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ACKNOWLEDGEMENTS

I would like to express my sincere gratitude and appreciation to the person who made all this possible, Prof. Dr. Mohamed Amar. You always dedicated me your time, expertise, and good proposal. I would like to express my special thanks for giving me the opportunity to do this work at the Laboratory of Microbiology and Molecular Biology of the National Center for Scientific and Technical Research. This study was only possible thanks to you.

My genuine gratitude and gratefulness are extended to Prof. Peter Vandamme. Your tremendous guidance, your expertise, thoughtful insights into microbiology, understanding, kindness and helpfulness would be the unforgettable blessing of this stage. I am highly indebted to you for giving me the opportunity to carry out my Ph.D study at the Laboratory of Microbiology of the University of Gent.

I also would like to thank Prof. Dr. Omar El Farricha for his enthusiastic guidance and for his support and assistance during my Ph.D study in the Faculty of Sciences and Technology of the University Sidi Mohamed Ben Abdellah.

Sincere thanks and gratitude are extended to my friends and colleagues from the Laboratory of Microbiology and Molecular Biology, Dr. Tarik Aaanniz, Prof. Mouna Ouadghiri, Mr. Lahcen Ouchari, Mr. Mohamed Chenaoui, Mrs. Bahia Rached, Mrs. Sanae Karmouna, Mr. Azzedine Badouri and Mrs. Leila Bouchane for their collaboration in different parts of this work. Your support and assistance would be the unforgettable blessing of this Ph. D study.

I would like to give my special thanks to Margo Cnockaert, from the Laboratory of Microbiology of the University of Gent, who has always shared everything with me and encouraged me all the time. She helped me both with knowledge in researching and everyday living. She has been a very good friend and colleague.

I would also like to thank Dr. Freek Spitaels from the Laboratory of Microbiology of the University of Gent, for his enthusiastic guidance. I appreciate your willingness to help me.

I am thankful to all the staff of the Laboratory of Microbiology and of LMG Bacteria Collection for all collaboration to this project. Their research experience and thoughtful explanations are highly appreciated.

I would like to thank the members of the examination committee (Prof. Dr. Peter Vandamme, Prof. Dr. Mohamed Amar, Prof. Dr. Omar El Farricha, Prof. Dr. Ir. Jean Swings, Prof. Dr. Saad Ibnsouda Koraichi, Prof. Dr. Khalid Sendide, Prof. Dr. Guissi Sanae, Prof. Dr. Jamal Ibijbijen and Prof. Dr. Kurt Houf) for their evaluation and comments on the thesis.

I thank my parents, Mr. Mohamed Kadri and Mrs. Sanae Kettani, my brother Ahmed, my sister Nada, my aunt Oumama Kadri, and all members of family for their continued support and inspiration throughout this project.

Lastly, thanks to my friends O.C, I.B, T.L and Z.B for trusting me and for their friendship and assistance.

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LIST OF ABBREVIATIONS

°D	Degree dornic
α-CHCA	A-cyano-4-hydroxycinnamic acid
AA	Acetic acid
ACA	All culture agar
Aci.	*Acinetobacter*
acmA	Gene encoding the lactococcal N-acetylmuramidase
ACN	Acetonitrile
AE	Aerobic conditions
Aer.	*Aeromonas*
AFLP	Amplified fragment length polymorphism
AFNOR	Association Française de Normalisation
AN	Anaerobic conditions
ANI	Average nucleotide identity
API	Analytical profile index
ARDRA	Amplified ribosomal DNA restriction analysis
ATCC	American type culture collection, manassas, virginia, usa
ATP	Adenosine triphosphate
atpA	Gene encoding ATP synthase α-subunit
atpD	Gene encoding atpase β-subunit
BCCM/LMG	Belgian Coordinated Collections of Micro-organisms/Laboratory of Microbiology, Ghent University
BHI	Brain heart infusion
Bif.	*Bifidobacterium*
Blast	Basic local alignment search tool
Bp	Base pair
BTC	Belgian technical cooperation
CBA	Columbia blood agar
CCMM/LMBM	Moroccan Coordinated Collections of Micro-organisms /Laboratory of Microbiology and Molecular Biology
CCUG	Culture Collection of the University of Göteborg, Sweden
CHCA	cyano-4-hydroxycinnaminic acid

Chr.	*Chryseobacterium*
CFU	Colony forming units
Cit.	*Citrobacter*
CO_2	Carbon dioxide
cpn60	Gene encoding the *groel* chaperonin
Da	Daltons
DDH	DNA-DNA hybridization
Ddl	Gene encoding D-alanine ligase
DGGE	Denaturing gradient gel electrophoresis
DMSO	Dimethylsulfoxide
DNA	Deoxyribonucleic acid
dnaJ	Gene encoding chaperone protein dnaj
dNTPs	Deoxynucleotides
DSM	Deutsche Sammlung von Mikroorganismen
E.	*Enterococcus*
E-M	Embden-meyerhof
Emp.	*Empedobacter*
Ent.	*Enterobacter*
ERIC	Enterobacterial repetitive intergenic consensus sequence
Esch.	*Escherichia*
FA	Formic acid
FAO	Food and agriculture organization
G+C	Guanine + cytosine
GES	Guanidium-thiocyanate-EDTA-sarkosyl
GIT	Gastro-intestinal tract
GLIPHA	Global livestock production and health atlas
GRAS	Generally recognized as safe
gyrB	Gene encoding DNA gyrase *B*-subunit
HPLC	High performance liquid chromatography
ITS	Intergenic transcribed spacer
IU	International unit
Kg	Kilograms

Koc.	*Kocuria*
L.	*Lactococcus*
LA	Lactic acid
LAB	Lactic acid bacteria
Lb.	*Lactobacillus*
Leuc.	*Leuconostoc*
LM-UGent	Laboratory of Microbiology-Ghent University
LSU	Large subunit
lytA	Gene encoding autolysin
Mac.	*Macrococcus*
MALDI-TOF MS	Matrix- assisted laser desorption/ionization-time of flight mass
MEGA	Molecular evolutionary genetic analysis
Mg	Milligrams
Min	Minutes
Mle	Gene encoding the malolactic enzyme
MLSA	Multilocus sequence analysis
mM	Millimolar
Mol	Mole
Mor.	*Moraxella*
MRS	De Man, Rogosa and Sharpe
MSA	Mannitol salt agar
MUMs	Maximal unique and exact matches
m/z	Mass to charge ratio
NCBI	National Center for Biotechnology Information
NCDO	National Collection of Dairy Organisms
NCTC	National Collection of Type Cultures
ND	Not determined
nM	Nanomolar
nm	Nanometer
O.	*Oenococcus*
OD	Optical density
P.	*Pediococcus*

Pan.	*Pantoea*
PCA	Plate count agar
PCR	Polymerase chain reaction
pepT	Gene encoding tripeptidase
pepV	Gene encoding dipeptidase
PFGE	Pulsed field gel electrophoresis
pH	Potential of hydrogen
PheS	Gene encoding phenylalanyl-trna synthase
PK	Phosphoketolase
ppm	Parts per million
PPMCC	Pearson product-moment correlation coefficient
Ps.	*Pseudomonas*
QPS	Qualified presumption of safety
R.	*Raoultella*
RAPD	Randomly amplified polymorphic DNA
rDNA	Ribosomal deoxyribonucleic acid
RDO	Registered designation of origin
RecA	Gene encoding recombinase enzyme
Rep-PCR	Repetitive extragenic palindromic-PCR
RFLP	Restriction fragment length polymorphism
rnpB	Gene encoding the RNA subunit of endoribonuclease P
Rot.	*Rothia*
rpoA	Gene encoding DNA-directed RNA polymerase α-subunit
rpoB	Gene encoding the bacterial RNA polymerase
rpoC	Gene encoding DNA-dependent RNA polymerase β-subunit
RNA	Ribonucleic acid
rRNA	Ribosomal ribonucleic acid
S.	*Streptococcus*
Ser.	*Serratia*
SEs	staphylococcal enterotoxins
SLSA	Single locus sequence analysis
SDS-PAGE	Sodium dodecyl sulphate polyacrylamide gel electrophoresis

sp. nov.	Species nova
St.	*Staphylococcus*
subsp.	Subspecies
SodA	Gene encoding manganese-dependent superoxide dismutase
TAE	Tris-acetate EDTA
TBC	Total bacterial content
tDNA-PCR	tRNA intergenic length polymorphism analysis
TSA	Trypticase soy agar
Tuf	Gene encoding elongation factor Tu (*EF-Tu*)
UPGMA	Unweighted pair group method using arithmetic average
Var	Variable
VRBG	Violet red bile glucose
WR	Weak reaction

PART I
INTRODUCTION & OBJECTIVES

INTRODUCTION GÉNÉRALE

Le dromadaire (*Camelus dromedarius*, chameau à une bosse) est l'animal d'élevage le plus important des déserts du Maroc. C'est un animal polyvalent, utilisé pour son approvisionnement en lait, viande, cuir et transport (Kappeler, 1998). Le lait de chamelle est l'une des ressources alimentaires les plus précieuses pour les populations pastorales dans les zones arides et semi-arides, où il couvre une partie substantielle des besoins nutritionnels quantitatifs et qualitatifs. Les populations autochtones croient que le lait de chamelle cru a des vertus thérapeutiques par rapport à celui d'autres espèces animales (Barbour et al. 1985, Benkerroum et al. 2003). Néanmoins, le lait de chamelle est produit de manière traditionnelle, et est habituellement manipulé et transporté dans des conditions sanitaires déplorables. En outre, les troupeaux de chameaux bénéficient rarement de soins vétérinaires. Par conséquent, la mammite est commune chez les femelles allaitantes (Obeid et al. 1996). Le lait produit est alors susceptible de provoquer des infections d'origine alimentaire et les facteurs antimicrobiens naturels ne peuvent fournir qu'une protection limitée contre les agents pathogènes spécifiques et ce pour une courte période. Un tel risque est plus élevé lorsque le lait est consommé à l'état cru, chose que les producteurs locaux ont l'habitude de faire.

Alors que de nombreuses études ont étudié la microbiologie du lait de vache, de brebis et de chèvre, seules quelques études se sont intéressées à la microbiologie du lait de chamelle. Une des principales raisons du manque d'intérêts aux études microbiologiques vis-à vis de ce produit est que la production mondiale du lait de chamelle destiné à la consommation humaine a été récemment estimée à seulement 1,3 millions de tonnes / an (FAO, 2004).

En effet, le Maroc produit seulement 3.900 tonnes / an de lait de chamelle, alors que d'autres pays sont de grands producteurs tels que la Somalie (850.000 tonnes/an) et l'Arabie saoudite (89.000 tonnes/an). La majorité des études scientifiques portant sur les chameaux ont été principalement axées sur leurs caractéristiques anatomiques et physiologiques d'adaptation à des climats défavorables. Par conséquent, les informations concernant le lait de chamelle sont très limitées. Plus particulièrement, seulement quelques études portant sur les bactéries lactiques du lait de chamelle produit au Maroc ont été réalisées. Néanmoins, aucune de ces études n'a appliqué des techniques moléculaires modernes pour explorer la diversité des bactéries lactiques ou la diversité du microbiote total de ce produit.

L'objectif de la présente étude est de présenter d'abord une description plus complète et détaillée de la diversité des bactéries lactiques, et de décrire d'une autre part le microbiote global d'échantillons de lait de chamelle cru provenant de différentes régions du Maroc en utilisant des méthodologies contemporaines. L'approche polyphasique utilisée consiste en la réaction de polymérisation en chaine, en utilisant des éléments répétés (rep-PCR), (plus spécifiquement, (GTG_5-PCR)) et la spectrométrie de masse par désorption laser assistée par matrice / ionisation à temps de vol (MALDI-TOF MS) (Ghyselinck et al. 2011) en tant qu'outils de déréplication, combinée avec une analyse de séquence de l'ARN ribosomal 16S (De Bruyne et al. 2008) et / ou d'autres marqueurs moléculaires pour l'identification précise des isolats représentatifs. L'analyse de séquences du gène *rpoB* a été effectuée pour identifier les membres de la famille des *Enterobacteriaceae* (Mollet al. 1997; Nhung et al. 2007, Spitaels et al. 2014), du gène *gyrB* pour l'identification des membres la famille des *Pseudomonacae* (Kupfer et al. 2006; Yanez et al . 2003), du gène *dnaJ* pour les espèces du genre *Staphylococcus* (Shah et al. 2007), et du gène *pheS* pour identifier les bactéries lactiques (De Bruyne et al. 2007, et 2008; Naser et al. 2005, et 2007; Spitaels et al. 2014). Les isolats qui n'ont pas pu être identifiés en utilisant cette approche, ont été par ailleurs étudiés en utilisant la technique d'hybridation d'ADN (Ezaki et al. 1989), la détermination de la composition en bases d'ADN (% en G+C) (Mesbah et al. 1989) et ont été par la suite soumis à une caractérisation phénotypique détaillée.

OBJECTIFS

Objectifs globaux

Le lait est la principale source de nutrition pour les veaux nouveau-nés et fournit tous les nutriments essentiels à la croissance et au développement tels que les protéines, les minéraux, les glucides, les acides gras, les facteurs de croissance et les modulateurs immunitaires. Le lait de chamelle est apprécié pour ses caractéristiques anti-infectieuses, anti-cancéreuses, anti-diabétiques, et plus généralement comme étant un agent de rétablissement chez les patients convalescents (Agrawal et al. 2003; Magjeed, 2005; Shabo et al. 2005). Les composants tels que la lactoperoxydase, la lactoferrine, les immunoglobulines, le lysozyme ou la vitamine C jouent un rôle central dans la détermination de ces propriétés (Konuspayeva et al. 2007). Le lait de chamelle a été utilisé à l'état frais ou fermenté dans différents pays. De nouvelles sources de nutriments tels

que le lait de chamelle devraient être exploitées le plus souvent pour faire varier l'alimentation humaine et bénéficier de nouveaux ingrédients fonctionnels et de composants d'aliments naturels. Le microbiote bénéfique du lait de chamelle, représenté par les bactéries lactiques, est une source potentielle de matériaux biologiques pouvant être utilisés dans la technologie laitière. Cependant, la transformation du lait de chamelle par fermentation n'est pas facile à réaliser et davantage de recherches élucidant le processus sont nécessaires. Bien que le lait de vache a été largement étudié au Maroc, jusqu'à maintenant, aucune étude utilisant des méthodes moléculaires modernes n'a été entreprise pour caractériser le microbiote du lait de chamelle, en particulier la diversité des bactéries lactiques y existantes. Ainsi, la présente étude vise à déterminer les propriétés biochimiques, physiologiques et phénotypiques de souches de bactéries lactiques isolées à partir du lait de chamelle produit au Maroc. Par conséquent, afin d'étendre avec succès la gamme d'applications du lait de chamelle Marocain à l'échelle industrielle, une connaissance plus approfondie de sa composition bactérienne est alors nécessaire.

L'objectif global de la présente étude est d'appliquer des techniques polyphasique appropriées, pour déterminer pleinement la diversité des bactéries lactiques des échantillons de lait de chamelle cru, collectés à partir de différentes régions du Maroc en tant que première partie de cette étude, suivi par la détermination du microbiote global en seconde partie. Par conséquent, la génération d'une collection de souches de bactéries lactiques bien caractérisées, qui serviront de ressource pour la recherche de levains, ayant des caractéristiques technologiques optimales, et pouvant être utilisées pour la production de dérivés de lait de chamelle.

Objectifs spécifiques

- Dans la première partie de cette étude, la diversité des bactéries lactiques du lait de chamelle cru est explorée en se basant sur une approche de culture-dépendante. Initialement, la discrimination génotypique et l'identification préliminaire des bactéries lactiques repose sur l'utilisation de la réaction de polymérisation en chaine, en utilisant des éléments répétés et la $(GTG)_5$ comme amorce, soit la $(GTG)_5$-PCR. Par la suite, l'analyse de la séquence d'ARN ribosomal 16S a été utilisée pour identifier les isolats représentatifs.
- Dans la deuxième partie de cette étude, quatre nouvelles espèces de bactéries lactiques, appartenant au genre *Streptococcus*, ont été isolées dans le cadre de l'étude de la

diversité des bactéries lactiques du lait de chamelle, et ont été entièrement caractérisées par des études taxonomiques polyphasiques. La $(GTG)_5$-PCR et les données de séquences de gènes de l'ARNr 16S ont été complétées par une analyse de séquence des gènes codant pour des protéines, notamment l'analyse des séquences de gènes de la sous-unité alpha de la phénylalanyl-ARNt synthétase (*pheS*), la sous-unité alpha de l'ARN-polymérase (*rpoA*) et la sous-unité alpha de l'ATP synthase (*atpA*), des expériences d'hybridation d'ADN, la détermination de la composition en bases de l'ADN (% G+C), l'analyse MALDI-TOF MS et une caractérisation phénotypique détaillée, ce qui a permis la description formelle de quatre nouvelles espèces du genre *Streptococcus* dont les noms *Streptococcus moroccensis, Streptococcus rifensis, Streptococcus cameli* et *Streptococcus tangierensis* ont été attribués.

- La troisième partie de cette étude a consisté en l'étude de la diversité bactérienne globale du lait de chamelle en utilisant le MALDI-TOF MS comme étant un outil rapide et fiable pour la déréplication et l'identification des bactéries. A cet effet, un total de 808 isolats ont été analysés grâce à leurs empreintes digitales obtenues en utilisant le MALDI-TOF MS. Une identification finale a été obtenue en combinant les données du MALDI-TOF MS et le séquençage du gène d'ARNr 16S des isolats représentatifs. Ceci a été suivi par une analyse des séquences de gènes codant pour les protéines (*pheS, rpoB, dnaJ* ou *gyrB*) comme outil de validation.

