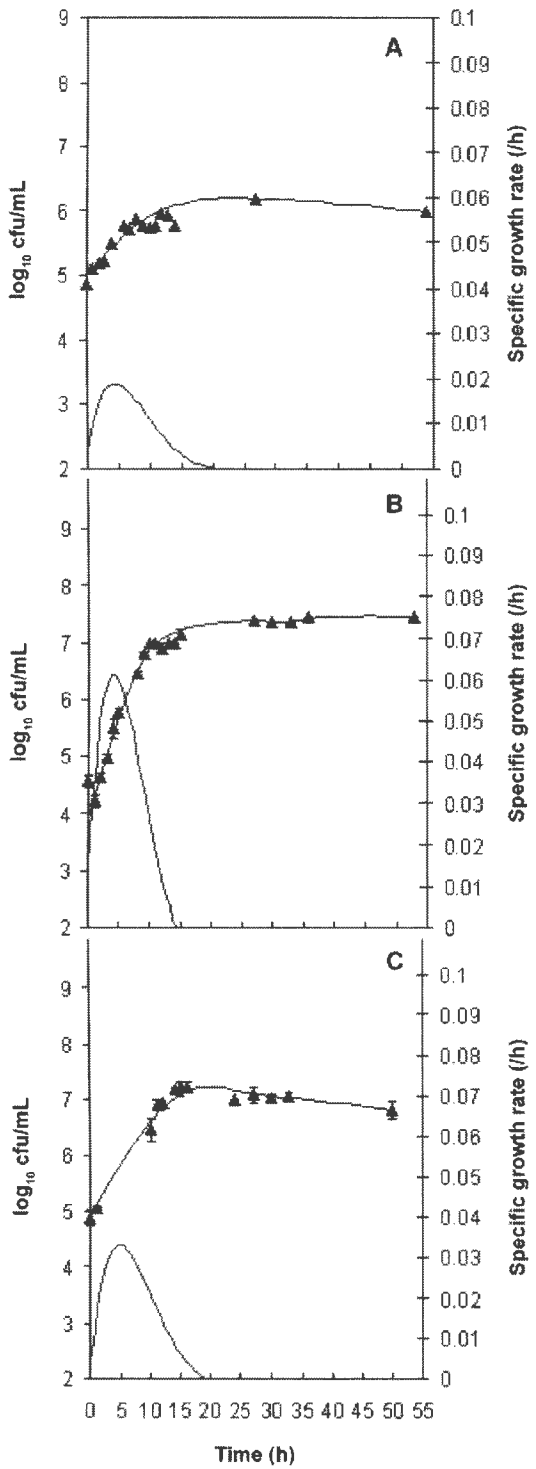

Figure 2. Growth of *Carnobacterium maltaromaticum* LMA 28 in MCGC minimal medium, along with specific growth rates cultivated in bioreactors under different dissolved oxygen concentrations: (A) 0% oxygen, (B) 50% air saturation, and (C) 90% air saturation. Curves with symbols (▲) represent cellular concentration, and curves without symbols represent the specific growth rate.

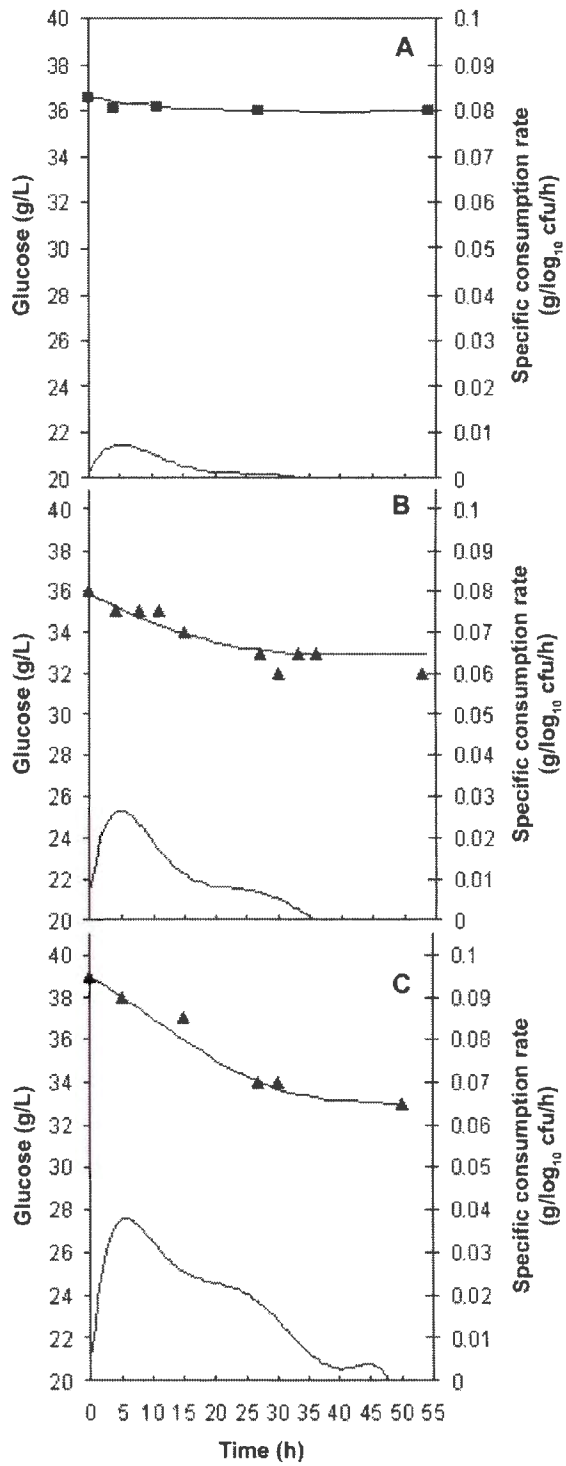

Figure 3. Consumption of glucose along with specific consumption rates during cultivation of *Carnobacterium maltaromaticum* LMA 28 in bioreactors under different dissolved oxygen concentrations: (A) 0% oxygen, (B) 50% air saturation, and (C) 90% air saturation. Curves with symbols (▲) represent residual glucose concentration, and curves without symbols represent the specific rate of glucose consumption.