- Dans la dernière partie de ce travail et dans le cadre d'une étude du microbiote total du lait de chamelle, plusieurs isolats de bactéries lactiques n'ont pas pu être identifiés après une comparaison de notre base de données interne des empreintes digitales du MALDI-TOF MS, combinée avec l'analyse des séquences de gènes d'ARNr 16S et les séquences de gènes codant pour les protéines *pheS*, *rpoA* et *atpA*. Ces isolats ont été, par ailleurs, étudiés en utilisant une approche d'identification polyphasique combinant l'analyse RAPD, les expériences d'hybridation d'ADN, la détermination de la composition de base d'ADN (% G+C), suivie d'une caractérisation phénotypique détaillée permettant ainsi la description de nouvelles espèces appartenant aux genres *Enterococcus* et *Lactococcus* nommées *Enterococcus bulliens* sp. nov., et *Lactococcus multifermentans* sp. nov.

APERÇU DE LA THÈSE

PARTIE I: une introduction à la présente étude et ses objectifs sont présentés.

PARTIE II: un aperçu de la littérature liée aux thèmes de recherche et au travail effectué. Tout d'abord, une description générale des chameaux (*Camelus dromedarius*) et du lait de chamelle incluant à la fois la composition chimique et les connaissances actuelles de sa microbiologie (chapitre 1). Ensuite, les caractéristiques générales des bactéries lactiques sont présentées, ainsi que les concepts généraux de la taxonomie et la définition des espèces bactériennes, suivis par une présentation détaillée des méthodes taxonomiques utilisées pour la classification et l'identification des bactéries lactiques (chapitre 2). De plus, une description détaillée des genres *Streptococcus, Enterococcus* et *Lactococcus* est présentée (chapitre 3). Enfin, une introduction au MALDI-TOF et ses applications actuelles pour l'identification et la déréplication des bactéries est fournie (chapitre 4).

PARTIE III: comprend le travail expérimental du présent projet de doctorat. Dans le chapitre 5.1, les bactéries lactiques isolées à partir des échantillons du lait de chamelle sont caractérisées en utilisant une approche de la culture-dépendante. Par la suite, quatre nouvelles espèces appartenant au genre *Streptococcus*, nommées *Streptococcus moroccensis, Streptococcus rifensis, Streptococcus cameli* et *Streptococcus tangierensis* sont décrites.

Le chapitre 6.1 valide l'utilisation du MALDI-TOF MS pour une déréplication rapide et une identification du microbiote du lait de chamelle cru, combinée avec l'analyse des séquences des gènes d'ARNr 16S, *pheS, rpoB, dnaJ* ou *gyrB.*

Les chapitres 6.2 et 6.3 rapportent la description de nouvelles bactéries lactiques isolées à partir du lait de chamelle cru produit au Maroc, en utilisant une approche polyphasique, comme décrit ci-dessus, permettant la description des espèces : *Enterococcus bulliens* sp. nov., et *Lactococcus multifermentans* sp. nov.

Dans **la partie IV,** les conclusions générales des résultats obtenus sont discutées et les perspectives des applications futures sont décrites.

GENERAL INTRODUCTION

The dromedary camel (*Camelus dromedarius*, one-humped camel) is the most important livestock animal in African deserts including the deserts of Morocco. It is a multipurpose animal, used for its supply of milk, meat and hides and for transport (Burgemeister, 1974; kappeler, 1998). Camel milk is one of the most valuable food resources for pastoral people in arid and semi-arid areas, where it covers a substantial part of the quantitative and qualitative nutritional needs. It is also the main source of nutrition for neonate calves and provides all essential nutrients for growth and development such as proteins, minerals, carbohydrates, fatty acids, growth factors and immune modulators. The indigenous populations believe that raw camel milk is safe and even has therapeutic virtues as compared to that of other animal species (Barbour et al. 1984, Benkerroum et al. 2003). Camel milk is believed to modulate the immune system (Shabo et al. 2005) and is becoming popular due to its claimed therapeutic properties (Agrawal et al. 2003; Magjeed, 2005; Rao et al. 1970; Yagil, 1982). Components such as lactoperoxidase, lactoferrin, immunoglobulins, lysozyme or vitamin C have been reported to play a central role in the determination of these properties (Konuspayeva et al. 2007).

Nonetheless, camel milk is produced in a traditional way, and is usually collected, handled and transported in poor sanitary conditions. Moreover, camel herds rarely benefit from veterinary care and, hence, mastitis (Obied et al. 1996) and subclinical mastitis (Barbour et al. 1985; Abdurahman et al. 1995; Obied et al. 1996; Almaw and Molla, 2000) are common among lactating females. The bacteria causing these infections are similar to those reported in mastitis of cows or other animals kept in a traditional nomadic environment or on camel farms (Barbour et al. 1985; Almaw and Molla, 2000). These infections cause colossal losses in terms of reduced milk production, cost of treatment, loss of milk, etc (Tuteja et al. 2003) and the milk produced is likely to cause food-borne diseases. Indeed, natural antimicrobial factors can only provide a limited protection against specific pathogens and for a short period. Such a risk is higher when the milk is consumed in its raw state as the local producers are used to do.

Yet raw milk contains a diverse bacterial population, which directly impacts on the subsequent development of dairy products. Many such bacteria can contribute to natural fermentations. In some situations, specific milk isolates have been so successfully applied that they are now used as starter cultures or adjuncts designed to confer desirable

traits on fermented products. The beneficial microbiota of camel milk, represented particularly by lactic acid bacteria (LAB), is a potential source of biological materials to be used in dairy technology (Jans et al. 2012; Khedid et al. 2009). Still, the transformation of camel milk by fermentation is not easy and more research for elucidating this process is needed (Fguiri et al. 2015) and would be boosted by the thorough description of the typical LAB community in this type of food and the availability of an extensive camel milk strain collection.

While many studies investigated the microbiology of cow, sheep, and goat's milk, only a few studies have focused on the microbiology of camel milk and these studies were generally limited to the LAB fraction. One of the main reasons for the underinvestigation of raw camel milk is that the world production of camel milk for human consumption was recently estimated to be only 1.3 million tons/year (FAO, 2004). Morocco produces only 3.900 tons/year of camel milk, but other countries are big producers like Somalia (850.000 tons/year) and Saudi Arabia (89.000 tons/year). The majority of scientific studies on camels have mainly focused on their anatomic characteristics and physiological adaptation to adverse climates. Consequently, information regarding camel milk is very limited. Specifically in Morocco, very few studies have addressed the characterization of the microbiota of raw camel milk and none of these involved the use of a modern polyphasic molecular identification approach.

OBJECTIVES

Camel milk has been used fresh or fermented in different regions of the world. New sources of nutrients such as camel milk should be exploited more often for varying the human diet and to benefit from new functional ingredients and natural food components. African and Arab countries, where the breeding conditions for cows are severe and fastidious, can circumvent this situation by developing a breeding system for local animals such as camel.Therefore, given the relevance of camel milk in countries with an arid climate, the many unknowns and the importance of its microbiota and the interest in biotechnological application of dairy product and camel milk derived LAB, the objective of the present study was to give first a more extensive and detailed description of the LAB diversity in camel milk, and to describe secondly the overall microbiota of raw camel milk samples collected from different regions in Morocco using state-of-the-art methodologies. The polyphasic approach consisted of repetitive element primed pol-

ymerase chain reaction (rep)-PCR (more specifically $(GTG)_5$-PCR)) and matrix-assisted laser desorption/ionization-time-of-flight mass spectrometry (MALDI-TOF MS) (Ghyselinck et al. 2011) as dereplication tools, combined with sequence analysis of the 16S ribosomal RNA (De Bruyne et al. 2008) and/or other molecular markers for definite identification of representative isolates. Sequence analysis of the *rpoB* gene was performed to identify members of the *Enterobacteriaceae* (Mollet al. 1997; Nhung et al. 2007, Spitaels et al. 2014), of the *gyrB* gene for the *Pseudomonacae* (Kupfer et al. 2006; Yanez et al. 2003), of the *dnaJ* gene for the genus *Staphylococcus* (Shah et al. 2007), and of the *pheS* gene to identify LAB (De Bruyne et al. 2007, and 2008; Naser et al. 2005, and 2007; Spitaels et al. 2014). Isolates which failed to be identified using this approach were further studied by DNA-DNA hybridization (Ezaki et al. 1989) and determination of the DNA base composition (% G+C content) (Mesbah et al. 1989) and were subjected to a detailed phenotypic characterisation.

A thorough identification of the microorganisms present in camel milk collected from different regions of Morocco will furthermore enable to: (i) establish and preserve the microbial species diversity of Moroccan camel milk and (ii) select appropriate strains as starter cultures for dairy fermentation.

General objectives

The beneficial microbiota of camel milk represented by LAB is a potential source of biological materials to be used in dairy technology. The transformation of camel milk by fermentation is not easy and more research for elucidating the process is needed. While cow milk has been widely investigated, up until now, no studies using modern molecular methods have been undertaken to characterize the microbiota of camel milk, in particular its LAB community. Therefore, the present study aims to determine the biological diversity of LAB strains isolated from the dromedary camel milk produced in Morocco and their biochemical, physiological and phenotypic properties.

Although the camel milk microbiota is poorly known, it is at present clear that poor farming and sanitary conditions during milking are responsible for the presence of pathogens and other unwanted micro-organisms. An understanding of the camel milk natural microbiota will also be useful as a reference for evaluating measures aimed at the improvement of hygienic conditions and thus milk quality. The availability of a collection of camel milk derived LAB strains can form the basis for the development of safer and, potentially fermented, camel milk derived dairy products.

The global objective of the present Ph.D was to apply appropriate polyphasic techniques to fully determine the LAB diversity in raw camel milk samples collected from different locations in Morocco as a first part of this study and the overall microbiota in a second part thus generating a collection of well characterised LAB strains which will serve as a resource for searching starter cultures with optimal technological characteristics that can be used for the production of camel milk derivates in future research.

Specific objectives

- **In the first phase of this study** the LAB diversity in raw camel milk was explored using a culture-dependent approach. Genotypic discrimination and preliminary identification of LAB relied on the use of $(GTG)_5$-PCR. Subsequently, 16S rRNA gene sequence analysis was used to further identify representative isolates **(chapter 5.1)**.
- **In the second phase of this study** four novel LAB species within the genus *Streptococcus*, which were detected in the frame of the camel milk LAB diversity study, were fully characterised using polyphasic taxonomic studies. The $(GTG)_5$-PCR and 16S rRNA gene sequence data were complemented with sequence analysis of protein encoding genes including the phenylalanyl-tRNA synthase alpha subunit (*pheS*), the RNA polymerase alpha subunit (*rpoA*) and the ATP synthase alpha subunit (*atpA*) gene sequences analysis, DNA-DNA hybridization experiments, determination of the DNA base composition (% G+C), MALDI-TOF MS analyses and a detailed phenotypic characterization. This allowed the formal description of four novel *Streptococcus* species for which the names *Streptococcus moroccensis, Streptococcus rifensis, Streptococcus cameli* and *Streptococcus tangierensis* were proposed **(chapters 5.2 and 5.3).**
- **The third phase of this study** consisted of the investigation of the overall bacterial diversity of camel milk by a culture-dependent analysis using MALDI-TOF MS as a fast and reliable tool for dereplication and identification of bacteria. For this purpose a total of 808 isolates were analyzed with MALDI-TOF MS fingerprinting. A final identification was obtained through the combination of the MALDI-TOF MS data with 16S rRNA gene sequencing of representatives strains followed by sequence analysis of protein encoding genes (*pheS, rpoB, dnaJ* or *gyrB*) as a validation tool **(chapter 6.1).**
- **In a final phase of this work**, several additional novel LAB taxa that remained unidentified after MALDI-TOF MS and sequence analyses of 16S rRNA and the protein encoding genes *pheS, rpoA* and *atpA,* were further

studied. Their characterization included RAPD analysis, DNA-DNA hybridization experiments, determination of the DNA base composition (% G+C) and a detailed phenotypic analysis which allowed the description of a novel *Enterococcus* and *Lactococcus* species named *Enterococcus bulliens* sp. nov., and *Lactococcus multifermentans* sp. nov. **(Chapters 6.2 and 6.3).**

OUTLINE OF THE THESIS

PART I is an introduction to the present study and its objectives are presented.

PART II comprises a literature overview related to the research topics and the work performed. First, a general description of camels (*Camelus dromadarius*) and camel milk including both the chemical composition and current knowledge of its microbiology **(chapter 1).** Next, general characteristics of lactic acid bacteria are presented, along with general concepts of bacterial taxonomy and species definition, followed by a detailed presentation of taxonomic methods used for the classification and identification of LAB. Subsequently, the use of dereplication techniques in microbial diversity study is discussed **(chapter 2).** Furthermore, a detailed description of the genera *Streptococcus, Enterococcus* and *Lactococcus* **(chapter 3)** and, finally, an introduction to MALDI-TOF mass spectrometry and its current applications for the identification and dereplication of bacteria are presented **(chapter 4).**

PART III comprises the experimental work of the present Ph.D project. **In chapter 5.1,** the LAB of camel milk samples are characterised using a culture-dependent approach. Subsequently, four new *Streptococcus* species, named *Streptococcus moroccensis, Streptococcus rifensis, Streptococcus cameli and Streptococcus tangierensis* are described and formally named **(chapters 5.2, and 5.3).**

Chapter 6.1 presents the isolation and identification of the cultivable bacterial microbiota of raw camel milk samples through the use of MALDI-TOF MS combined with 16S rRNA and *pheS, rpoB, dnaJ* or *gyrB* gene sequence analysis. Again, several novel LAB were isolated, characterized and formally named as *Enterococcus bulliens* sp. nov., and *Lactococcus multifermentans* sp. nov. (**Chapters 6.2 and 6.3**).

In **PART IV,** general conclusions of the results obtained are discussed and an outlook on future applications is given.

The summary of the thesis is given in **PART V.**

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PART II
LITERATURE

CHAPTER 1: THE DROMEDARY CAMEL AND CAMEL MILK

1.1 The dromedary camel

The dromedary camel (*Camelus dromedarius*, one-humped camel) is the most important livestock animal that resides in the semi-arid areas of Northern and Eastern Africa. It is a multipurpose animal used for its supply of milk, meat and hide and for transport (Burgemeister, 1974; Kappeler, 1998).

1.2 Dromedaries as milking animals

1.2.1 Taxonomy and breeds

On the basis of zoological taxonomy, camelids are classified in the suborder Tylopoda (pad-footed animals) that represents with the suborders Suiformes (pig-like) and Ruminantia (ruminants) the order Artiodactyla (even-toed ungulates). Camelids (family Camelidae) as ruminating animals are evidently classified in proximity to ruminants but developed in parallel and are not part of the suborder Ruminantia. Some differences with regards to foot anatomy, stomach system and the absence of horns underline this (Fowler, 1998; Schwartz & Dioli, 1992; Wernery, 2003). The family Camelidae is divided into three genera comprising the old world (genus *Camelus*) and the new world camels (genus *Lama* with the species *Lama glama, Lama guanicoe* and *Lama pacos* and genus *Vicugna* with the species *Vicugna vicugna*) (Wilson & Reeder, 2005). There are two domesticated species of camels. The first, the dromedary or one humped camel (*Camelus dromedarius*) has its distribution in the hot deserts of Africa and Asia. The name “dromedary” is derived from the Greek word “dromeus” which means runner or “droma” - running (Jassim & Naji, 2002). The second, the bactrian or two-humped camel (*Camelus bactrianus*) that can be found in the cold deserts and dry steppes of Asia and was named after the area of Bactriana in central Asia.

1.2.2 The reproduction in dromedary camels

According to Puschman (1989), the sexual cycle of dromedary camels begins at 24 months. Different from ruminants, camels are seasonal polyoestrous animals. Usually the ovulation of the female is induced by copulation (Wilson, 1984). Camel bulls show their sexual cycle during 3-4 months in winter season, beginning in December (Fazil &

Hofman, 1981; Rao et al. 1970) and the mean gestation period is reported to be between 315-360 days (Puschmann, 1989) up to 370 -375 days (Arthur, 1992; Fazil & Hofman, 1981; Rao et al. 1970).

1.2.3 Camel population in the world

In most countries, the camel population is increasing after a period of decreasing number due to the introduction of modern transport facilities (Farah, 2004; Brezovecki et al. 2015). However, the camel population is thought to be decreasing slightly in Morocco. It has gone from 37,000 in 1995 to 36,000 in 2003 (Glipha, 2006; Brezovecki et al. 2015). According to FAO statistics (2008), there are about 19 million camels in the world, of which 15 million are found in Africa and 4 million in Asia. Of this estimated population, 17 million are considered a single hump camels (*Camelus dromedarius*) and 2 million with two-humped (*Camelus bactrianus*). Approximately 15 million dromedaries, representing two-thirds of the world camel population, are living in the arid areas of Africa, particularly in Northeast Africa (Somalia, Sudan, Ethiopia, and Kenya) (El-Agamy et al. 2008; Yamina et al. 2013). In the following text, the term "camel" without further details will be used exclusively for dromedary camels.