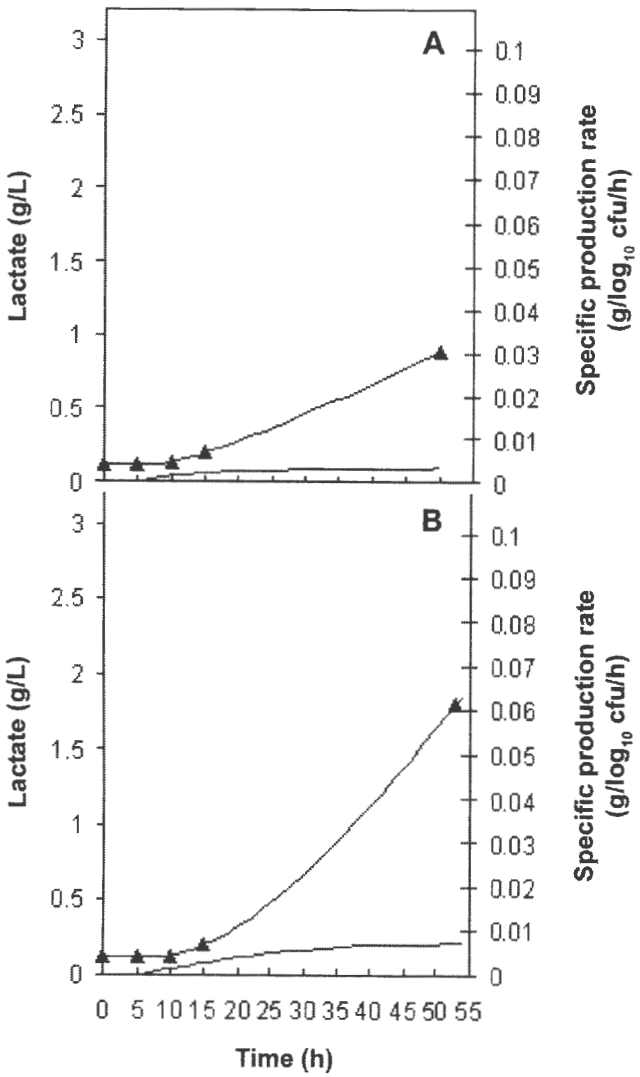

Figure 4. Production of lactate along with specific production rates during cultivation of *Carnobacterium maltaromaticum* LMA 28 in bioreactors under different dissolved oxygen concentrations: (A) 50% air saturation and (B) 90% air saturation. Curves with symbols (▲) represent lactate concentration, and curves without symbols represent the specific rate of lactate production.

might be more active under high DOC. The same tendency was observed for KADH activity: 1.67 ± 0.01 nmol/mg per minute in the absence of oxygen and 3.5 ± 0.05 nmol/mg per minute with a DOC at 90% of air saturation. The presence of oxygen in the culture medium clearly induced an increase in the activity of both enzymes (which could be related to an increase in enzyme synthesis).

It was previously thought that extracellular redox potential might affect KADC and KADH activities. Under oxidative conditions, the production of 3-meth-

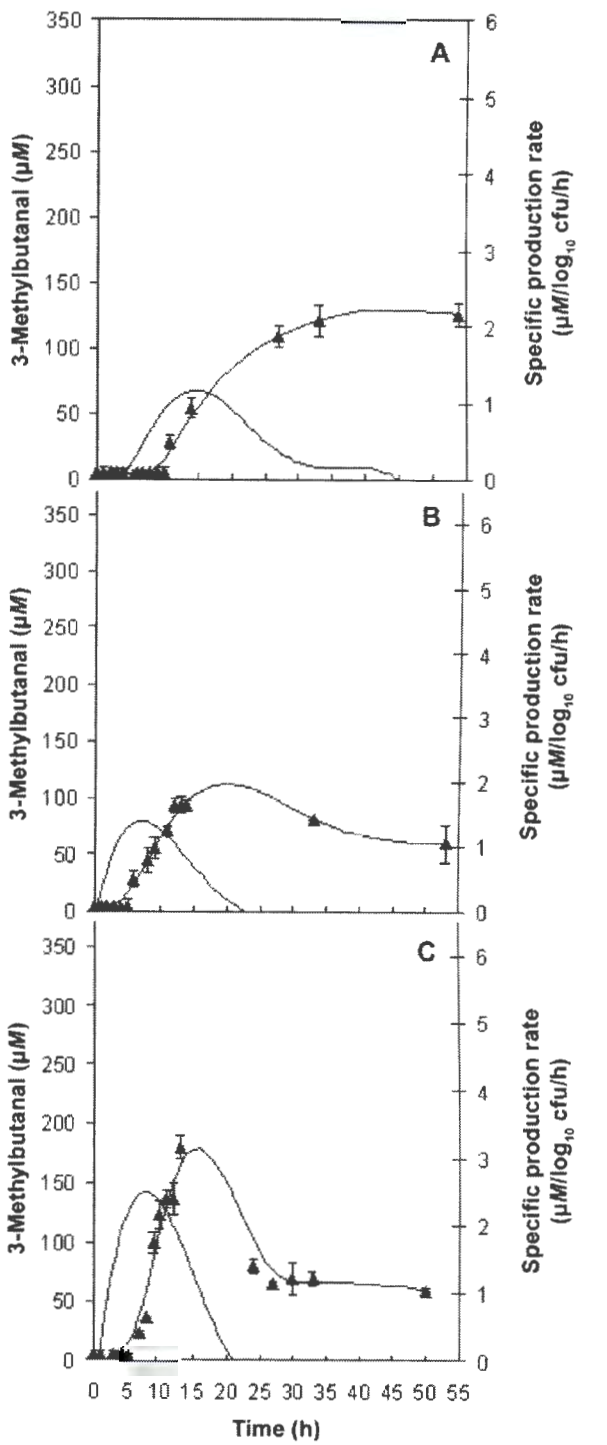

Figure 5. Biosynthesis of 3-methylbutanal along with specific production rates during cultivation of *Carnobacterium maltaromaticum* LMA 28 in bioreactors under different dissolved oxygen concentrations: (A) 0% oxygen, (B) 50% air saturation, and (C) 90% air saturation. Curves with symbols (▲) represent 3-methylbutanal concentration, and curves without symbols represent the specific rate of 3-methylbutanal production.

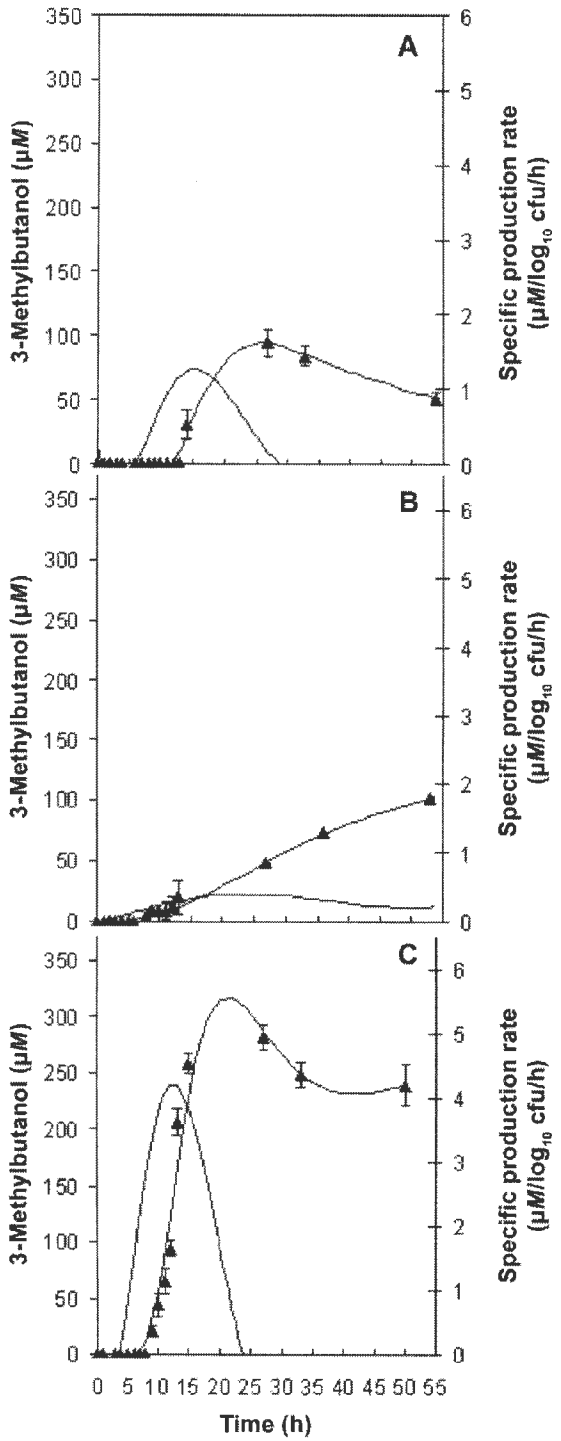

Figure 6. Biosynthesis of 3-methylbutanol along with specific production rates during cultivation of *Carnobacterium maltaromaticum* LMA 28 in bioreactors under different dissolved oxygen concentrations: (A) 0% oxygen, (B) 50% air saturation, and (C) 90% air saturation. Curves with symbols (▲) represent 3-methylbutanol concentration, and curves without symbols represent the specific rate of 3-methylbutanol production.

ylbutanal would mainly be due to KADC (Kieronczyk et al., 2006), whereas in reducing conditions, 3-methylbutanal would be synthesized mainly via the KADH pathway (Yvon and Rijnen, 2001). The determination of both KADC and KADH activities in the presence and absence of oxygen provided no answers concerning the respective roles of these 2 enzymes in *C. maltaromaticum* LMA 28, and further studies are required to clarify the role of these enzymes in synthesis of 3-methylbutanal. For instance, it has been shown that the ratio of NAD^+ to NADH, H^+ affects pyruvate dehydrogenase activity, which is responsible for the oxidative decarboxylation of aromatic and branched-chain keto acids in *Lactococcus lactis* (Yvon and Rijnen, 2001). We could anticipate a similar effect on KADH enzyme activity.

CONCLUSIONS

In conclusion, DOC has a significant influence on the growth and biosynthesis of 3-methylbutanal and 3-methylbutanol in *C. maltaromaticum* LMA 28. The highest DOC tested resulted in increased production of both flavor compounds. This increase might be due to stimulation of both KADC and KADH pathways as revealed by the determination of enzyme activities under different DOC states. To control the desired formation of 3-methylbutanal by *C. maltaromaticum* LMA 28 in food applications, external parameters, such as oxygen or redox potential, should be considered and controlled

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III.3 Contributions de l'article

Le maintien des différents niveaux de saturation en air (0, 50 et 90 %) au cours de cultures discontinues de *C. maltaromaticum* LMA 28 a permis de montrer une influence de ce paramètre sur la croissance des cellules viables, la consommation de glucose, la production de lactate et la biosynthèse de 3-méthylbutanal et de 3-méthylbutanol.

Au niveau de la croissance bactérienne, en présence de 50 et 90 % de saturation en air, la biomasse atteint des valeurs de 10^7 ufc.mL^{-1} en fin de culture, avec des μ_{max} de 0,06 et 0,035 h^{-1} respectivement. En absence d'oxygène, la croissance est ralentie avec un μ_{max} de 0,02 h^{-1}.

Cette augmentation du taux de croissance en aérobiose a été également démontrée chez *Leuconostoc* et *Brochothrix* et a été attribuée à la consommation d'oxygène et à la production d'une quantité importante d'ATP (Borch and Molin, 1989; Plihon et al., 1995).