1.3 Characteristics of lactation and camel milk

1.3.1 Characteristics of camel milk

Camel milk is usually opaque-white in colour and has an acceptable taste (Alwan et al. 2014; Alwan and Igwegbe, 2014; Yagil et al. 1980). The milk normally has a faintly sweetish odour producing a sweet and sharp taste. Sometimes it can also have a salty taste due to the species of desert plants eaten (Alwan et al. 2014; Alwan and Igwegbe, 2014; Khaskheli et al. 2005; Rao et al. 1970). It is thinner than cow milk (Abdurahman, 1996; Ohri & Joshi, 1961) and has a much slower natural creaming rate than cow milk, both in its raw state and heat treated (Farah, 1993; Farah & Ruegg, 1991).

1.3.2 Lactation and milk yield

Camels are capable of producing more milk and for a longer period of time than any other milk producing animal held under the same conditions (Farah, 1993; Knoess, 1977; Yagil, 1982). The length of the lactation period depends on race, parturition, climatic and food conditions and has been reported to be between 12 and 24 months (Farah, 2004; Rao et al. 1970; Yagil, 2000). The milk yield varies between the different

dromedary breeds or types and between individual camels of the same breed. High milking frequency and adequate feed also have a positive effect on milk yield. Farah (1993) reported that daily milk yield varied from 3.5 litres for camels under desert conditions to 18.0 liters for those on irrigated lands. On the other hand, Wardeh (1998) reported that some dairy breeds are characterised by a milk production capability of more than 2.090 kg up to 4.000 kg per lactation (305 days) under natural grazing conditions.

1.3.3 Milk contents and pH

The milk composition of dairy animals has been widely studied throughout the world and thousands of references are available especially with regard to milk consumed by humans. In the last years, milk consumption among urban populations has been increasing (Chaibou, 2005; Farah, 2004). The published data are mainly dedicated to cow milk, which represents 85% of the milk consumed in the world and, to a lesser extent, goat and sheep milk. Other studies related to dairy animals, especially for camels, are scarce in spite of their nutritional interest.

Camel milk differs from other ruminant animal milk. It has high mineral contents such as sodium, potassium, iron, copper, zinc, cobalt, manganese, iodine and magnesium (Attila et al. 2000; Gorban and Izzeldin, 1997; Yagil, 1982, Tab.1.1). The total amount of minerals is generally presented as total ash, for which values range from 0.60 to 0.90% (Alwan and Igwegbe, 2014; Konuspayeva et al. 2009), while Al-Haj et al. (2010) reported that the mean value of ash is 0.79±0.07%. In addition, the mean values of protein, lactose, and total solids (%) reported over the last 30 years were: 3.1±0.5, 4.4±0.7, and 11.9±1.5, respectively (Al-Haj et al. 2010). Moisture content is reported within the range of 86.0 and 90.5 % (Gaili et al. 2000). Fat content is reported with values between 2.0 and 4.2 % (Alshaik & Salah, 1994; Hassan, 1967), while Al-Haj et al. (2010) reported a mean value of 3.5±0.1.

Tab. 1. 1: Some oligoelements composition of camel milk compared to cow milk (mg/l)

Milk origin	Iron	Cobalt	Manganese	Iodine
Camel milk	0.4-2.64	0.02-1.63	0.1-2	0.1
Cow milk	0.2-0.5	0.02-0.15	0.03-0.05	0.01-0.05

The variations observed in camel milk composition have been attributed to different factors such as analytical techniques used, geographical location, feeding regime, size

of samples analyzed, and breeds. Other variations include milking frequency, stage of lactation and parity (Al-Haj and AlKanhal, 2010; Aljumaah et al. 2011; Ayadi et al. 2009; Hammadi et al. 2010; Konuspayeva et al. 2009). Additional reasons for variation in milk contents were age and race (Farah, 1993) and husbandry conditions (El-Hatmi et al. 2004; Merin et al. 1998).

As compared to cow milk, camel milk fat contains few short-chain fatty acids (C4 - C12). The fatty acid pattern contains more long-chain fatty acids (C14:0, C16:0 and C18:0) (Farah, 1993). Camel milk is a rich source of unsaturated fatty acids which contributes to its overall dietary quality (Konuspayeva et al. 2009; Tab.1.2). It may lower human serum lipids and decrease the incidence of lipid-related cardiovascular diseases (Ereifaj et al. 2011; Karray et al. 2005; Konuspayeva et al. 2008).

Tab. 1. 2: Fatty acid composition of camel milk compared to cow milk (%)

Categories	Common name	Camel milk	Cow milk
Saturated fatty acids	butyric	**0.1-0.6**	3-4
	caproic	**0.2-0.4**	2-5
	Caprylic	**0.1-0.2**	1-1.5
	capric	**0.1-0.9**	2
	lauric	**0.7-0.9**	3
Monounsaturated fatty acids	lauroleic	**0.1**	0.05
	palmitoleic	**9.4-11**	2
	oleic	**19.1-26.3**	3
Polyunsaturated fatty acids	linoleic	**2.9- 3.6**	2
	linolenic	**1.4-3.5**	0.5

Camel milk is rich in vitamin C, vitamin E and vitamin B3 compared to cow milk (Field et al. 1997; Jassim & Naji, 2002; Kappeler, 1998; Tab.1.3), making it a good source of this vitamin for the people of the desert where fruits and vegetables are lacking (Alwan and Igwegbe, 2014).

Tab. 1. 3: Some vitamins composition of camel milk in comparison to cow milk

Nature of vitamins	Camel milk	Cow milk
B3 (Niacin) (µg/kg)	4600-4610	500-800
E (Tocopherol) (µg/kg)	530-560	100-200
C (Ascorbic acid) (mg /kg)	24-37	3-23

The average pH values of camel milk were reported to be between 6.2 and 6.8 (Gnan & Sheriha, 1986), while El-Bahay (1962) and Field et al. (1997) reported the average value of 6.6. In addition, Tuteja et al. (2003) reported that the pH value of camel milk can increase up to 7.2 in case of clinical mastitis.

1.3.4 Camel milk: a remedy for medical problems

Historically camel milk has been used as a remedy for medical problems (Dickson 1951). The lactoferrin, immunoglobulins, lysozyme, α-lactalbumin, acidic and basic whey proteins (Beg et al. 1986; Ochirkhuyag et al. 1998), acidic serum albumin, vitamin C and peptidoglycan recognition protein (Kappeler et al. 1994) which are all present in camel milk, have been recognised as components of interest (El-Agamy et al. 1996; Ereifej et al. 2011; Konuspayeva et al. 2007) and contributes to the beneficial effect of its consumption. Clinical trials in human diabetes type 1 have shown that the daily consumption of 0.5 litre camel milk reduces the need for insulin medication by an average of 30% (Agrawal, 2005). In addition, comparative physiological studies carried out in Israel (Zagorski, 1998) and Germany (El-Mahdi, 1997) demonstrated the anti-diabetic properties of camel milk. Also, a study of eight children showed its ability to ameliorate allergies in children (Shabo et al. 2005) and clinical trials showed that recovery from infectious desease was signifantly faster in patients consuming camel milk regularly (Patel et al. 2016).

1.4 Microbiological quality of camel milk

1.4.1 Introduction

Camel milk has properties that it can be kept for long periods than cow milk due to its high contents of proteins acting as inhibitory properties against bacteria (Mullaicharam 2014; Younan, 2004), which makes raw camel milk a marketable commodity, even under conditions of high temperatures (Younan, 2004). As camel milk is usually consumed in its raw state, the presence of pathogenic bacteria may not only be important for animal health but also of concern for public health (Adugna et al. 2013; Saad & thabet, 1993; Younan, 2004). Adugna et al. (2013) isolated members of the genera *Staphylococcus, Streptococcus, Acinetobacter, Enterobacter* and *Escherichia* (*Esch.*) *coli* from raw camel milk and attributed the risk of milk contamination with pathogens

to the practice of mixing milk from different sources, poor hygiene, handling practice of camel milk along the chain and absence of cooling facilities.

1.4.2 Total bacterial content (TBC)

The TBC of camel milk was reported to be between 10^2 and 10^8 cfu/ml (Sela et al. 2003; Teshager and Bayleyegn, 2001; Wernery et al. 2002; Younan, 2004). Benkerroum et al. (2003) reported an average TBC for Moroccan camel milk of 6.2 10^7 cfu/ml. The differences between studies underline that the TBC depends on several parameters including the camel milk itself, contamination of the camel udder, the contamination by milking personnel and storage containers. The importance of the different sources of contamination varies according to the storage and milking conditions of the camels (Benkerroum et al. 2003).

1.4.3 *Enterobacteriaceae*

All genera of the family *Enterobacteriaceae*, except *Erwinia*, *Obesumbacterium*, *Xenorhabdus*, *Rahnella*, *Cedecea* and *Tatumella* and possibly *Edwardsiella* and *Providencia* have been reported in milk (Robinson, 1990). *Enterobacteriaceae* are gram-negative rods with an aerobic or facultative anaerobic metabolism that inhabit the intestine of humans and other animals, and sometimes cause disease (Joklik et al. 1992). Some of them can act as opportunistic pathogens. None of the *Enterobacteriaceae* are particularly heat resistant and thus all are easily eradicated from milk by pasteurization or equivalent heat treatments (Joklik et al. 1992; Robinson, 1990). Coliforms have been reported in camel milk (Omer and Eltinay, 2008; Saad and Thabet, 1993). Abdelgadir et al. (2005) and Adugna et al. (2013) reported the presence of *Esch. coli* in raw camel milk samples taken from healthy camels. In addition, *Klebsiella pneumoniae* and *Citrobacter freundii* have been reported in camel milk as well (Abdelgadir et al. 2005; Barbour et al. 1985; Eberlein, 2007; Semereab & Molla, 2001). According to Adugna et al. (2013), members of the genera *Enterobacter* were also isolated from raw camel milk. Most of these species originate from soil or water and some from faecal contamination (Robinson, 1990; Schmidt-Lorenz & Spillmann, 1988). They are considered as indicator organisms, which are closely associated with the presence of pathogens but not necessarily pathogenic themselves. However, their ability to ferment lactose, which results in the production of acid and gas, and to degrade milk proteins can cause rapid spoilage of milk (Robinson, 1990; Schmidt-Lorenz & Spillmann, 1988).

1.4.4 Pathogens in camel milk

Besides the above mentioned bacteria, many pathogenic species belonging to different genera were identified in raw camel milk, including *Micrococcus* spp. (Abdelgadir et al. 2005; Barbour et al. 1985), *Staphylococcus* (*St*.) *aureus* (Abdurahman et al. 1995; Tuteja et al. 2003); *Brucella* spp. (Hamdy et al. 2002), *Pseudomonas aeruginosa* and *Helicobacter pylori* (Hafez et al. 1987; Rahimi et al. 2012, respectively).

Staphylococci are small Gram-positive cocci belonging to the family of *Micrococcaceae* and can be subdivided into coagulase positive and coagulase negative species (Kloos & Schleifer, 1986). Among the predominant bacteria involved in food-borne diseases, *St. aureus* produces a wide variety of toxins including staphylococcal enterotoxins (SEs). SEs are a major cause of food poisoning, which typically occurs after ingestion of different foods, particularly processed meat and dairy products, contaminated with *S. aureus* by improper handling and subsequent storage at elevated temperatures (Argudin et al. 2010, Le Loir et al. 2003), leading to nausea, violent vomiting, abdominal cramping, with or without diarrhea. This opportunistic pathogen has been isolated from raw camel milk from different parts of the world, including Morocco (Benkerroum et al. 2003; Ismaili et al. 2015; Khedid et al. 2009), Saudi Arabia (Zahran and Al-Saleh, 1997), Ethiopia (Semereab and Molla, 2001), Egypt (Aly and Abo-Al-Yazeed, 2003), Sudan (Shuiep et al. 2009) and Kenya (Jans et al. 2012).

Brucella melitensis is a pathogenic bacterium that can cause human, camel and cattle's brucellosis (Gwida et al. 2012; Shimol et al. 2012). Shimol and colleagues showed that *Brucella melitensis* was cultivable from milk samples of infected camel (Shimol et al. 2012).

The species *Listeria monocytogenes*, *Salmonella* spp., and *Clostridium perfringens* were absent in camel milk samples studied by Omer and Eltinay (2008). The authors suggested that this absence was due to the activities of protective proteins (lysozyme, lactoferrin, lactoperoxidase, immunoglobulins G and A) in raw camel milk. Barbour et al. (1984) reported a similar result. Contrary to this finding, Matofari et al. (2007) reported that from 196 samples tested, 84 contained *Salmonella* spp. and explained the presence of *Salmonella* spp. through the poor handling practice. In the other hand, Matofari and colleagues reported that the camel milk lactoperoxidase was bacteriostatic against Gram-positive bacteria and bactericidal against Gram-negative bacteria while the immunoglobulins had little effect against the bacteria tested (Matofari et al. 2007).

1.4.5 Bifidobacteria

Bifidobacteria such as *Bifidobacterium* (*Bif.*) *angulatum, Bif. longum, Bif. bifidum* and *Bif. breve* have all been identified in camel milk (Abu-Taraboush et al. 1998).

1.4.6 Lactic acid bacteria

One of the most important groups of bacteria in milk are LAB. Khedid et al. (2009) reported that raw camel milk could be an additional source of typical dairy-LAB species but until now few studies have characterized LAB from camel milk in comparison to LAB from cow milk. Nowadays, the interest in LAB from camel milk is increasing. Biochemical tests (Benkerroum et al. 2003; Fguiri et al. 2015; Karam and Karam, 2005; Khelid et al. 2009) and 16S rRNA gene sequence analysis have been used to identify these LAB isolates (Jans et al. 2012). Several authors reported the dominance of coccus-shaped LAB in camel milk as compared to other kinds of milk (Akhmetsadykova et al. 2014; Jans et al. 2012; Khedid et al. 2009).

Lactobacillus (*Lb.*) *helveticus, Lb. casei, Lb. plantarum, Lb. pentosus* and *Lactococcus* (*L.*) *lactis* strains have been isolated repeatedly from raw camel milk (Drici et al. 2010; Fguiri et al. 2015; Khedid et al. 2009). *Leuconostoc* (*Leuc.*) *mesenteroides* strains have been isolated from raw camel milk in two southwest Algerian arid zones (Benmechernene et al. 2013; 2014). In addition, Khedid et al. (2009) reported the isolation of *Leuc. lactis, Leuc. mesenteroides* subsp. *mesenteroides, Leuc. mesenteroides* subsp. *cremoris*, and *Leuc. mesenteroides* subsp. *dextranicum* and of *Pediococcus* (*P.*) *acidilactici, P. damnosus, P. pentosaceus, Enterococcus* (*E.*) *casseliflavus, E. faecalis* and *Streptococcus* (*S.*) *salivarius* subsp. *thermophilus* from raw camel milk in Morocco using various culture media. In another study, Benkerroum et al. (2013) concluded that *P. acidilactici, P. halophilus, E. faecalis, E. faecium, S. salivarius* and *S. bovis* were present in camel milk. *S. equi* subsp. *zooepidemicus* was found in camel milk collected from Kenya and Somalia (Younan et al. 2005), and *S. agalactiae* was reported in raw camel milk (Jans et al. 2012) in Kenya.

1.4.7 *Camelimonas*

Camelimonas lactis is a recently reported organism that was isolated from camel milk in the United Arab Emirates (Kampfer et al. 2010). It has been shown to belong to the *Alphaproteobacteria* and was most closely related to *Chelatococcus asaccharovorans* and *Chelatococcus daeguensis.* The DNA G+C content of the type strain is 65 mol%. This novel bacterium was initially cultivated from Brucella agar and incubated at 37°C. It is a Gram-negative rods, non-motile, non-spore-forming, Oxidase-positive, showing an oxidative metabolism. The characteristic peptidoglycan diamino acid is *meso*-diaminopimelic acid. The predominant compound in the polyamine pattern is spermidine, and *sym*-homospermidine is absent. The quinone system is ubiquinone Q-10. Until now, however, there is no information on its origin or biological role.

1.4.8 Fungi in camel milk

Fungi occur in soil, barn dust, feeds, manure and unclean utensils and are capable of producing extremely toxic components in foods including milk and milk products, which can pose health problems for the consumer. Benkerroum et al. (2003) reported the presence of yeasts in Moroccan camel milk. Also, El-Ziney and Al-Turki (2006) reported that yeasts and moulds were detected in 19 samples out of 33 total camel milk samples and Adugna et al. (2013) reported the presence of yeasts in camel milk from Eastern Ethiopia. According to Njage et al. (2011), the yeasts commonly associated with camel milk belong to the genera *Saccharomyces, Rhodotorula, Cryptococcus, Trichosporon*, *Candida*, *Geotrichum* and *Issatchenkia*.

CHAPTER 2: LACTIC ACID BACTERIA

2.1. General characteristics

LAB encompass a heterogeneous group of microorganisms with as a common metabolic property the production of lactic acid as the end product of lactose metabolism (Carr et al. 2002). LAB are Gram-stain-positive, non spore-forming, catalase negative, acid tolerant, and anaerobic but tolerate the presence of oxygen (Vandevoorde et al. 1992; Yamamoto et al. 2011). Except for some streptococcal pathogens, LAB are generally non-pathogenic organisms and are considered as generally safe for public consumption (Leuschner et al. 2010). Based on sugar fermentation patterns, two broad metabolic categories of LAB exist: homofermentative and heterofermentative. The first, homofermentative LAB, include some lactobacilli and most enterococci, lactococci, pediococci, streptococci, tetragenococci and vagococci species. They ferment hexoses through the Embden-Meyerhof (E-M) pathway and produce lactic acid as major end product (> 85-90%) in glucose fermentation (Kleerebezem and Hugenholtz, 2003; Todar et al. 2004). The second, heterofermentative LAB, include leuconostocs, some lactobacilli, oenococci, and *Weissella* species. The apparent difference on the enzyme level between these two categories is the presence or absence of the key cleavage enzymes of the E-M pathway (fructose-1.6-bisphosphate aldolase) and the PK (phosphoketolase) pathway. In addition, apart from lactic acid the heterofermentative LAB yield a large variety of fermentation products such as acetic acid, ethanol, carbon dioxide and formic acid (Kleerebezem and Hugenholtz, 2003; Todar et al. 2004).