Dans nos conditions de culture, la consommation de glucose et la production d'acide lactique sont également supérieures en présence d'oxygène, même si elles restent limitées.

Les *Carnobacterium* sont des bactéries héterofermentatives facultatives, capables de métaboliser les hexoses en acide lactique, acétate, éthanol, CO_2 et acide formique. Chez *Leuconostoc*, en aérobiose, l'oxydation du pyruvate peut conduire à une augmentation de la synthèse d'acétate et d'ATP sans changement de production de lactate (Plihon et al., 1995). Lors de l'oxydation du pyruvate, l'oxygène va jouer le rôle d'accepteur d'électrons, ce qui permet la réoxidation du NAD(P)H formé pendant la glycolyse par l'activité de la NADH oxydase, présente chez les LAB.

La production de 3-méthylbutanal et 3-méthylbutanol est assez semblable à 0 et 50 % de saturation en air, tandis qu'une augmentation de 1,5 et 3 fois respectivement a été mesurée en présence de 90 %. Cela suggère que l'oxygénation dans le milieu de culture a un effet significatif sur la biosynthèse de ces deux composés aromatiques chez *C. maltaromaticum* LMA 28.

La mesure des activités KADC et KADH montre que la présence d'oxygène (90 %) dans le milieu de culture induit une augmentation de l'activité spécifique d'un facteur 3 pour la KADC et d'un facteur 2 pour la KADH par rapport à la condition anaérobie, en accord avec les valeurs détectées de métabolites.

Il a été supposé que dans des conditions oxydantes, la production de 3-méthylbutanal serait principalement due à la KADC (Kieronczyk et al., 2006), alors que dans des conditions réductrices, la voie de la KADH serait prédominante pour la biosynthèse de cet aldéhyde (Yvon

et Rijnen, 2001). Chez *C. maltaromaticum* LMA 28, l'oxygénation du milieu de culture permet l'activation simultanée des deux voies de biosynthèse de 3-méthylbutanal, ce qui laisse supposer que ces deux voies seraient impliquées dans la synthèse accrue de ce composé en présence d'oxygène.

3ème Chapitre

Conclusion générale et Perspectives

Chapitre III : Conclusion générale et perspectives

Ces travaux de thèse s'inscrivent dans la continuité des recherches menées au LIBio sur la signification écologique du genre *Carnobacterium*, bactérie lactique atypique et sur son impact sur les autres flores. Pour cela, deux approches ont été utilisées. La première consiste en l'étude des aptitudes technologiques de *C. maltaromaticum* en tant que flore d'affinage. La seconde approche repose sur l'identification des voies de biosynthèse d'un composé aromatique, le 3-méthylbutanal et des facteurs pouvant influencer sa production.

Afin d'étudier le rôle de *C. maltaromaticum* dans des fromages, les caractérisations phénotypiques et génotypiques de souches isolées de fromage à pâte molle ont été réalisées. Des fromages modèles ont ensuite été fabriqués dans des conditions de laboratoire avec et sans ajout d'une souche de *C. maltaromaticum*.

Les résultats de la caractérisation phénotypique et génotypique de six souches de *C. maltaromaticum* ont montré qu'elles n'étaient pas phylogénétiquement identiques. Bien que ces souches n'aient pas montré de différences significatives dans leur profil d'assimilation de carbone, leur spectre antibactérien et leur aptitude technologique, cette différenciation a été révélée par électrophorèse en champ pulsé (PFGE). Acidotolérantes, ces 6 souches se sont révélées incapables de coaguler rapidement le lait. En conséquence, *C. maltaromaticum* ne peut pas être utilisée comme starter dans l'industrie du fromage car elle ne possède pas une capacité d'acidification suffisante par rapport à des bactéries starters, comme *L. lactis* et *S. thermophilus*.

La transformation du lait en fromage nécessite plusieurs étapes impliquant diverses espèces bactériennes résultant de l'inoculation, la contamination de l'air ou résidant dans le lait et résistant aux traitements thermiques. L'étude de l'impact de *C. maltaromaticum* LMA 28 sur la microflore bactérienne d'un fromage à pâte molle a révélé que sa présence provoque une diminution de la concentration en *Psychrobacter* sp., micro-organisme qui pourrait être responsable de l'accélération des phénomènes de vieillissement du fromage.

La concentration cellulaire initiale en *C. maltaromaticum* LMA 28 est le facteur principal impliqué dans l'inhibition de *Psychrobacter* sp. et de *L. monocytogenes* CIP 82110. Les chemins optimaux montrent l'inhibition maximale de *Psychrobacter* sp. et *L. monocytogenes* pour les

quatre facteurs étudiés. Cependant, les mécanismes responsables de cette inhibition sont encore inconnus et mériteraient des investigations complémentaires.

La présence de *C. maltaromaticum* dans un écosystème bactérien complexe comme le fromage ne permet pas d'évaluer sa contribution dans la production de flaveurs maltées; il était donc intéressant d'étudier sa production de composés aromatiques *in vitro*. Le 3-méthylbutanal, le 3-méthylbutanol et l'acide 3-méthylbutanoïque, provenant du catabolisme de la leucine, ont un impact sur la qualité sensorielle des fromages en leur conférant une flaveur maltée (Curioni and Bosset, 2002; Avsar et al., 2004). En effet, la conversion de la leucine en 3-méthylbutanal par *S. lactis* var. *maltigenes* (renommé *C. maltaromaticum*) était responsable de la production de l'arôme malté dans le lait (Sheldon et al., 1971), cité par Larrouture et al. (2000). De même, dans des circonstances non déterminées, Miller et al. (1974) avaient isolé *Lb. maltaromicus* de lait présentant une odeur de malt.

Dans un premier temps, la biosynthèse du 3-méthylbutanal a été étudiée chez la souche *C. maltaromaticum* LMA 28. Les voies de biosynthèse ont été explorées et identifiées au niveau enzymatique et génétique. En fonction de l'espèce bactérienne considérée, la leucine peut être convertie en 3-méthylbutanal suivant deux voies métaboliques; soit directement par la voie de l'α-cétoacide décarboxylase (KADC) ou indirectement par la voie de l'α-cétoacide déshydrogénase (KADH).