LAB were first isolated from milk and have since been found in many food sources including meat, vegetables, beverages and sourdough breads (Caplice & Fitzgerald, 1999; Carr et al. 2002). LAB species are also commonly found among the resident microbiota of the gastrointestinal tract and genitourinary tract of humans and animals (Eckburg et al. 2005; Marchesi and Shanahan, 2007). LAB are considered essential components in these environments and exhibit a large variety of health-promoting functions, such as immunomodulation, intestinal integrity and pathogen resistance (Vaughan et al. 2005). The antimicrobial effect of LAB is mainly due to the production of organic acids, hydrogen peroxide, and bacteriocins (Caplice & Fitzgerald, 1999). For this reason, strains of some LAB are used as probiotics or as functional bacteria in various food commodities (Ljungh and Wadström, 2006). The commercial exploitation

of LAB as starter and probiotic cultures is economically significant. That is a key reason why research on their genetics, physiology and applications has been developed in the last 25 years (Gasson and De Vos, 2004; Wood and Warner, 2003).

2.2. Taxonomy and phylogeny of lactic acid bacteria

2.2.1. Bacterial taxonomy and the polyphasic species definition

Bacterial taxonomy is generally considered a synonym of systematics and is traditionally divided into three parts: 1) classification (i.e. the arrangement of organisms into groups on the basis of similarity), 2) nomenclature (i.e. the labelling of the units); and, 3) identification (i.e. the process of determining whether an unknown belongs to one of the units defined) (Vandamme et al. 1996). In the last decades, bacterial classification has come to reflect, as much as possible, the natural relationships between bacteria. These phylogenetic relationships are traditionally coded in highly conserved macromolecules such as 16S or 23S rRNA genes (Woese, 1987).

Currently, the explosion of available genomic information has impacted our phylogenetic view of the traditional species concept. The most accepted definition of a bacterial species today is the phylo-phenetic definition: "a species is considered a monophyletic and genomically coherent cluster of individual organisms that show a high degree of overall similarity with respect to many independent characteristics, which are diagnosable by a discriminative phenotypic property" (Felis and Dellaglio, 2007; Rossello-Mora and Amann, 2001). Prior to the genome sequencing boom, the criteria used to define species included a large variety of phenotypic (morphological, physiological and chemotaxonomic) as well as genotypic characteristics (% G+C content, level of DNA-DNA hybridization, ribosomal RNA gene restriction analysis, ribotyping, RAPD-PCR, multilocus sequence typing) (Carr et al. 2002; Felis and Dellaglio, 2007; Klein et al. 1998; Ludwig and Schleifer, 1994 ; Vandamme et al. 1996).

Despite the explosion of novel technologies for the identification and the characterization of novel specimens, DNA-DNA hybridization (DDH) still forms the cornerstone of species delineation, since 1987 (Stackebrandt et al. 2002; Wayne et al. 1987). For the classification and identification of unknown organism, the application of numerous other genotypic, chemotaxonomic, and phenotypic analyses for the delineation of bacteria at various hierarchical levels can be used (Vandamme et al. 1996). Additionally, the use of a DDH value of ≥70% was shown to correlate with ≥97% sequence identity

of the highly conserved 16S rRNA gene (Holzapfel and Goebel, 2014; Stackebrandt & Goebel, 1994). This threshold level was however recently revisited and replaced by threshold of 98.65% for differentiating two species (Kim et al. 2014).

Interesting developments in methodologies to define species also include multilocus sequence analysis (MLSA) which can serve as a suitable supplement to DDH (Adékambi et al. 2008; Vandamme et al. 2014; Pitulle et al. 2002). Especially for depicting relationships within and between closely related species, this MLSA approach has a resolution superior to that of traditional 16S rRNA gene sequence analysis (Li et al. 2014; Naser et al. 2005b; Vandamme et al. 2014). The MLSA approach is enticing, but the definition of the limits of species clusters requires the analysis of large numbers of strains of the bacteria of interest. A polyphasic approach which includes a combination of phenotypic and genotypic information is needed to interpret the observed clustering patterns, and to derive a consensus view of which clusters deserve species names (Hanage et al. 2006; Vandamme et al. 1996).

Today however, whole genome sequences provide a new resource for defining bacterial species (Konstantinidis et al. 2006; Deloger et al. 2009). The Average Nucleotide Identity (ANI) value is the average nucleotide identity of all orthologous genes shared between two strains (Konstantinidis et al. 2006) and is being introduced in a large number of recent taxonomic studies. To calculate this value a list of orthologous genes must be determined. These are then used to derive the overall divergence of the core genome by averaging the percentage of identity at the nucleotide level of all orthologs found. An ANI value of 95% generally corresponds to the DDH threshold of 70% (Cho and Tiedje, 2001; Goris et al. 2007) and thus represents a very robust measure of genetic and evolutionary relatedness between two strains.

2.2.2. Taxonomy and phylogeny of lactic acid bacteria

Based on 16S and 23S rRNA gene sequence data, the Gram-positive bacteria cluster in two bacterial phyla (Holzapfel et al. 2001; Bergey's Manual of Systematic Bacteriology, 2004, the outline is available at http://dx.doi.org/10.1007/bergeysoutline200405), i.e. one with a % (G+C) content of less than 50% (the so-called *Firmicutes*), and another with a % (G+C) content of more than 50% (the so-called *Actinobacteria*). LAB belong to the *Firmicutes* phylum of the Gram-positive bacteria and are therefore characterised by a % (G+C) content of less than 50 mol%. The main genera currently included in the

LAB group are *Carnobacterium, Enterococcus, Lactobacillus, Lactococcus, Leuconostoc, Oenococcus, Pediococcus, Streptococcus, Tetragenococcus, Vagococcus* and *Weissella* (Holzapfel et al. 2001).

Although bifidobacteria share some phenotypic features with typical LAB, they are phylogenetically distinct. The genus *Bifidobacterium* exhibits a relatively high G+C content of more than 50 mol% and belongs to the *Actinobacteria* (Holzapfel et al. 2001; Klein, 2003; Nguyen, 2012). Traditionally, bifidobacteria have been considered as LAB because of similar physiological and biochemical properties and also because they share some common ecological niches e.g. the gastro-intestinal tract (GIT) (Klein et al. 1998).

Traditionally, classification of LAB has long been based on morphological, ecological and physiological characteristics (Orla-Jensen, 1919). This methodology was expanded to include analysis of the cell wall composition, cellular fatty acids, and other characteristics of the cells (Stiles and Holzapfel, 1997). Generally, the taxonomical usefulness of phenotypic characters is limited because of the low discrimination between closely related organisms and many strain specific characteristics. The taxonomy of LAB underwent much change during the past two decades. A much broader, polyphasic, range of taxonomic studies has gradually replaced the former reliance upon morphological, physiological and biochemical characterisation. Molecular characteristics such as the mol% G+C content of the DNA, DDH studies and sequencing of ribosomal RNA (rRNA) genes have become important taxonomic tools and implied significant advances in LAB taxonomy (Stiles and Holzapfel, 1997; Vandamme et al. 2014). Especially in LAB taxonomy, several taxa which had been classified on the basis of phenotypic characteristics were phylogenetically not coherent (Hammes and Vogel, 1995), and many synonymous LAB taxa have been detected (Holzapfel et al. 2001).

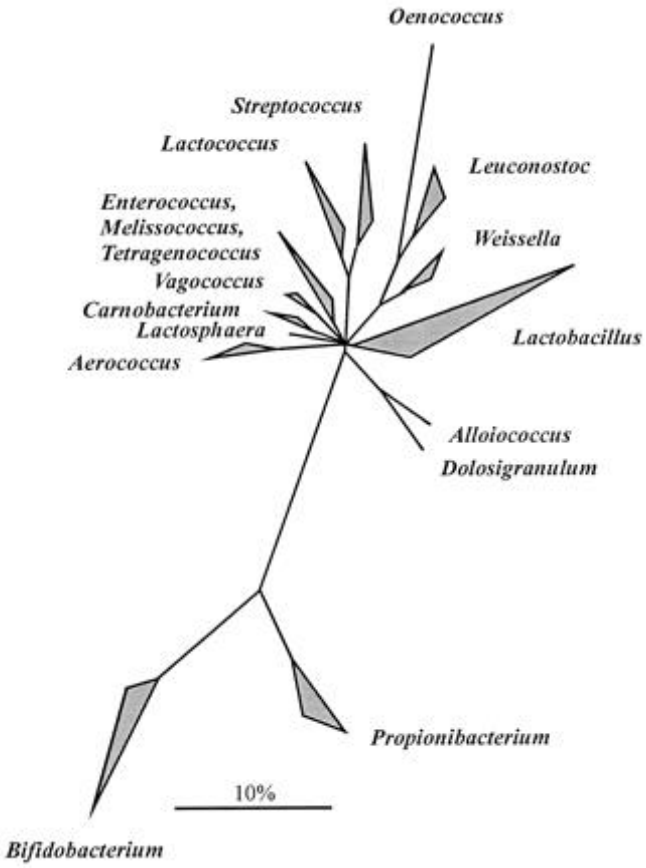

Fig 2. 1: Consensus tree based on comparative analysis of the 16S rRNA gene, showing major phylogenetic groups of lactic acid bacteria with low mol% of guanine plus cytosine in the DNA and the non-related Gram-positive genera *Bifidobacterium* and *Propionibacterium.*

The most important LAB in the context of food microbiology are the genera *Carnobacterium*, *Lactobacillus*, *Lactococcus*, *Leuconostoc*, *Oenococcus*, *Pediococcus*, *Tetragenococcus*, and *Weissella*. Although not solely food related but also clinically relevant, also the genera *Streptococcus* and *Enterococcus* are food related LAB genera, with *S. thermophilus*, *E. faecalis* and *E. faecium* as the most important food related species.

Within LAB, the 16S rRNA gene sequence variability has been analysed extensively. Aligned sequences of all type strains from all LAB genera are available in the silva database (http://www.arb-silva.de/). The low evolutionary rate of ribosomal RNA genes complicates the delineation of bacterial species and the resolution of the phylogenetic trees (Acinas et al. 2004; Coenye and Vandamme, 2003a; Woese, 1987). Many LAB species share more than 97% of their 16S rRNA gene sequences and there is no 16S rRNA gene similarity threshold value for LAB species delineation. Several LAB species exhibit identical or nearly identical 16S rRNA gene sequences (Björkroth et al. 2002; Cachat and Priest, 2005; Švec et al. 2001). From a 16S rRNA gene based phylogenetic tree (Yarza et al. 2008), it is furthermore clear that the current genus delineation

of various LAB taxa does not fit their phylogenetic structure. The most striking observations are: (i) the polyphyletic nature of the genus *Lactobacillus*, (ii) the clustering of the genera *Pediococcus* and *Paralactobacillus* among the lactobacilli, (iii) the relationships of members of the genus *Enterococcus* versus the genera *Tetragenococcus*, *Melissococcus* and *Catellicococcus*, and (iv) the family level classification of the genus *Bavariicoccus* (Vandamme et al. 2014).

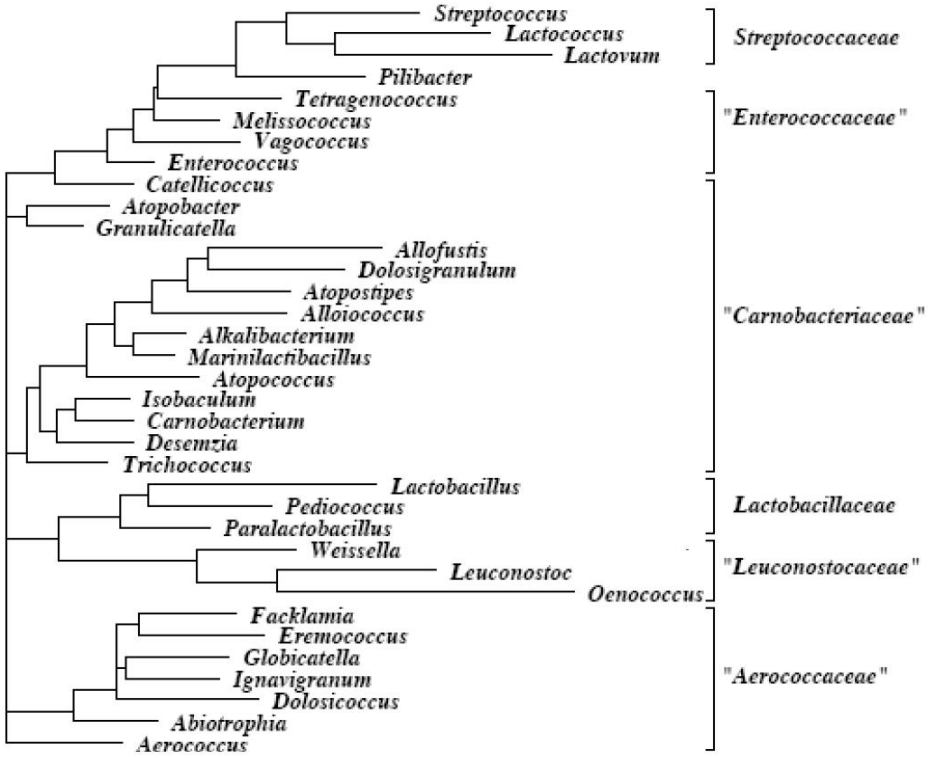

Fig 2. 2: The phylogenetic relationship of lactic acid bacteria based on 16S rRNA gene analysis (Garrity et al. 2004).

To compensate for the lack of taxonomic resolution of the 16S rRNA gene, the use of housekeeping genes, i.e. genes encoding metabolic functions, has been proposed for phylogenetic analysis (Stackebrandt et al. 2002). Different studies have reported on the use of protein-coding genes as phylogenetic markers for the classification and identification of LAB. These single-locus sequence analysis (SLSA) studies focus on protein-coding genes that evolve relatively slowly, though more rapidly than 16S rRNA genes, to obtain a higher taxonomic resolution. Moreover, these housekeeping genes encode products that are likely to be essential to the bacteria and consequently are expected to be ubiquitously present in the taxon of interest.

The first SLSA study within LAB has been published in 1996 by Morse and co-workers, who reported on the sequencing of the *rpoC* gene, encoding the β subunit of DNA-

dependent RNA polymerase, of *Leuconostoc* strains to study the phylogenetic relationship between *O. oeni*, *Leuconostoc sensu stricto*, and *Weissella* (Morse et al. 1996). In 1999, Groisillier and Lonvaud-Funel sequenced the *mle* gene, which encodes the malolactic enzyme for 13 LAB strains representing the genera *Pediococcus*, *Leuconostoc*, *Lactobacillus*, and *Oenococcus* (Groisillier and Lonvaud-Funel, 1999). Poyart and coworkers reported the use of *sodA* gene (encoding the manganese-dependent superoxide dismutase) sequences as an approach to the genotypic identification of streptococcal and enterococcal species (Poyart et al. 2000). *RecA* (gene encoding recombinase enzyme) is a small protein essential for the repair and maintenance of DNA and is implicated in homologous DNA recombinations. Due to its fundamental role, the *recA* gene is ubiquitous and its gene product has been proposed as a phylogenetic marker to distinguish closely related species including members of the *Lb. plantarum* and *Lb. casei* species groups (Felis et al. 2001; Torriani et al. 2001b).

The nucleotide sequences of the *atpD* gene encoding the ATPase β-subunit comparison showed that *O. oeni*, *Leuc. mesenteroides* subsp. *mesenteroides* form a group that is well-separated from *P. damnosus* and *P. parvulus* and from a group that comprises *Lb. brevis* and *Lb. hilgardii* (Sievers et al. 2003). The *rnpB* gene which is universally present in the low % G+C Gram-positive bacteria and encodes the RNA subunit of endoribonuclease P, proved suitable for phylogenetic analysis of closely related taxa and showed potential as a tool for species identification within the genus *Streptococcus* (Täpp et al. 2003). A study on the use of partial gene sequences of the *rpoB* gene, encoding the highly conserved subunit of the bacterial RNA polymerase, has been published by Drancourt and co-workers (Drancourt et al. 2004). The *rpoB* gene sequences showed a higher discriminative power than 16S rRNA gene sequences for identification of aerobic Gram-positive cocci belonging to the LAB genera *Streptococcus*, *Enterococcus*, *Gemella*, *Abiotrophia*, and *Granulicatella*.

It has been shown that classification of *Enterococcus* species based on sequence analysis of the housekeeping genes *pheS* (encoding phenylalanine tRNA synthetase), *rpoA* (encoding RNA polymerase α subunit), and *atpA* (encoding ATP synthase α subunit) is highly correlated with 16S rRNA gene phylogeny (Naser el al. 2005 a, b). Moreover, tripeptidase (*PepT*) and dipeptidase (*PepV*) encoding genes have been demonstrated to be distributed widely in LAB, especially in lactococci, and proved useful for a precise classification of *L. lactis* subspecies (Mori et al. 2004). Two different *tuf* gene sequences [*tufA* and *tufB*, encoding the elongation factor Tu (*EF-Tu*)], proved to be useful

for the identification of representative species of *Lactobacillus*, *Bifidobacterium*, *Lactococcus* and *Streptococcus* (Ventura et al. 2003).