Les deux activités enzymatiques (KADC et KADH) ont été détectées et quantifiées *in vitro* chez *C. maltaromaticum* LMA 28. La production de 3-méthylbutanal à partir de la leucine est légèrement réduite en présence d'un inhibiteur spécifique de la KADH, le méta-arsénite de sodium. Ceci suggère que les deux voies précitées sont impliquées *in vivo* dans la biosynthèse de 3-méthylbutanal. En outre, la présence des gènes codant pour l'aminotransférase (*BcaT*), la glutamate déshydrogénase (*gdh*), l'α-cétoacide décarboxylase (*KdcA*), l'α-cétoacide déshydrogénase (*bkdABCD*) et l'aldéhyde déshydrogénase (*adhE*) a été confirmée chez *C. maltaromaticum* LMA 28. Ces résultats révélant l'existence et la fonctionnalité de ces deux voies métaboliques, fait de cette souche un exemple unique parmi les bactéries lactiques. Cependant, le rôle respectif de ces deux enzymes lors de la synthèse *in vivo* de 3-méthylbutanal reste encore à préciser.

Le contrôle de la formation de 3-méthylbutanal, notamment pour apporter une touche aromatique à certains fromages, pourrait être envisagé. La fonctionnalité des voies métaboliques impliquées pourrait être influencée par divers facteurs tels que le pH, la température et l'environnement des cellules et plus particulièrement le niveau en oxygène ou le potentiel redox.

Dans une deuxième étape, une étude de l'influence de l'oxygène sur la croissance et la biosynthèse de ces composés aromatiques a été réalisée chez *C. maltaromaticum* LMA 28, lors d'une culture en mode discontinu réalisée dans un bioréacteur parfaitement contrôlé. L'application de différents niveaux de saturation en air du milieu de culture a permis de révéler une influence significative de la concentration en oxygène sur la croissance et la biosynthèse de 3-méthylbutanal et de 3-méthylbutanol chez *C. maltaromaticum* LMA 28. Le taux de croissance spécifique maximal a été mesuré lors de la culture avec une oxygénation du milieu de culture permettant d'obtenir 50 % de la saturation en air. La vitesse spécifique de consommation du glucose et la vitesse spécifique de production de lactate étaient plus élevées en présence d'oxygène qu'en absence. De plus, *C. maltaromaticum* LMA 28 a produit des quantités élevées de 3-méthylbutanal et de 3-méthylbutanol au cours d'une culture avec un niveau d'oxygène maintenu à 90 % de la saturation en air, ce qui suggère que l'oxygène a un effet significatif sur la formation de ces composés aromatiques. La production importante des composés aromatiques dans un environnement riche en oxygène a été attribuée à l'activation/stimulation simultanée de la voie de la KADC et de la voie de la KADH. Pour obtenir une formation souhaitée de 3-méthylbutanal, le contrôle de paramètres extérieurs tels que le niveau en oxygène ou le potentiel redox constitue donc une piste intéressante. L'influence de ces paramètres mériterait une étude plus approfondie avant d'envisager une utilisation de *C. maltaromaticum* LMA 28 dans le cadre d'une application alimentaire.

A l'issue de ce travail, de nombreuses perspectives peuvent être envisagées. Ainsi, si la production de l'arôme malté/chocolaté, 3-méthylbutanal, par *C. maltaromaticum* LMA 28 peut constituer un avantage intéressant dans le cadre d'une application fromagère, il apparaît essentiel d'étudier également l'impact de cette espèce bactérienne sur la protéolyse et la lipolyse au cours de l'affinage du fromage.

L'utilisation de *C. maltaromaticum* en technologie fromagère afin de limiter le développement d'espèces pathogènes ou d'altération pourrait être limitée en raison de sa capacité

à produire de la tyramine à partir de la tyrosine ou de l'histamine. Cependant, dans un fromage à pâte molle artificiellement inoculé avec *C. maltaromaticum* LMA 28, ni la tyramine ni l'histamine n'ont été détectées (Edima et al., 2007).

La présence de *C. maltaromaticum* a été montrée de façon quasi permanente dans certains fromages à pâte molle, fabriqués à partir de lait cru de vache, de brebis ou de chèvre (Millière et al., 1994; Cailliez-Grimal et al., 2005). Ses niveaux de population sont quelquefois très élevés en fin d'affinage en raison de valeurs de pH basiques, favorables à sa croissance, et en raison de périodes de stockage relativement longues en réfrigération. Cette espèce de bactérie lactique n'est pas commercialisée en tant que levain pour les industries alimentaires de fermentation. Sa présence dans des fromages est en relation avec son habitat (herbe, fourrages) et est liée à certaines étapes de la fabrication, affinage puis stockage au froid, qui la favorisent. Certainement présentes à un niveau très faible de population dans le lait cru, l'espèce *C. maltaromaticum* est donc capable progressivement de devenir la flore lactique dominante dans des fromages affinés.

Ainsi, ses caractéristiques psychrotrophes lui confèrent la capacité de s'implanter aux températures d'affinage (12-14 °C). De plus, son caractère alcalinophile lui permet de croître à des pH alcalins. Cette remontée de pH est provoquée par la flore d'affinage, *G. candidum* et *P. camemberti* ensemencés lors de la fabrication fromagère. Ainsi, *C. maltaromaticum* peut jouer un rôle comme flore lactique d'affinage.

Étant non toxique, psychrotrophe et alcalinophile, possédant une activité antibactérienne et la capacité de génération d'un arôme intéressant, *C. maltaromaticum* LMA 28 pourrait être considérée comme «Generally Recognized As Safe (GRAS)» et proposée comme culture bio-protectrice. Il peut ainsi être imaginé que cette souche joue un rôle principalement lors de l'affinage et du stockage au froid préalablement à la commercialisation de fromages à pâte molle. Il est à noter que depuis 2005, une souche de *C. maltaromaticum*, la souche CB1, a déjà été classée comme GRAS (GRn 00159) pour une utilisation dans des plats carnés «ready-to-eat».