Consequently, several MLSA studies of LAB bacteria have now been published. The classification and identification of LAB by MLSA was initiated in 2005 by Naser and co-workers. They evaluated the use of three housekeeping genes, *atpA*, *pheS* and *rpoA* as phylogenetic and species identification tools within the genus *Enterococcus* and used two of these genes, *pheS* and *rpoA*, within the genus *Lactobacillus* (Naser et al. 2005a, b; Naser et al. 2007). The same three housekeeping genes were subsequently used to study the genera *Leuconostoc* (De Bruyne et al. 2007), *Pediococcus* (De Bruyne et al. 2008) and *Streptococcus* (Huch et al. 2013). Also, three functional genes involved in the production of flavour compounds were used to study the genus *Lactococcus* (Rademaker et al. 2007). These studies demonstrated the superior capacity of MLSA for reconstructing a robust phylogeny of LAB in which all species can readily be distinguished (De Bruyne et al. 2010; Ehrmann et al. 2010; Naser et al. 2005a, 2005b). In addition, a growing number of studies include housekeeping gene sequence analyses as part of the description of novel species. Clearly, the extent of intra and inter species divergence of protein encoding genes has to be documented extensively before they can be reliably used for reconstructing the phylogeny of individual strains and species (Vandamme et al. 2014).

2.3. Classification and identification methods for lactic acid bacteria

2.3.1. Phenotypic methods

Phenotypic characterisation is an important part of the standard description and the analysis of several new species, including LAB taxa. The standard characteristics of LAB taxa include a Gram reaction (positive), presence of endospores (no), oxidase and catalase activity (typically absent), glucose fermentation products, carbohydrate fermentation patterns, lactic acid isomer production, hydrolysis of aesculin and arginine, reduction of nitrate, gelatine liquefaction, growth at different temperatures, pH range values and NaCl concentrations and tolerance to oxygen (Vandamme et al. 2014). In the past, the classification and identification of LAB through traditional phenotypic methods was mainly based on morphological, biochemical and physiological characters. For example, the genus *Leuconostoc* could be divided into the obligatory homofermentative species (no pentoses fermented, hexoses fermented through the Embden

Meyerhof pathway), obligatory heterofermentative species (hexoses and pentoses fermented via the phosphogluconate pathway) and facultative heterofermentative species (hexoses fermented via the Embden Meyerhof pathway and pentoses via the phosphogluconate pathway), based on the fermentation of hexose and pentose sugars (Thunnel 1995). In addition, to facilitate and standardize some of these tests, miniaturized biochemical test systems were developed such as the API systems, which contain enzyme assays and an array of substrate utilization tests. For exemple, Ouadghiri and coworkers used a polyphasic approach combining the API 50 CHL microtest system (bioMérieux), SDS-PAGE of whole-cell proteins and rep-PCR using the $(GTG)_5$ primer to identify LAB from traditional Moroccan soft white cheese, named "Jben" (Ouadghiri et al. 2005). In another study, Khedid et al. (2009) used traditional physiological and biochemical tests and the API 50 CHL microtest system to identify LAB from Moroccan camel milk. It is very important to note that sugar fermentation profiles obtained by the API 50 CHL microtest system do not always yield an acceptable identification result and that some carbohydrate fermentation based identification results are simply wrong because strain dependent fermentations often occur (Andrighetto et al. 1998; Champomier et al. 1987; Shaw and Harding, 1984).

The presence of meso-diaminopimelic acid in the cell wall was one of the key parameters in the earlier biochemical identification keys (Hammes et al. 1991; Hammes and Vogel, 1995). Cell-walls of Gram-positive bacteria contain various peptidoglycan types (Schleifer and Stackebrandt, 1983), which differ in the amino acid sequence of the peptide moiety of the peptidoglycans and the cross-linkage type. For some species the peptidoglycan type is species specific but there is generally limited diversity among species, with the Lys-D-Asp type being the predominant type within the genera *Lactobacillus* and *Pediococcus* and several other LAB genera (Hammes and Hertel, 2009; Holzapfel et al. 2009). Determination of the peptidoglycan composition is for example useful to differentiate the genus *Weissella* from other LAB (Björkroth and Holzapfel, 2003), and some species, for instance *Lb. sanfranciscensis* and *Lb. rossiae*, have rather specific peptidoglycans, i.e. Lys-Ala and Lys-Ser-Ala_2, respectively (Hammes and Hertel, 2009).

Also sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) of whole-cell proteins proved particularly useful for the accurate species level identification of LAB and provided a database of digitised and normalized protein patterns of all known species of LAB (Benito et al. 2008; De Bruyne et al. 2008; Groth Laursen et al.

2005; Ouadghiri et al. 2005, 2009; Pot et al. 1994). This technique has widely been applied as a first line screening method for grouping large numbers of strains, of which subsequently a limited number of representative strains was selected for further analysis by genotypic or phenotypic methods (Dalgaard et al. 2003). However, this method proved problematic concerning portability and time efficacy. A novel high-throughput identification method also based on the analysis of whole cell proteins, MALDI-TOF MS, has been introduced and successfully applied in bacterial taxonomy (Bizzini and Greub, 2010). In 2010, Tanigawa and colleagues used MALDI-TOF mass spectra for the differentiation of *Lactococcus* species and subspecies (Tanigawa et al. 2010) and De Bruyne et al. (2011) developed a standard protocol to generate MALDI-TOF mass spectra of *Leuconostoc*, *Fructobacillus* and *Lactococcus* strains (De Bruyne et al. 2011). An introduction to MALDI-TOF mass spectrometry and its current applications for the identification and dereplication of bacteria is presented below in chapter 4.

2.3.2. Genotypic methods

During the last two decades, advances in molecular biology resulted in numerous genotypic methods for the analysis of the bacterial genome or its transcription products. As a consequence of their use in taxonomy, our knowledge of the microbial diversity of complex mixed microbial communities has improved considerably. Numerous genotypic methods are in use today. As described above, DNA-DNA reassociation experiments are still considered the gold standard for species delineation with strains sharing at least 70% DNA-DNA relatedness attributed to a single species (Stackebrandt & Goebel, 1994). **The DNA base composition,** expressed as mol% guanine plus cytosine (% G+C) is one of the required characteristics for the description of new species. In general, the range observed within one species does not exceed 3% (Stackebrandt & Liesack, 1993). Identification of LAB through 16S rRNA gene sequencing is widespread, but this approach often fails to discriminate between closely related LAB species or subspecies (Dellaglio and Felis, 2005). Many studies therefore use sequence analysis of one or more protein encoding genes as a more effective approach for accurate species level identification and classification of LAB.

Plasmid profiling and pulsed-field gel electrophoresis (PFGE) of macrorestriction fragments of genomic DNA were developed and used in taxonomic studies of LAB. These techniques were, however, first developed as typing methods to trace individual strains

in epidemiological studies (Van Belkum et al. 2007) and not as species level identification tools. Especially since the introduction of PCR amplification (Saiki et al. 1988), a large number of DNA-based fingerprinting methods have been developed based on the selective amplification of DNA fragments using oligonucleotide primes. The increased sensitivity and the reduced analysis time are the main advantages of PCR-based assays. Below, some genotypic techniques that are commonly used in polyphasic taxonomy of LAB are briefly discussed.

Pulsed field gel electrophoresis (PFGE) analysis

PFGE requires the digestion of genomic DNA with rare-cutting restriction enzymes to yield a few relatively large DNA fragments, which are then separated in an alternating electric field. This fingerprint represents the complete genome and thus can detect specific changes (DNA deletion, insertions, or rearrangements) within a particular strain over time (Ben Amor et al. 2007). PFGE was, for instance, used to study the diversity of LAB from fermented meat (Tran et al. 2011), to differentiate *Lb. plantarum* strains (Pepe et al. 2004) and important probiotic bacteria, such as *Bif. longum* and *Bif. animalis* (Ben Amor et al. 2007; Roy et al. 1996). However, this fingerprint technique is very laborious and time-consuming.

Amplified Fragment Length Polymorphism (AFLP) analysis

This method is a high-resolution fingerprint technique for intra species identification and genotyping of LAB and bifidobacteria from various fermented food products as well as from the human gastrointestinal microbiota (Ben Amor et al. 2007) (Borgen et al. 2002; Torriani et al. 2001; Vancanneyt et al. 2002). Many studies demonstrated the successful application of AFLP to identify new LAB taxa (e.g. Valcheva et al. 2005). The AFLP technique is based on the selective PCR amplification of restriction fragments from a total digest of genomic DNA. The technique involves three steps: i) restriction of the DNA and ligation of oligonucleotide adapters, ii) selective amplification of sets of restriction fragments, and iii) gel analysis of the amplified fragments (Vos et al. 1995).

Amplified Ribosomal DNA Restriction Analysis (ARDRA)

ARDRA is a commonly used tool to study microbial diversity (Deng et al. 2008; Sklarz et al. 2009). 16S rDNA gene fragments, obtained by applying either universal or genus-

specific primer sets, are amplified and digested by restriction endonucleases (REs), followed by separation of the resulting fragments on high-density agarose or acrylamide gels. The emerging profiles are then used either to cluster the community into genotypic groups or for strain typing (Sklarz et al. 2009; Tiedje et al. 1999).

Ribotyping

Ribotyping was developed as one of the first DNA fingerprinting techniques used in bacterial taxonomy (Grimont and Grimont, 1986). This method combines the restriction enzyme analysis of chromosomal DNA with recombinant DNA probe hybridisation to differentiate between species (Bjorkroth and Korkeala, 1996; Lyhs et al. 1999; Zhong et al. 1998). The discriminatory power of this method depends on the number and type of oligonucleotide probes and restriction enzymes used. Ribotyping has been used in taxonomic studies of *Lactobacillus*, *Streptococcus*, and *Leuconostoc* (Björkroth et al. 2000; Endo et al. 2007; Schlegel et al. 2000). This technique is labour intensive but a commercialised automated system comprising a limited database for the identification of LAB is available (http://www2.dupont/Qualicon/en_US/products/RiboPrinted_System/index.html).

Typing methods based on the Polymerase Chain Reaction (PCR)

Both randomly amplified polymorphic DNA (RAPD) and repetitive sequence-based (rep)-PCR represent DNA fingerprinting methods that reply on PCR and subsequent electrophoresis of the obtained amplicons (Cocolin et al. 2008). RAPD has been widely reported as a rapid, sensitive, and inexpensive method for genetic typing of different strains of LAB (Ben Amor et al. 2007). This PCR based technique employs short arbitrary primers and low-stringency annealing to amplify a range of DNA fragments which are electrophoretically separated to give a fingerprint. RAPD analysis has been applied extensively for genotyping of thermophilic streptococci and lactobacilli in several Italian, French and Spanish cheese types such as Mozzarella cheese (De Candia et al. 2007; Morea et al. 1998; Moschetti et al. 1998). Also, the genetic diversity of lactococcal strains isolated from raw milk in Registered Designation of Origin (RDO) Camembert cheese (Corroler et al. 1998; Desmasures et al. 1998) has been successfully evaluated by RAPD analysis. Despite RAPD being a simple and rapid technique, careful optimization and standardisation are needed to achieve sufficient reproducibility. Differences

in thermal cycles, DNA polymerases and their concentrations, DNA preparation methods, primer to template ratios and magnesium concentrations can cause variations in the RAPD patterns, and consequently, patterns obtained in different laboratories are not always comparable (Randazzo et al. 2009).

Repetitive element primed Polymerase Chain Reaction (rep-PCR)

Repetitive element primed polymerase chain reaction (rep-PCR) provides a high taxonomic resolution and may act as a rapid detector of diversity and evolution of the microbial genomes being studied (Busch and Nitschko, 1999; Versalovic et al. 1993). Rep-PCR is a genomic fingerprinting technique that takes advantage of the presence of interspersed intra or extragenic elements [like REP, $(GTG)_5$ and BOX sequences)] found throughout the bacterial chromosome. PCR with primers targeting these repetitive elements generates specific DNA fingerprints (Busch and Nitschko, 1999; Gevers et al. 2001; Olive and Bean, 1999; Lupski and Weinstock, 1992; Rademaker et al. 2002). Selective amplification of the regions between these conserved sequences generates amplicons of varying sizes that are separated by agarose gel electrophoresis and then compared to profiles of reference strains for identification (Heyndrickx et al. 2007). For lactic bacteria, the $(GTG)_5$ primer has been used for typing and identification of *Lactobacillus* and *Enterococcus* strains (Gevers et al. 2001; Švec et al. 2005).

Rep-PCR fingerprinting methods has been successfully applied to the identification and taxonomic classification of a high number of LAB. Rep-PCR was, for instance, used to describe LAB from "Tarhana", a traditional Turkish fermented food (Sengun et al. 2009), from Azerbaijani traditional dairy products (Terzic-Vidojevic et al. 2009), from traditional fura processing in Ghana (Owusu-Kwarteng et al. 2012), from a Ghanaian fermented milk product (Akabanda et al. 2013), from raw cow milk in Morocco (Ouadghiri et al. 2009) and from African raw and fermented camel milk products (Jans et al. 2012). Despite its limitations, rep-PCR can be used to rapidly compare a large number of isolates in order to remove isolates with identical fingerprints from further studies (see below)

2.4. Fingerprinting methods as tools for dereplication

Several diversity studies use a dereplication step to reduce the work load once cultures of bacterial isolates are obtained. The term is referring to the process of recognizing identical isolates at a specific taxonomic level and grouping them accordingly (Ghyselinck et al. 2011). The selection of representatives of each group reduces subsequently the number of isolates to be examined in further analyses. Dereplication originally referred to the grouping of bacterial isolates at the lowest taxonomic level, the strain level (Bull et al. 1992). However, the term is somewhat ambiguous and nowadays used in a broader sense, also indicating grouping at subspecies (De Clerck and De Vos, 2002), species or any higher taxonomic level (Coorevits et al. 2008).

A variety of tools have been used to dereplicate and sometimes simultaneously identify bacteria. In general, morphological and biochemical characterisation can be used as primarily screening tools for descriptive purposes, not dereplication or identification (De Bruyne, 2009). For exemple, to select LAB isolates from primary isolation plates, the Gram reaction type (positive), cell morphology (cocci or bacilli) and the absence of catalase activity are commonly used (Scheirlinck et al. 2007). Next, techniques that can differentiate strains on the basis of fingerprinting profiles, and that allow the grouping of isolates based on their profiles can be performed to reduce the number of strains requiring further analyses (Cocolin et al. 2008). A dereplication technique should conform with the following criteria: i) it should possess an universal character, i.e. being applicable to many bacterial species, ii) it should be robust, iii) it should produce easy to interpret data, iv) it should have a high taxonomic resolution, and v) it should provide the possibility of high-throughput application/automation with low operational cost and labour intensity (Ghyselinck et al. 2011). Fingerprinting techniques such as rep-PCR (Cocolin et al. 2004; Gevers et al. 2001; Scheirlinck et al. 2009, Švec et al. 2005), RAPD (Ercolini et al. 2009; Franciosi et al. 2009; Kostinek et al. 2008), AFLP (Giraffa & Neviani, 2000; Portier et al. 2006; Rademaker et al. 2000), and ribotyping (Barney et al. 2001; Endo et al. 2007; Satokari et al. 2000), but also MALDI-TOF MS (see below) (Nguyen et al. 2012; Nguyen et al. 2013; Spitaels et al. 2014) have been used for this purpose in studies of LAB diversity.

CHAPTER 3: THE GENERA *STREPTOCOCCUS, LACTOCOCCUS* AND *ENTEROCOCCUS*.

In the frame of the microbial diversity studies performed in the present PhD thesis, we isolated novel bacterial taxa belonging to the genera *Streptococcus, Lactococcus* and *Enterococcus*. We therefore provided a concise description of each of these genera below.

3.1. The genus *Streptococcus*

3.1.1. Taxonomy of the genus *Streptococcus*

The name *Streptococcus* was first used by Rosenbach (1884) to describe the chain-forming, coccus-shaped bacteria associated with wound infections. The genus *Streptococcus* embraces a broad range of Gram-positive, catalase-negative, chain-forming and coccus-shaped organisms (Hardie and Wiley, 1997; Du Toit et al. 2014). Species are non-motile and non-sporing, facultatively anaerobic (some require CO2), chemo-organotrophs with complex nutritional requirements and a fermentative metabolism resulting in L (+)-lactic acid as the major product of glucose fermentation (Hardie and Wiley, 1997; Du Toit et al. 2014). The majority of streptococcal species have a mol % G+C content in the range of 34 to 46 (Spellerberg & Brandt, 2007; Tomida et al. 2010). Three types of haemolysis can be distinguished: α-haemolytic, β-haemolytic and non (γ)-haemolytic streptococci. Since 1984, many changes in the nomenclature and taxonomy of the *Streptococcus* genus have taken place and the genus *Streptococcus* has been subdivided into three separate genera: *Streptococcus*, *Lactococcus* and *Enterococcus* (Devriese et al. 1993; Devriese and Pot, 1995; Franz et al. 2003; Schleifer and Kilpper-Bälz, 1984), by using 16S rRNA cataloguing (Ludwig et al. 1985; Bentley et al. 1991; Williams & Collins, 1991), DNA-DNA and DNA-rRNA hybridizations (Garvie & Farrow, 1981; Kilpper-Bälz & Schleifer, 1981, 1984; Kilpper-Bälz et al. 1982; Schleifer & Kilpper-Bälz, 1984; Schleifer et al. 1985) and serological studies with superoxide dismutase antisera (Schleifer et al. 1985).