Actuellement, il n'existe pas de réglementation claire concernant l'utilisation de bactéries bioprotectrices dans les aliments. Bourdichon et al. (2012) presente une liste de micro-organismes d'utilisation technologique bénéfique pour l'alimentation proposée par des associations de professionnels des ferments, où figure *C. maltaromaticum*. L'approche QPS utilisée par l'EFSA permet d'évaluer l'innocuité de micro-organismes entrant dans la chaine

alimentaire mais ce n'est pas suffisant pour que son utilisation soit autorisée. Le cahier des charges pour l'obtention de ce statut inclue notamment la nécessité de bien connaître la bactérie. Dans cet objectif, le séquençage et l'exploitation des données du génome de *C. maltaromaticum* LMA 28 a été entrepris. L'exploitation des séquences génomiques permettra: 1) d'améliorer et de justifier les applications du potentiel antagoniste des cultures de *Carnobacterium* contre les flores pathogènes ou d'altération des aliments, 2) d'identifier les facteurs de virulence et de résistance aux antibiotiques (cahier des charges QSP), 3) d'identifier et d'étudier les voies métaboliques impliquées dans les propriétés organoleptiques des aliments, 4) de faciliter la caractérisation des réponses cryoadaptatives impliquées dans la psychrotolérance et dans la remarquable stabilité de survie à la congélation qui font de *Carnobacterium* un organisme modèle pour ces propriétés. La psychrophilie de cette souche pourrait notamment être intéressante afin de limiter l'émergence de bactéries pathogènes telles que *Listeria* dans les aliments.

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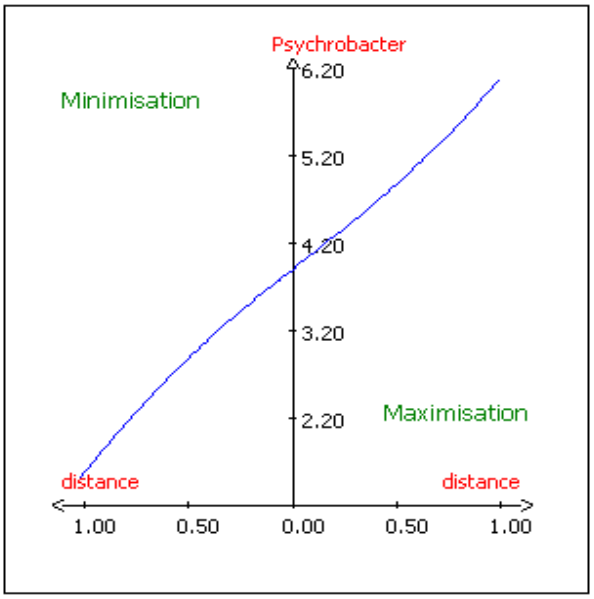
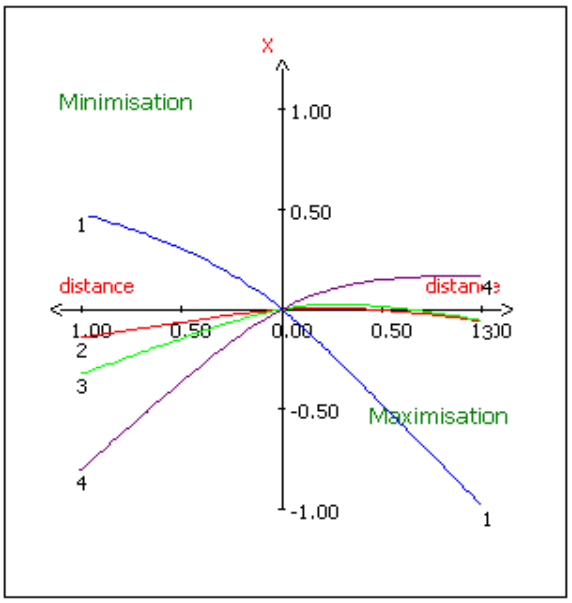
1

Annexes

Chemin optimal pour la réponse de *Psychrobacter* sp.

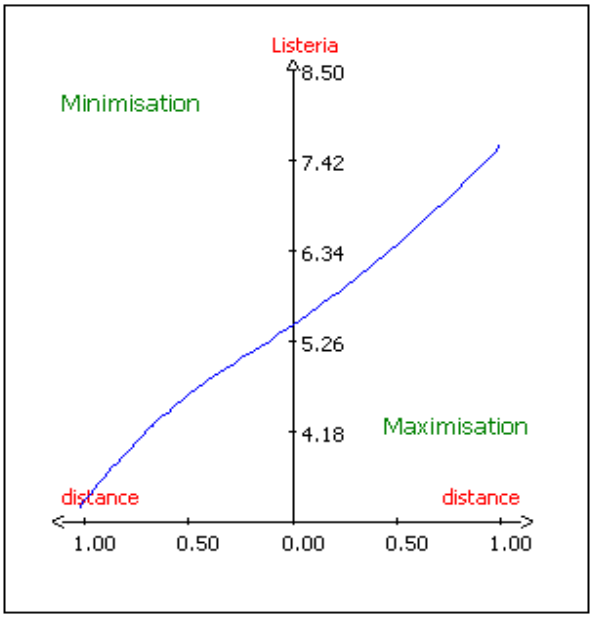
1 : *C. maltaromaticum* ; 2 : NaCl ; 3 : pH ; 4 : Durée

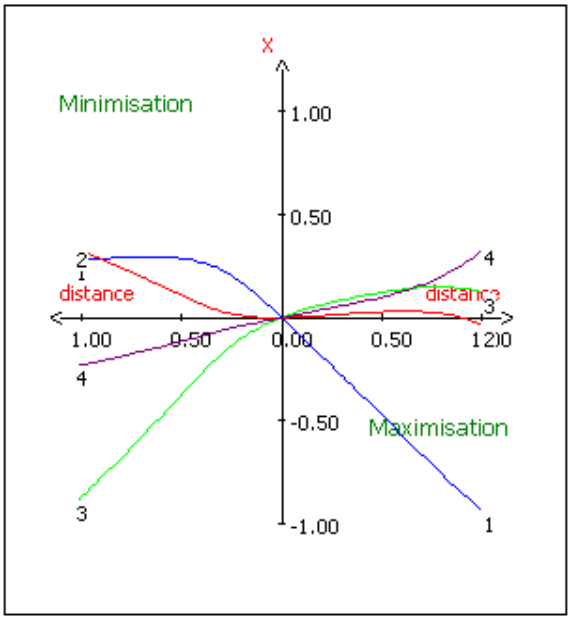




Chemin optimal pour la réponse de la souche *L. monocytogenes* CIP 82110

1 : *C. maltaromaticum* ; 2 : NaCl ; 3 : pH ; 4 : Durée





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Complete Chromosome Sequence of *Carnobacterium maltaromaticum* LMA 28

AQ: au Catherine Cailliez-Grimal,[a] Stéphane Chaillou,[b,c] Jamila Anba-Mondoloni,[b,c] Valentin Loux,[d] Muhammad Inam Afzal,[a] Abdur Rahman,[a] Gilles Kergourlay,[e,f] Marie-Christine Champomier-Vergès,[b,c] Monique Zagorec,[b,c,e,f] Paw Dalgaard,[g] Jorgen J. Leisner,[h] Hervé Prévost,[e,f] Anne-Marie Revol-Junelles,[a] Frédéric Borges[a]

Université de Lorraine, Laboratoire d'Ingénierie des Biomolécules (LIBio), Vandoeuvre-lès-Nancy, France[a]; INRA, UMR1319 Micalis, Jouy-en-Josas, France[b]; AgroParisTech, UMR Micalis, Jouy-en-Josas, France[c]; Unité de Mathématique, Informatique et Génome, INRA, Jouy-en-Josas, France[d]; INRA, Nantes, France[e]; L'Université Nantes Angers Le Mans (L'UNAM), Oniris, UMR 1014 Secalim, Nantes, France[f]; National Food Institute (DTU Food), Technical University of Denmark, Kgs. Lyngby, Denmark[g]; Department of Veterinary Disease Biology, Faculty of Health and Medical Sciences, University of Copenhagen, Copenhagen, Denmark[h]

Within the lactic acid bacterium genus *Carnobacterium*, *Carnobacterium maltaromaticum* is one of the most frequently isolated species from natural environments and food. It potentially plays a major role in food product biopreservation. We report here on the 3.649-Mb chromosome sequence of *C. maltaromaticum* LMA 28, which was isolated from ripened soft cheese.

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Address correspondence to Catherine Cailliez, catherine.cailliez@univ-lorraine.fr.

The genus *Carnobacterium* belongs to the lactic acid bacteria, and currently consists of 11 species. *Carnobacterium maltaromaticum* strains are widely found in foods, including dairy products (1). This species has potential for application as a protective culture in foods. Most research has focused on the production of bacteriocins, their roles in the inhibition of *Listeria monocytogenes*, and on the regulation of metabolic pathways of sensory importance (2). *C. maltaromaticum* LMA 28 was isolated from a soft ripened cheese (3).

The genome sequence of *C. maltaromaticum* LMA 28 was determined using 454 pyrosequencing GS-FLX system (Roche 454 Life Sciences, Mannheim, Germany) and Illumina sequencing. Pyrosequencing runs, including shotgun and paired-end runs, resulted in 15 scaffolds containing 123 contigs and 55-fold coverage. A subsequent Illumina sequencing run performed with a paired-end library corrected 923 indels. PCR-base techniques and Sanger sequencing of the products were used to close the remaining gaps. The manually curated sequence of LMA 28 comprises one chromosome of 3,649,737 bp with an overall G+C content of 34.5%. Coding sequence (CDS) predictions and annotations were performed with Integrative Services for Genomics Analysis (ISGA) (4) and provided 3,933 predicted CDSs, 59 tRNA genes, 6 rRNA operons, and a single 5S rRNA gene.

So far, three genomic sequences of *Carnobacterium* have been published: the complete genome sequence of *Carnobacterium* spp. 17 to 4 (5) isolated from permanent cold seawater; the draft genome sequences of *C. maltaromaticum* ATCC 35586, isolated AQ: A from a diseased salmon (6); and the draft genome of *Carnobacterium* sp. AT7, a piezophilic strain isolated from the Aleutian trench (7). The genome size of the strain ATCC 35586 (3.5 Mbp) is similar to that of LMA 28, and both are approximately 1 Mbp larger than the genomes of *Carnobacterium* spp. 17 to 4 and AT7 (2.6 Mbp and 2.4 Mbp, respectively). The larger chromosomal size of *C. maltaromaticum* might explain the ability of this species to adapt to multiple and diverse environments compared to the other carnobacterial species. This genomic trait is illustrated by the presence of genes involved in the metabolism of branched-chain amino acids.

Indeed, the species *C. maltaromaticum* is well known for its ability to produce the flavor compound 3-methylbutanal, which is the result of leucine catabolism. In lactic acid bacteria, the more prevalent pathway is the α–keto acid dehydrogenase (KaDH) pathway (8). A less common alternative pathway is the α–keto acid decarboxylase pathway, encoded by gene *kdcA*. This gene is only described for two strains of *L. lactis* (8). In the genome sequences of *C. maltaromaticum* LMA 28 and ATCC 35586, an orthologous gene of *kdcA* was found (2) that is absent from the genomes of the other *Carnobacterium* strains.

Nucleotide sequence accession numbers. The complete chromosome sequence of *Carnobacterium maltaromaticum* LMA 28 has been deposited at EMBL/GenBank under the accession number no. HE999757.