The currently recognized phylogenetic relationships within the genus *Streptococcus* have been determined by comparative sequence analysis of their 16S rRNA gene sequences (Bentley et al. 1991; Kawamura et al. 1995; Kilpper-Bälz and Schleifer, 1984; Schleifer and Kilpper-Bälz, 1987). Based on these data, six phylogenetic species groups

were distinguished (Bentley et al. 1991; Kawamura et al. 1995): the anginosus, bovis, mitis, mutans, pyogenes and salivarius species groups (Kawamura et al. 1995).

The genus *Streptococcus* underwent considerable expansion and revision in the past decade and novel taxa are continuously being discovered (Camelo-Castillo et al. 2014; Milinovich et al. 2008; Nomoto et al. 2015; Saito et al. 2014; Shewmaker et al. 2007; Takada et al. 2013; Takada & Hirasawa, 2007 and 2008; Vela et al. 2009; Vela et al. 2014; Zbinden et al. 2012; Zhang et al. 2013). Currently (October 2015), the genus *Streptococcus* consists of 113 species and 22 subspecies (http://www.bacterio.cict.fr/s/*streptococcus*.html). However, in this genus too several species proved to be synonymous or generically misclassified necessitating additional classification changes (Avendano-Herrera et al. 2014; De Maesschalck et al. 2014; Kawamura et al. 1995; Nomoto et al. 2014; Schleifer et al. 1985; Du Toit et al. 2014).

3.1.2. Identification methods of the genus *Streptococcus*

In 1998, Poyart and colleagues demonstrated that the sequencing of the *sodA* gene allowed the identification of *Streptococcus* species. In fact the pairwise comparison of two given streptococcal species always revealed a lower percentage of sequence similarity between their *sodA* fragments than between their 16S RNA gene sequences (Poyart et al. 1998). In addition to *sodA*, the *rnpB* gene which encodes the RNA subunit of endoribonuclease P has been shown to be suitable for phylogenetic analysis of closely related streptococci and gives it superior potential for species discrimination compared to the 16S rRNA (Täpp et al. 2003). The applicability of sequence analysis of the 16S-23S ribosomal RNA (rRNA) has been evaluated by Chen et al. (2004). Their results indicated that the ITS region might constitute a more discriminative target sequence than 16S rRNA for the differentiation of closely related species of viridans streptococci. It was found that considerable variation in both the lengths and the sequences of the ITS regions can occur between species (Chen et al. 2004; Gurtler and Barrie, 1995). The ITS region has been suggested to be a suitable target from which useful taxonomic information for bacteria can be derived, particularly with regard to identification to the species level (Chen et al. 2004; Hamid et al. 2002; Suffys et al. 2001) and genotyping (Chen et al. 2004; Gurtler, 1993; Gurtler and Barrie, 1995).

Picard et al. (2004) demonstrated that streptococcal *tuf* genes are generally more variable and offer more discriminatory power than the 16S rRNA gene and should allow identification at the species level of even the most closely related streptococcal species.

Subsequently Naser et al. (2006) demonstrated that the combined use of partial *pheS*, *rpoA* and *atpA* gene sequences offered a reliable identification system for all *Streptococcus* species and provided a higher resolution than the 16S rRNA gene. The *pheS* and *atpA* gene sequence analyses provided interspecies gaps typically exceeding 6% and 5% divergence, and intraspecies variations up to 3% and 2%, respectively. The *rpoA* gene sequences revealed a lower resolution with an interspecies gap typically exceeding 3% and an intraspecies variation up to 1%. They recommended the use of the *pheS* gene as the first choice for screening during a biodiversity studies (Naser el al. 2006). Additionaly, the genetic diversity of *Streptococcus* can be analysed by PFGE (Brzychczy-Wloch et al. 2010; Huch et al. 2013; Poyart et al. 2002; Vela et al. 2014), AFLP (Huch et al. 2013), ribotyping (Schlegel et al. 2000), tDNA-PCR (Vancanneyt et al. 2004), RAPD (Brzychczy-Wloch et al. 2010), or rep-PCR (Cheon et al. 2011; Moser et al. 2010; Švec et al. 2008). SDS-PAGE analysis of whole cell proteins and MALDI-TOF MS are among the most commonly used phenotypic methods for the delineation of streptococcal species (Avendano-Herrera et al. 2014; Collins et al. 2002; Collins et al. 2004; Lawson et al. 2005; Täpp et al. 2003).

3.2. The genus *Lactococcus*

3.2.1. Taxonomy of the genus *Lactococcus*

The genus *Lactococcus* was proposed by Schleifer and colleagues in 1985 to reclassify some species of the genera *Streptococcus* (i.e. the Lancefield group N lactic streptococci) and *Lactobacillus*. It has been defined on the basis of chemotaxonomic data that were corroborated by 16S rRNA sequencing (Collins et al. 1989; Schleifer et al. 1985; Schleifer and Killper-Bälz, 1987). Currently (October 2015), the genus *Lactococcus* comprises nine validly named species and four subspecies (Euzéby, 1997; last full update 5 May 2015, http://www.bacterio.net/*lactococcus*.html); two additional *Lactococcus* species were recently described, *L. hircilactis* and *L. laudensis* (Meucci et al. 2015). All members of this genus are characterized as being facultatively anaerobic, catalase-negative, Gram-positive cocci that occur singly, in pairs, or in chains (Facklam and Elliott, 1995). They are not β- hemolytic and they are poorly α- hemolytic. The % G+C content of DNA ranges from 34.4 to 43.0 mol% (Cai et al. 2011; Kim, 2014; Pérez et al. 2011; Schleifer et al. 1985; Williams et al. 1990). Species of the genus *Lactococcus*

have been isolated from various food, plant and animal sources (Cai et al. 2011; Williams et al. 1990). Members of the genus *Lactococcus* have been identified as lactic acid bacteria that contribute significantly to the properties of fermented dairy products while others produce antimicrobial compounds. They have been isolated largely from food-related sources and are generally regarded as safe organisms (Salminen et al. 1998). Their use for the production of probiotic foods has also been considered, since they contribute to the maintenance of human intestinal health (Steidler et al. 2000; Tanigawa et al. 2010). However, rare cases of invasive disease in humans, sometimes severe, have been reported in association with *L. garvieae* (Vinh et al. 2006; Wang et al. 2007; Yiu et al. 2007) and *L. lactis* (Mannion & Rothburn, 1990).

3.2.2. Identification methods of the genus *Lactococcus*

The identification of the most common *Lactococcus* species found in dairy products can be successfully performed by rapid and accurate molecular techniques. PCR-DGGE analyses can be used for differentiating *L. lactis* from other lactic acid bacteria (Coppola et al. 2001). rRNA oligonucleotide probes have been designed for identifying *L. lactis* subsp. *cremoris* (Salama et al. 1991). Several PCR or multiplex PCR assays can be applied for the identification of *L. lactis* and *L. garvieae* by using primers targeting the 16S ribosomal RNA gene sequences (Pu et al. 2002; Pogačić et al. 2011; Zlotkin et al. 1998), and exploiting the polymorphism of the 16S-23S rDNA spacer region (Blaiotta et al. 2002) or other functional genes like the histidine biosynthesis operon (Corroler et al. 1999), the *acmA* gene encoding the lactococcal N-acetylmuramidase (Garde et al. 1999) and the *sodA* gene (Fihman et al. 2006). The genetic diversity of *Lactococcus* can be analysed by RAPD (Tailliez et al. 1998) or multiple locus microsatellite analysis (Quénée et al. 2005) for *L. lactis* or by PFGE for *L. garvieae* (Vela et al. 2000). The study of Tanigawa et al. (2010) demonstrated that MALDI-TOF MS allowed to identify species and subspecies of *L. lactis;* the technique was considered attractive because of its speed, accuracy, simplicity, and low cost. It has been shown that this approach produced nearly the same results as did molecular techniques, such as phylogenetic analysis based on housekeeping gene sequences or AFLP profiles (Tanigawa et al. 2010).

3.3. The genus *Enterococcus*

3.3.1. Taxonomy of the genus *Enterococcus*

The genus *Enterococcus* constitutes together with the genera *Melissococcus*, *Tetragenococcus* and *Vagococcus* the family *Enterococcaceae*. The genus *Enterococcus* was first proposed by Schleifer and Kilpper-Bälz in 1984 to include the Lancefield group D faecal streptococci, i.e. *Steptococcus* (*S.*) *faecalis* and *S. faecium*. The species *S. faecium* and *S. faecalis* were separated from other streptococci using DNA–DNA hybridization and 16S rRNA gene sequencing studies (Devriese et al. 1993; Devriese and Pot, 1995; Schleifer and Kilpper-Bälz, 1984). The new genus *Enterococcus* was constituted by the 'faecal streptococci' associated with the gastrointestinal tract of humans and animals, and those from some fermented foods and a range of other habitats (Franz et al. 2003; Foulquie Moreno et al. 2006; Hardie and Whiley, 1997). Currently (October 2015), fifty-four *Enterococcus* species and two subspecies have been recognized (http://www.bacterio.cict.fr/e/enterococcus.html). Since 2010, fourteen new enterococcal species and two subspecies were described, e.g., *Enterococcus* (*E.*) *alcedinis* (Frolkov et al. 2013), *E. diestrammenae* (Kim et al. 2013), *E. olivae* (Lucena-Padrós et al. 2014), *E. saccharolyticus* subsp. *taiwanensis* (Chen et al. 2013), and *E. xiangfangensis* (Li et al. 2014). Concomittantly, *E. porcinus*, *E. seriolicida* and *E. solitarius* were reclassified as *E. villorum*, *Lactococcus garvieae* and *Tetragenococcus solitarius* (De Graef et al. 2003; Ennahar & Cai, 2005; Teixeira et al. 1996), respectively. Nonetheless, *E. faecium* and *E. faecalis* remain the two most prominent species and they play the most important roles in human disease, in fermented foods and in probiotics (Foulquie Moreno et al. 2006; Franz et al. 1999; Kayser, 2003).

Enterococci are chemoorganotrophic and produce L (+)-lactic acid as major product of glucose fermentation. Enterococci are catalase-negative although some strains exhibit catalase or pseudo-catalase activity (Hardie & Wiley, 1997; Švec et al. 2001; Švec & Devriese, 2009). They are facultatively anaerobic cocci, which are arranged in pairs or short chains and have a mol % G+C content between 38 and 45 (Hardie and Whiley, 1997). They comprise an important group of lactic acid bacteria found ubiquitously in the environment, the gastrointestinal tract, traditional fermented foods, and in large numbers in dairy products.

3.3.2. Identification methods of the genus *Enterococcus*

Phenotypic methods to delineate enterococcal species include the determination of carbohydrate fermentation (Hardie and Whiley, 1997; LeBlanc, 2006; Švec et al. 2005; Naser et al. 2005a), enzyme patterns (Švec et al. 2005; Manero and Blanch, 1999; Naser et al. 2005a), SDS-PAGE analysis of whole-cell proteins (De Graef et al. 2003; Devriese et al. 2002; Vancanneyt et al. 2004), and cellular fatty acid analysis (Fortina et al. 2004; Tyrrell et al. 2002; Law-Brown and Meyers, 2003). The physiological and biochemical characteristics are often analyzed by means of commercially available test systems such as API 50 CHL, API 20 Strep and API ZYM (API systems, BioMérieux, France). Although useful as standardized methods, they present several inconveniencies as they are still labour-intensive and they show variations within species and between laboratories. For identification purposes, as described above, the physiological and biochemical methods have their limitations because of their poor reproducibility and their relatively low taxonomic resolution, which often only allows differentiation at the genus level (Corsetti et al. 2001; Nguyen et al. 2012; Pot et al. 1994). MALDI-TOF MS is a relatively new approach that is fast, reliable, and cost effective for the identification of LAB (De Bruyne et al. 2011; Nguyen et al. 2012), including enterococci (Griffin et al. 2012; Quintela-Baluja et al. 2013).

Genotypic methods used in the description of the enterococcal species include rep-PCR (Švec et al. 2005, 2006), tRNA intergenic length polymorphism analysis (tDNA-PCR) (De Graef et al. 2003; Naser et al. 2005a; Švec et al. 2001), ribotyping (Koort et al. 2004; Švec et al. 2005) and PFGE (Teixeira et al. 2001; Koort et al. 2004). Although applicable for species level identification, these methods may present problems concerning portability and inter-laboratory reproducibility. The phylogenetic relationships of the different species within the genus *Enterococcus* have been determined by comparative sequence analysis of their 16S rRNA genes and different species groups can be distinguished (Hardie and Whiley, 1997; Klein 2003) (see below). Other genomic markers applied for the identification of *Enterococcus* species include *sodA* and *cpn60* (encoding 60-kDa chaperonin protein subunits) partial gene sequences (Poyart et al. 2000 and Goh et al. 2000). Furthermore, Naser et al. (2005a, b) demonstrated that multilocus sequence analysis of partial *pheS* (395–455 bp), *rpoA* (413–793 bp) and *atpA* (862–963 bp) gene sequences allows the delineation of enterococci species and the detection of novel species. Enterococcal strains of the same species had highly similar

rpoA, *pheS* and *atpA* gene sequences having at least 99 % *rpoA*, 97 % *pheS* and 96.3 to 100 % *atpA* gene sequence similarities. In contrast, at the inter-species level, all enterococcal species were clearly differentiated on the basis of *rpoA*, *pheS*, and *atpA* gene sequences, with at maximum 97 %, 86 % and 92 % similarity between any pair of enterococcal species, respectively.

CHAPTER 4. THE IDENTIFICATION OF BACTERIA USING MALDI-TOF MS

4.1 General introduction

MALDI-TOF MS was first described in 1985. It appeared well suited and was successfully applied in the analysis of polar biomolecules, including peptides and proteins (Karas et al. 1987). Tanaka et al. (1988) reported the ionization of the protein carboxypeptidase-A (34 kDa) by means of soft laser desorption and Karas and Hillenkamp (1988) subsequently reported MALDI-TOF MS of even larger proteins, up to 67 kDa. MALDI-TOF MS progressed rapidly and earned much attention in the field of proteomics and later in bacteriology and bacterial taxonomy. It has an outstanding performance as compared with other analytical methods. Indeed, the simplicity, mass accuracy, high resolution, speed, sensitivity and diversity of its applications stand out as its most important advantages (Aebersold & Mann, 2003; Hoffmann and Stroobant, 2007).

4.2 Soft ionization

MALDI is a soft ionization technique (Hillenkamp & Karas, 2007) and enables the ionization of large biomolecules such as proteins and carbohydrates through the use of an organic matrix substance that absorbs light energy at a specific wavelength. This energy is subsequently used for the desorption and ionization of an analyte (Liyanage & Lay, 2006). In MALDI-TOF MS, the analyte solution (sample) is mixed with an equal volume of matrix solution and deposited on a stainless steel MALDI target (Liyanage and Lay, 2006). The matrix, which is important in MALDI analyses, serves to absorb the laser energy and for energy transport (Fig 4.1). In addition, it helps to separate the analyte molecules from each other, which prevents cluster formation and avoids direct laser “hits” on the analyte that could result in fragmentation. The matrix compound is typically a weak acid (e.g. α-cyano-4-hydroxycinnamic acid [α-CHCA]), sinapic acid, ferulic acid or 2,5-dihydroxybenzoic acid) that absorbs photons at the wavelength of the laser used in the experiment, typically around 355 nm (Liyanage and Lay, 2006). This matrix can also improve the spectra mainly by increasing the sensitivity and reducing background signals (Pan et al. 2007). The choice of matrix depends on

the type of biomolecule analyte. For example, α-CHCA and sinapic acid are good matrices for peptides and proteins, whereas 2,5-dihydroxybenzoic acid has higher preference for oligonucleotides (Vargha et al. 2006).

After a brief laser pulse, the radiated spot is rapidly heated and becomes vibrationally excited. The laser energy is absorbed, and the vibrational energy results in expulsion of material in a plume just above the irradiated site. Due to minimal dissociative vibrational excitation of the analyte, intact proteins can be transferred into the gas phase. Because the matrix compound is also a weak acid, some of the polar analytes (like proteins) will receive protons from the carboxylic acid groups of the matrix in an acid-base equilibrium process via proton transfer mechanisms just above the site of desorption (Liyanage and Lay, 2006).

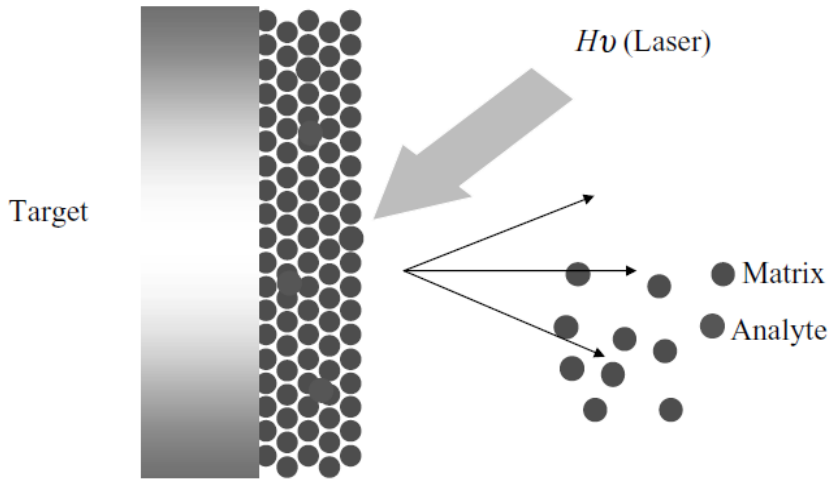

Fig 4. 1: Principle of MALDI process (Liyanage and Lay, 2006).

4.3 Applications, advantages and limitations

Nowadays, MALDI-TOF MS is finding applications in all areas of biochemistry and proteomics due to the simplicity of sample preparation for routine analysis of proteins and peptides, and the high sensitivity of modern MALDI-TOF MS instruments. Mass spectral analysis determines the molecular mass of chemical compounds by separating molecular ions according to their mass-to-charge ratio (m/z). Generally, in mass spectrometry, ions are formed in the ion source, separated in mass analyser and one-by-one detected (Liyanage and Lay, 2006) (Fig 4.1).

In the ion sources, the analysed samples are ionised before the analysis in the mass analyser. Ions are mainly produced by ionising a neutral molecule in the gas phase

through electron ejection, electron capture, protonation, cationisation, and deprotonation or by the transfer of a charged molecule from the condensed phase to the gas phase. A variety of ionisation techniques are used for mass spectrometry such as electron ionisation, chemical ionisation, field ionisation, matrix-assisted laser desorption ionisation (MALDI) and electrospray ionisation (Hoffmann and Stroobant, 2007).

Mass analyzers, when the gas-phase ions have been generated, separate the ions based on their mass-to-charge ratio (m/z). There are many different types of mass analyzers, however, MALDI is mostly combined with a time-of-flight (TOF) tube as mass analyzer, since both operate in a pulsed manner (Liyanage & Lay, 2006). In the TOF tube, the time needed to reach the detector is inversely proportional to the m/z ratio of the ions. The m/z ratio can be calculated by the use of a calibration curve. The TOF time measurement is initiated by a pulse of the laser light.

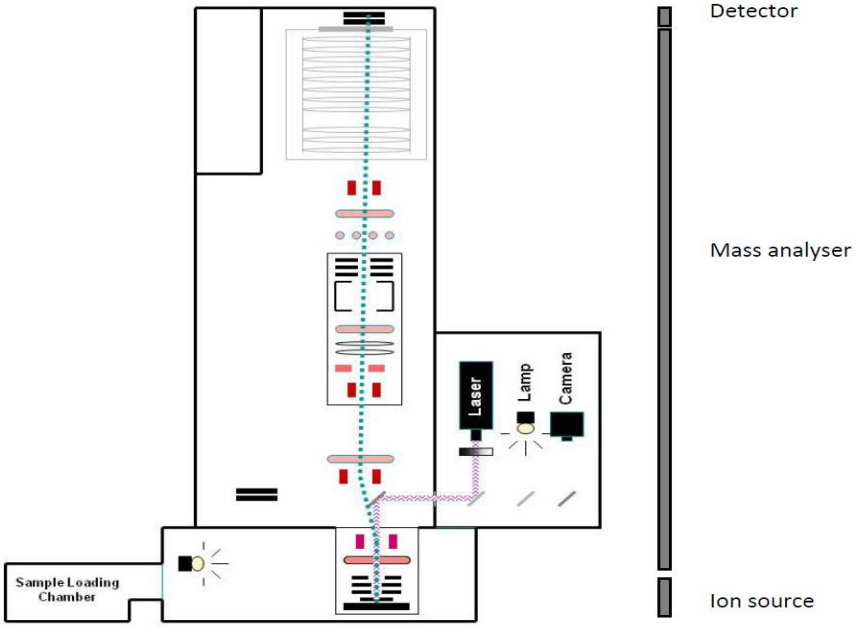

Fig 4. 2: Representation of the MALDI-TOF system (Applied Biosystems).

The advantage of TOF separation is that spectra are obtained very quickly (in a few seconds) and typically, several spectra are averaged to produce a final spectrum with high signal-to noise ratios (Liyanage and Lay, 2006). The resolution of a TOF analyser is relatively low, but there is virtually no upper mass limitation. To improve resolution, TOF reflectron mass analysis and delayed extraction MALDI have been developed. Although higher resolution is achieved, the former is only applicable in a limited mass range (Siuzdak, 1996).

The ions pass through the mass analyser and are finally detected and transformed into a usable signal by the **detector**. Although a variety of approaches are used to measure ions, the detection of ions is always based on their charge, mass, and concomitant velocity (Hoffmann and Stroobant, 2007).

MALDI-TOF MS is commonly used for bacterial identification in the field of medical microbiology (Croxatto et al. 2012; Pfleiderer et al. 2013). Compared with conventional phenotype or PCR based identification wich cannot be easily applied for rapid classification and identification of large numbers of isolates, MALDI-TOF MS shows short analysis time, ease of sample handling, modest reagent costs and superior identification capacity (Cherkaoui et al. 2010; Croxatto et al. 2012). This explains its rapid adoption in a variety of microbiological identification studies (Bizzini and Greub, 2010; Dieckmann et al. 2005; Tanigawa et al. 2010). Recently MALDI-TOF MS has been introduced in the field of food microbiology for a range of applications including the detection and identification of food pathogens, the identification of LAB and probiotic bacteria (De Bruyne et al. 2011; Dušková et al. 2012; Kuda et al. 2014; Nguyen et al. 2012; Sedo et al. 2013; Snauwaert et al. 2013; Tanigawa et al. 2010; Zeller-Péronnet et al. 2013), the detection and quantification of microbial spoilage in milk and pork (Nicolaou et al. 2012), the identification of beer spoilage bacteria (Kern et al. 2013; Wieme el al. 2014), the identification of beneficial bacteria, such as acetic acid bacteria used in the production of vinegar (Andres-Barrao et al. 2013), the identification of starter cultures for the production of cheese, yoghurt, or other fermented food (Burns et al. 2008; Hansen, 2002; Milesi et al. 2008) and for the identification of fungal and yeast species (Marklein et al. 2009; Spitaels et al. 2014; Wieme et al. 2014).

Nevertheless, the implementation of this fast and accurate methodology for routine identification of microorganisms in food microbiology faces some outstanding challenges including an adequate reproducibility and the need to develop ideally a single, universal sample preparation protocol (Keys et al. 2004; Liu et al. 2007; Vargha et al. 2006). Actually, two procedures are widely used for the sample preparation. The first protocol consists of a protein extraction from a small amount of cells using formic acid and acetonitrile, preceded by an ethanol inactivation step (FA/ACN extraction) (Freiwald & Sauer, 2009). The second consists of smearing the bacterial colony on the MALDI plate, after which it can additionally be lysed on the plate using formic acid. The latter protocol has been considered more straightforward and reduces handling time

(McElvania TeKippe et al. 2013). Moreover, this technique presents problems concerning the reproducibility. Different experimental factors, including sample preparation, cell lysis method, matrices, and organic solvents, affect the quality and reproducibility of bacterial MALDI-TOF MS fingerprints (Liyanage & Lay, 2006; Ruelle et al. 2004; Smole et al. 2002; Vargha et al. 2006, Williams et al. 2003). Other parameters such as bacterial growth conditions and the MALDI-TOF MS instrument used can also have a clear impact on species level identification purposes (Karger et al. 2013; Wunschel et al. 2005). In contrast, De Bruyne et al. (2011), Sedo et al. (2013) and Wieme et al. (2014) minimize this effect. In addition, the quality assessment of the mass spectra obtained differs widely. Some studies focus on specific characteristics of the mass spectra, such as signal-to-noise ratio, signal intensity and peak resolution (Goldstein et al. 2013; Schumaker et al. 2012; Toh-Boyo et al. 2012), while others focus on the applicability of the mass spectra for species identification (McElvania TeKippe et al. 2013; Sedo et al. 2013). The inter-laboratory reproducibility can be analysed more easily through the use of commercial systems that facilitate the use of the same instrument and parameter settings (Garner et al. 2013; Karger et al. 2013; Mellmann et al. 2009).

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PART III
EXPERIMENTAL WORK

CHAPTER 5: CLASSIFICATION AND IDENTIFICATION OF CAMEL MILK LAB

5.1 Occurrence of lactic acid bacteria in Moroccan raw camel milk.

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In preparation for submission

PREAMBLE

In recent years, the use of molecular techniques has considerably increased our knowledge on microbial diversity in food ecosystems. The classical approach to study LAB present in raw (unfermented) foods starts with cultivation and identification mainly by molecular biology techniques of purified isolates. In the present study, we used a stepwise approach using $(GTG)_5$-PCR fingerprinting and 16S rRNA gene sequencing in order to identify the LAB diversity associated with raw camel milk produced in different regions in Morocco.

OUTLINE CONTRIBUTIONS

Experimental design: Prof. Mohamed AMAR.
Experimental work: KADRI Zaina.
Data analysis: KADRI Zaina and Prof. Mohamed AMAR.
Writing of manuscript: KADRI Zaina.
Critically reviewing of manuscript: Prof. Mohamed AMAR and Prof. Peter Vandamme.

Abstract

Raw camel milk plays an important role in the daily diet of pastoralists in dry area of African countries like Morocco. The aim of the present study was to identify lactic acid bacteria (LAB) isolated from camel milk produced in Morocco, using molecular methods. Twelve samples were collected from eight different locations in Morocco. A total of one hundred twenty nine LAB isolates were obtained from nine of the analyzed samples and were subjected to a stepwise identification approach combining $(GTG)_5$-PCR fingerprinting and 16S rRNA gene sequence analysis. LAB associated with camel milk were identified as *Leuconostoc* (*Leuc.*) *mesenteroides* subsp. *mesenteroides* (41.1%), *Lactococcus* (*L.*) *lactis* subsp. *lactis* (31.8%), *Leuc. pseudomesenteroides* (6.9%), *L. lactis* subsp. *cremoris* (6.2%), *Leuc. citreum* (5.4%), *Leuc. mesenteroides* subsp. *dextranicum* (2.3%), *Enterococcus* (*E.*) *faecium* (1.6%), *E. durans* (0.8%) and *L. raffinolactis* (0.8%); in addition, four isolates (3.1%) represented four new *Streptococcus* species which were classified as *Streptococcus* (*S.*) *moroccensis* sp. nov., *S. rifensis* sp. nov., *S. cameli* sp. nov., and *S. tangierensis* sp. nov., (see chapters 5.2 and 5.3). *Leuc. mesenteroides* subsp. *mesenteroides* and *L. lactis* subsp. *lactis* were common LAB isolated from most of analyzed samples.

Keywords: raw camel milk, lactic acid bacteria, stepwise identification approach**,** **(**$(GTG)_5$-PCR fingerprinting, 16S rRNA gene sequence analysis.

Introduction

The camel (*Camelus dromedarius*) is of a significant socio-economic importance in many arid and semi-arid parts of the world and its milk constitutes an important component of human diet in these regions **(**El-Hatmi et al. 2007). It is known that camel milk is an important source of proteins for the people living in the arid lands of the world. Also, camel milk is appreciated for its medicinal properties, which are widely exploited for human health, as in several countries from the ex-Soviet Union (Kenzhebulat et al. 2000) and developing countries (Mal et al. 2006). Camel milk is considered to have anti-cancer (Magjeed, 2005), hypo-allergic (Shabo et al. 2005) and anti-diabetic properties (Agrawal et al. 2003). A high content in unsaturated fatty acids contributes to its overall dietary quality (Karray et al. 2005; Konuspayeva et al. 2008). Other components such as lactoferrin, immunoglobulins, lysozyme or vitamin C were reported to play a key role in the determination of these properties (El-Agamy et al. 1996; Konuspayeva et al. 2007). The low quantity of β-casein and the lack of β-lactoglobulin are linked to its hypo-allergic effect (El-Agamy et al. 1996; Konuspayeva et al. 2007). Raw camel milk could be an additional source of typical dairy LAB species (Jans et al. 2012). Roughly, LAB contribute to sensorial as well as textural properties of fermented dairy products and microbial safety through the production of bacteriocins and organic acids (Leroy & De Vuyst, 2004). Although some opportunistic and obligate LAB pathogens are responsible for human infections and diseases (Carr et al. 2002) many LAB are generally recognized as safe (GRAS) and approved by the qualified presumption of safety (QPS) for food production and human consumption (Leuschner et al. 2010).

Few studies have been reported on camels and camel milk (Ereifej et al. 2011). In Morocco, a small number of studies addressing the diversity and dynamics of LAB in camel milk research have been carried out using phenotypic characterization only (Benkerroum et al. 2003; Khedid et al. 2009). The purpose of the present study was to genotypically describe the LAB diversity of raw camel milk produced in Morocco. It is the first detailed investigation of the LAB microbiota of Moroccan raw camel milk, sampled from several sites belonging to different Moroccan areas, and identified using modern molecular biology techniques.

Materials and methods

Collection of milk and physico-chemical analyses

Twelve samples of fresh dromedary camel milk were collected from eight different locations in Morocco in sterile bottles and kept at 4°C until arrival at the laboratory (Fig.5.1). The pH was measured using a calibrated pH meter (8521 Hanna Instruments, Amorim, Portugal). The titratable acidity was measured by pipetting 20 ml and titrating the acidity against 0.05 mol l^{-1} NaOH to 1% phenolphthalein end point. Total Kjeldahl nitrogen and fat contents were determined according to the French standard AFNOR T90-110 (Afnor, 1975) and the Röse-Gottlieb method respectively. One sample of pasteurized milk was included in this study as a control.

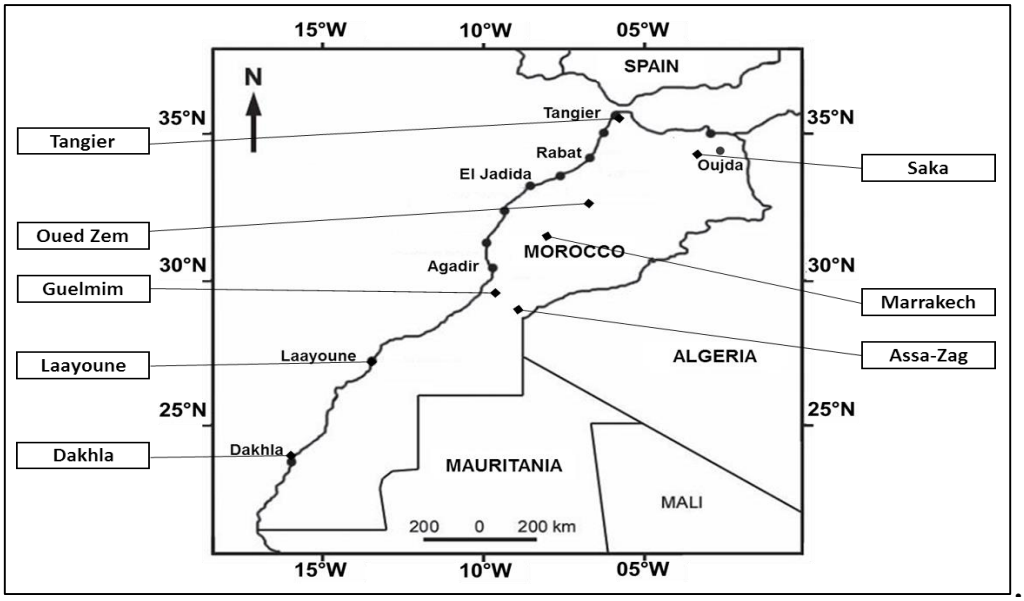


Fig 5. 1: Location of sampling sites.

Fig 5. 2: Two pictures of sampling process.

Enumeration and isolation of lactic acid bacteria

For microbial analysis, each camel mik sample (1ml) was homogenized in 9 ml of saline water (8.5 g/l) to make an initial dilution (10^{-1}). This suspension was used for making serial dilutions which were plated on Plate Count Agar (PCA) (Biokar Diagnostics) for enumeration of total bacteria, on Man-Rogosa-Sharp (MRS) agar (Biokar Diagnostics) and M17 agar (Biokar Diagnostics) for enumeration and isolation of presumptive LAB, supplemented with sorbic acid (1.4 g l^{-1}) (Panreac Quimica, Barcelona, Spain) to prevent fungal growth. The plates were incubated under aerobic conditions at 30°C for 48h. Following incubation, different colonies from MRS and M17 plates containing between 30 and 300 colonies were picked up randomly and streaked again for purification. All isolates were initially examined for Gram reaction and production of catalase and oxidase. Only Gram-positive, catalase and oxidase negative isolates (n=129) (Tab. 5.1) were considered as LAB and were stored at -80°C in MRS and M17 broths (Biokar Diagnostics) supplemented with 20% glycerol for further analysis.

Identification of lactic acid bacteria isolates

Genomic DNA extraction

Genomic DNA of bacterial isolates was extracted using the GES pitcher method (Pitcher et al. 1989). Quality and purity of the obtained DNA were checked by spectrophotometric measurements at 230, 260 and 280 nm (Thermo Scientific NanoDrop™ 1000 Spectrophotometer, Wilmington, Delaware USA) and visually by electrophoresis of 6 µL DNA mixed with 1 µL loading dye on a 1% w/v agarose (Result LE General Purpose Agarose, BIOzym group, Landgraaf, Holland) gel for 30 min at 70 V in TAE buffer (40 mM Tris-acetate, 1 mM EDTA, pH 8.0).

$(GTG)_5$-PCR genomic fingerprinting

All 129 isolates were subjected to $(GTG)_5$-PCR analysis as previously described (Gevers et al. 2001; Švec et al. 2005). The resulting fingerprints were analyzed using the BioNumerics software package version 6.1 (Applied Maths, Sint- Martens-Latem, Belgium). The similarity among the digitized profiles was calculated using the Pearson Correlation Coefficient, and an average linkage (UPGMA) dendrogram was derived from the profiles. Clusters containing profiles with a similarity higher or equal to 80% were considered to belong to the same species (Gevers et al. 2001). The profiles of the LAB isolates were compared to those in a LAB database available in CCMM/LMBM

Bacteria Collection. Based on cluster analysis, unidentified isolates and representatives of each obtained cluster were subjected to 16S rRNA gene sequencing.