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AUTORISATION DE SOUTENANCE
DU DOCTORAT DE L'UNIVERSITE DE LORRAINE

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Monsieur LUBBERS Samuel, Maître de Conférences HDR, AGROSUP Dijon

Le Président de l'Université de Lorraine, autorise :

Monsieur AFZAL Muhammad Inam

à soutenir devant un jury de l'UNIVERSITE DE LORRAINE, une thèse intitulée :

"Physiologie et aspects technologiques de *Carnobacterium maltaromaticum* lma 28 en biopréservation alimentaire"

en vue de l'obtention du titre de :

DOCTEUR DE L'UNIVERSITE DE LORRAINE

Intitulé du doctorat : **"Procédés biotechnologiques et alimentaires"**

Fait à Vandoeuvre, le **12 Octobre 2012**

Le Président de l'Université de Lorraine,

Pierre MUTZENHARDT

Université de Lorraine

UNIVERSITÉ DE LORRAINE
34 COURS LÉOPOLD - CS 25233
54052 NANCY CEDEX
TÉL : 03 54 50 54 00
CONTACT@UNIV-LORRAINE.FR
WWW.UNIV-LORRAINE.FR

Physiologie et aspects technologiques de *Carnobacterium maltaromaticum* LMA 28 en biopréservation alimentaire

Résumé :

Carnobacterium maltaromaticum est une bactérie lactique atypique isolée de fromages à pâte molle. Les caractéristiques majeures de cette bactérie sont i) sa capacité à produire un arôme de type malté ou chocolaté, le 3-méthylbutanal à partir du catabolisme de la leucine ii) sa capacité à produire des bactériocines, peptides à activité antibactérienne vis-à-vis de flores pathogènes et/ou d'altération fréquemment rencontrées en industries alimentaire, telle que *Listeria monocytogenes*. Une caractérisation phénotypique et génotypique réalisée sur six souches de *C. maltaromaticum* isolées du même biotope a montré que celles-ci ne sont pas phylogénétiquement identiques. Même si ces souches n'ont pas la capacité à coaguler rapidement le lait, elles sont acidotolérantes. Elles n'influent pas sur la capacité ni la rapidité de coagulation des starters, *Lactococcus lactis* et *Streptococcus thermophilus* utilisés en industrie laitière. L'étude de l'impact de *C. maltaromaticum* sur la flore bactérienne du fromage a révélé que sa présence provoque la diminution de la concentration de *Psychrobacter*, germe pouvant être responsable d'une accélération du phénomène de vieillissement du fromage. Un plan d'expérience a été réalisé pour mettre en évidence ces inhibitions et les éventuelles interactions entre différents facteurs (concentration cellulaire initiale, concentration en NaCl, pH, durée d'incubation). Deux modèles ont été choisis i) *Psychrobacter* sp. et ii) *L. monocytogenes*. La concentration cellulaire de *C. maltaromatiucm* est le facteur le plus important pour inhiber les bactéries testées. Cette espèce opportuniste pourrait être considérée comme un auxilliaire de fabrication intéressant et pourrait être retenue comme flore bactérienne d'affinage. Toutes les souches de *C. maltaromaticum* testées sont capables de produire du 3-méthylbutanal pouvant conférer une flaveur maltée au fromage. Les études menées sur la biosynthèse du 3-méthylbutanal ont montré la présence et la fonctionnalité de deux voies métaboliques; la voie directe impliquant l'α-cétoacide décarboxylase et la voie indirecte passant par l'α-cétoacide déshydrogénase. L'oxygénation du milieu de culture a un impact positif sur la formation du 3-méthylbutanal et du 3-méthylbutanol avec la stimulation des voies directes et indirectes.

Mots clés : *Carnobacterium maltaromaticum*, bactéries lactiques, arômes, leucine, fromage, biopréservation, interactions bacterienne, *Listeria*, *Psychrobacter*.

Physiology and technological aspects of *Carnobacterium maltaromaticum* LMA 28 in food biopreservation

Abstract :

Carnobacterium maltaromaticum is a lactic acid bacterium isolated from atypical soft cheeses. The major characterstics of this bacterium are i) its ability to produce a malty or chocolate-like flavor 3-methylbutanal from leucine catabolism ii) its ability to produce bacteriocins against pathogenic and spoilage bacteria for instance, *Listeria monocytogenes*. Phenotypic and genotypic characterization of six strains of *C. maltaromaticum* isolated from the same habitat showed that they were not phylogenetically identical. Although these strains lacked the ability to coagulate the milk quickly, they were acid tolerant. They did not affect the milk coagulation capacity of startes, *Lactococcus lactis* and *Streptococcus thermophilus* used in dairy industry. The study on the impact of *C. maltaromaticum* on the bacterial flora of cheese showed that its presence resulted in the decrease of the concentration of *Psychrobacter*, which might be responsible for accelerating the aging phenomenon of cheese. An experimental plan was realized to highlight these inhibitions and possible interactions between factors (cell concentration, NaCl, pH, incubation time) by choosing two models i) *Psychrobacter* sp. and ii) *L. monocytogenes*. The cell concentration of *C. maltaromatiucm* was the factor more significant for the inhibitions of the tested bacteria. Being psychrotrophic, alkalinophilic, malty or chocolate flavor producing and biopreservative agent, this species could play a role as a ripening flora of cheese. All strains of *C. maltaromaticum* tested were able to produce 3-methylbutanal conferring any malt flavor to cheese. The results on the physiology involved in the biosynthesis of 3-methylbutanal showed the presence and functionality of both metabolic pathways; the direct by α-ketoacid dacarboxylase enzyme and indirect comprising α-keto acid dehydrogenase enzyme. The oxygenation of culture medium had a positif impact on the formation of 3-methylbutanal and 3-methylbutanol with the stimulation of both direct and indirect metabolic routes.

Keywords : *Carnobacterium maltaromaticum*, lactic acid bacteria, flavor, leucine, cheese, biopreservation, bacterial interactions, *Listeria*, *Psychrobacter*.