16S rRNA gene sequencing

16S rRNA genes of unidentified and representative LAB isolates were amplified by PCR using the universal primers PA (5'-AGAGTTTGATCCTGCTCAG-3') and PH (5'-AAGGAGGTGATCCAGCCGCA-3') as described previously (Edwards et al. 1989). PCR was performed with 150 ng DNA, 0.5 U KAPA 2G fast DNA polymerase (KAPA Biosystems), 16 KAPA 2G Buffer, 1.5 mM $MgCl_2$, 0.2 mM each dNTP and 300 nM of each primer in a 25 ml reaction volume under the following conditions: preheating at 95°C for 2 min, then 35 cycles of denaturation at 95°C for 15 s, annealing at 60 °C for 15 s and extension at 72 °C for 2 s, followed by a final extension of 30 s at 72 °C. The amplified products were purified using EXOSAP-IT (Affymetrix) and sequence reactions were performed with the primers PDR (5'-GTATTAC-CGCGGCTGCTG-3'), PCF (5'-CTACGGGAGGCAGCAGTGGG-3') and PEF (5'-CATGGCTGTCGTCAGCTCGT-3') (Edwards et al. 1989) by using a BigDye terminator cycle sequencing kit version 3.1 (Applied Biosystems). Sequencing products were purified by using G50 gel filtration (Sephadex G50 superfine; Sigma Aldrich) and loaded onto an ABI 3130 XL capillary sequencer. Analysis of the produced electropho-regrams was done with the sequencing analysis software version 5.3.1 (Applied Biosystems). Sequence assembly was performed by Geneious software (Drummond et al. 2011). The consensus sequences were edited using MEGA software version 5.2.2 (Tamura et al. 2011) and compared with publicly available sequences using the EzTaxon-e database software (Kim et al. 2012). Isolates were regarded as tentatively identified to the species level when sequence similarity towards the corresponding type strain was at least 98.65% (Kim et al. 2014).

Nucleotide sequence accession numbers

The 16S rRNA gene sequences, determined in this study, have been deposited in the Genbank database under accession numbers KF879107-KF879188 and KF999654-KF999657.

Results

Physico-chemical composition of samples

In the present study, twelve samples of fresh dromedary milk collected from eight different locations in Morocco were used (Fig. 5.1). The physico-chemical composition of

raw milk varied slightly between all investigated samples (Tab. 5.1). For all raw milk samples analyzed, the pH values ranged from 6.1 to 6.8, with an average value of 6.4 and the acidity values ranged from 18 to 22.2°D with an average value of 20.1°D (Tab. 5.1). The protein content ranged from 29.2 to 34.5 g l^{-1} with an average of 32.5 g l^{-1} while the fat content ranged from 23.7 to 37.4 g l^{-1} with an average of 31.7 g l^{-1} (Tab. 5.1). With regards to the pasteurized milk, the pH and the acidity values were 6.2 and 21.2 respectively. Fat and protein contents were standardized to the value of 30 g l^{-1} (Tab. 5.1).

Tab. 5. 1: Occurrence of LAB in Moroccan raw camel milk.

	Raw milk from region of							P-milk
	A-Z	**Dak**	**G**	**M**	**OZ**	**S**	**T**	**L**
N° of samples (N° of isolates)	1 (18)	2 (18)	1 (16)	1 (12)	2 (30)	1 (0)	2 (32)/2 (0)	1 (3)
Food characteristic of camel	DA	DA	DA	DA	GA	GA	GA	DA
pH range	6.2	6.1 /6.5	6.1	6.5	6.2/6.4	6.5	6.5/6.5/6.8/6.8	6.2
°D	21.2	22.2/19.5	22.5	19.4	21.8/20.1	19.9	18.2/18.6/18.1/ 18.0	21. 2
Protein content (g. l^{-1})	31.4	34.1/33.2	32.2	31.1	33.5/32.1	29.2	33.5/33.8 /34.1/34.5	30.0
Fat content (g. l^{-1})	28.2	33.1/32.5	30.9	25.3	32.7/31.9	23.7	34.3/ 34.8/37.0/37.4	30.0
CFU ml^{-1} (PCA)	1.8 10^8	1.8 10^8/6.5 10^8	3 10^7	6 10^5	2.6 10^8/2.8 10^8	2.8 10^5	7.3 10^2/7.8 10^2/8.5 10^2/9 10^2	1 10^7
CFU ml^{-1} (M17)	1.5 10^8	7 10^8 / 6.5 10^6	2.4 10^8	1.6 10^5	2.1 10^8/2.7 10^8	10 10^2	4.8 10^2/5 10^2/4 10^2/3.8 10^2	3.2 10^8
CFU ml^{-1} (MRS)	1.2 10^8	6 10^8/ 4.5 10^5	2.8 10^8	6 10^2	2 10^8/2.9 10^8	1.8 10^4	2.7 10^3/2.1 10^3/1.9 10^3/1.5 10^3	7.6 10^8
Identified species (N° of isolates)								
E. durans **(1)**					1			
E. faecium **(2)**					2			
L. lactis **subsp.** ***cremoris*** **(8)**			1		4		3	
L. lactis **subsp.** ***lactis*** **(41)**	8	2	9	12			10	
L. raffinolactis **(1)**								1
Leuc. citreum **(7)**	7							
Leuc. mesenteroides **subsp.** ***dextranicum*** **(3)**							3	
Leuc. mesenteroides **subsp.** ***mesenteroides*** **(53)**	2	10	6		21		12	2
Leuc. pseudomesenteroides **(9)**	1	6			2			
S. moroccensis **(1)**							1	
S. rifensis **(1)**							1	
S. tangierensis **(1)**							1	
S. cameli **(1)**							1	
% of total LAB isolates	18	18	16	12	30		32	3

Where **D**: Dornic degree (1°D corresponds to 0.1 mg of lactic acid per litre). **DA**: Desert area.**GA**: Green area. **A-Z**: Assa-Zag, **DAK**: Dakhla, **G**: Goulemime, **M**: Marrakech, **OZ**: Oued Zem, **S**: Saka, **T**: Tangier and **L**: Laayoune. **P-milk**: pasteurized milk.

Enumeration and isolation of LAB

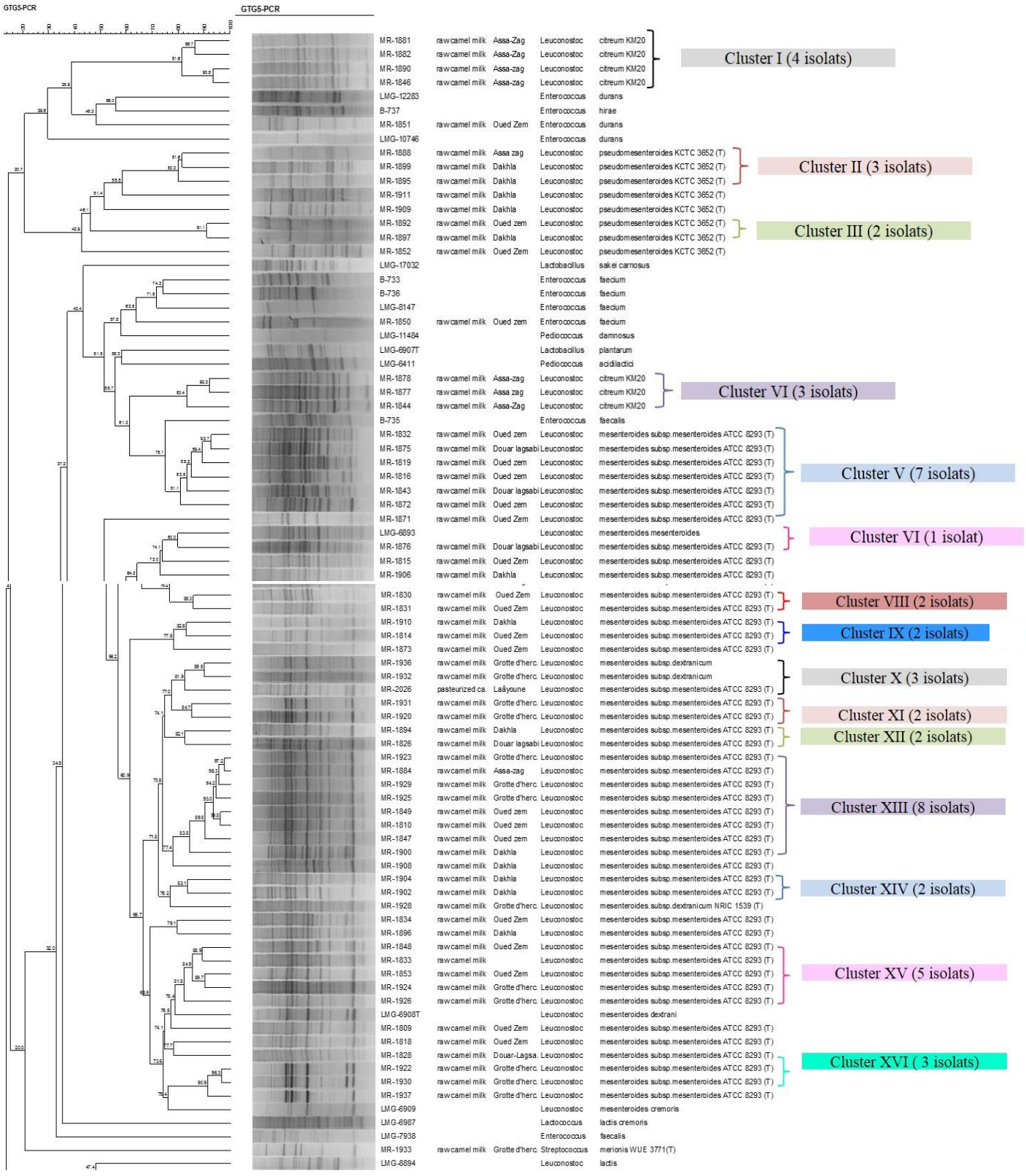
The viable counts of total bacteria on PCA ranged from 7.3 10^2 to 6.5 10^8 CFU/ml (Tab. 5.1). The highest count was observed for the sample collected from Dakhla with 6.5 10^8 CFU/ ml, whereas the lowest number, 7.3 10^2 CFU/ ml was associated with a sample collected from Tangier. LAB were present in all samples analyzed except three of

them [one sample from Saka, and two samples from Tangier because all isolated bacteria were catalase positive]. LAB counts on M17 agar ranged from 3.8 10^2 to 7 10^8, while LAB counts on MRS agar ranged from 6 10^2 to 6 10^8 CFU/ ml (Tab. 5.1). With regards to the pasteurized milk, the viable counts of total bacteria on PCA were 1 10^7 and the LAB counts on M17 agar and MRS agar were 3.2 10^8 and 7.6 10^8, respectively. One hundred and twenty nine Gram-positive and catalase negative isolates were considered to be presumptive LAB.

Identification of LAB

The $(GTG)_5$-PCR analysis of 129 isolates yielded only 108 profiles; for 21 isolates no DNA fragments were amplified. The latter isolates were therefore all examined by 16S rRNA gene sequencing. The dendrogram derived from the $(GTG)_5$-PCR fingerprints (Fig 5.2) showed 26 clusters, while 26 single isolates were unclustered. Only 7 isolates were identified when the $(GTG)_5$-PCR profiles obtained were compared with those of LAB reference strains available in the CCMM/LMBM bacteria collection. Six isolates were identified as *L. lactis subsp. lactis* (cluster 24) and one isolate was identified as *Leuc. mesenteroides.* subsp *mesenteroides* (cluster 6) (Fig 5.2). This identification was confirmed by further 16S rRNA gene sequencing.

Subsequently, 86 representatives of the 26 clusters and ungrouped isolates were subjected to 16S rRNA gene sequencing. Sequence analysis of the 16S rRNA gene of 82 isolates showed a high similarity value ($\geq$ 99.5%) with those of reference strains available in the EzTaxon-e database. We considered those isolates as tentatively identified. Moreover, four isolates, i.e. CCMM B831, CCMM B833, CCMM B832 and CCMM B834 had rather low 16S rRNA sequence similarities with their nearest phylogenetic neighbours, i.e. *S. rupicaprae* was the nearest phylogenetic neighbour species of CCMM B831 and CCMM B833 with 95.9% and 95.7%, similarities respectively, while *S. ovis* LMG 19174^T and *S. minor* LMG 21734^T were the closest established relatives for CCMM B832 and CCMMB834 with 96.86% and 97.41% similarities, respectively. These four isolates were further subjected to a polyphasic study resulting of the identification and the characterization of four novel LAB species, named *S. moroccensis* sp. nov., *S. rifensis* sp. nov., *S. tangierensis* sp. nov., and *S. cameli* sp. nov. (Chapters 5.2 and 5.3).


GTG5-PCR
GTG5-PCR
MR-1881 raw camel milk Assa-Zag Leuconostoc citreum KM20
MR-1882 raw camel milk Assa-Zag Leuconostoc citreum KM20
MR-1890 raw camel milk Assa-zag Leuconostoc citreum KM20
MR-1846 raw camel milk Assa-zag Leuconostoc citreum KM20
Cluster I (4 isolats)
LMG-12283 Enterococcus durans
B-737 Enterococcus hirae
MR-1851 raw camel milk Oued Zem Enterococcus durans
LMG-10746 Enterococcus durans
MR-1888 raw camel milk Assa zag Leuconostoc pseudomesenteroides KCTC 3652 (T)
MR-1899 raw camel milk Dakhla Leuconostoc pseudomesenteroides KCTC 3652 (T)
MR-1895 raw camel milk Dakhla Leuconostoc pseudomesenteroides KCTC 3652 (T)
Cluster II (3 isolats)
MR-1911 raw camel milk Dakhla Leuconostoc pseudomesenteroides KCTC 3652 (T)
MR-1909 raw camel milk Dakhla Leuconostoc pseudomesenteroides KCTC 3652 (T)
MR-1892 raw camel milk Oued zem Leuconostoc pseudomesenteroides KCTC 3652 (T)
MR-1897 raw camel milk Dakhla Leuconostoc pseudomesenteroides KCTC 3652 (T)
Cluster III (2 isolats)
MR-1852 raw camel milk Oued Zem Leuconostoc pseudomesenteroides KCTC 3652 (T)
LMG-17032 Lactobacillus sakei carnosus
B-733 Enterococcus faecium
B-736 Enterococcus faecium
LMG-8147 Enterococcus faecium
MR-1850 raw camel milk Oued zem Enterococcus faecium
LMG-11484 Pediococcus damnosus
LMG-6907T Lactobacillus plantarum
LMG-6411 Pediococcus acidilactici
MR-1878 raw camel milk Assa-zag Leuconostoc citreum KM20
MR-1877 raw camel milk Assa zag Leuconostoc citreum KM20
MR-1844 raw camel milk Assa-Zag Leuconostoc citreum KM20
Cluster VI (3 isolats)
B-735 Enterococcus faecalis
MR-1832 raw camel milk Oued zem Leuconostoc mesenteroides subsp.mesenteroides ATCC 8293 (T)
MR-1875 raw camel milk Douar lagsabi Leuconostoc mesenteroides subsp.mesenteroides ATCC 8293 (T)
MR-1819 raw camel milk Oued zem Leuconostoc mesenteroides subsp.mesenteroides ATCC 8293 (T)
MR-1816 raw camel milk Oued zem Leuconostoc mesenteroides subsp.mesenteroides ATCC 8293 (T)
MR-1843 raw camel milk Douar lagsabi Leuconostoc mesenteroides subsp.mesenteroides ATCC 8293 (T)
MR-1872 raw camel milk Oued zem Leuconostoc mesenteroides subsp.mesenteroides ATCC 8293 (T)
MR-1871 raw camel milk Oued Zem Leuconostoc mesenteroides subsp.mesenteroides ATCC 8293 (T)
Cluster V (7 isolats)
LMG-6893 Leuconostoc mesenteroides mesenteroides
MR-1876 raw camel milk Douar lagsabi Leuconostoc mesenteroides subsp.mesenteroides ATCC 8293 (T)
Cluster VI (1 isolat)
MR-1815 raw camel milk Oued Zem Leuconostoc mesenteroides subsp.mesenteroides ATCC 8293 (T)
MR-1906 raw camel milk Dakhla Leuconostoc mesenteroides subsp.mesenteroides ATCC 8293 (T)
MR-1830 raw camel milk Oued Zem Leuconostoc mesenteroides subsp.mesenteroides ATCC 8293 (T)
MR-1831 raw camel milk Oued Zem Leuconostoc mesenteroides subsp.mesenteroides ATCC 8293 (T)
Cluster VIII (2 isolats)
MR-1910 raw camel milk Dakhla Leuconostoc mesenteroides subsp.mesenteroides ATCC 8293 (T)
MR-1814 raw camel milk Oued Zem Leuconostoc mesenteroides subsp.mesenteroides ATCC 8293 (T)
Cluster IX (2 isolats)
MR-1873 raw camel milk Oued Zem Leuconostoc mesenteroides subsp.mesenteroides ATCC 8293 (T)
MR-1936 raw camel milk Grotte d'herc. Leuconostoc mesenteroides subsp.dextranicum
MR-1932 raw camel milk Grotte d'herc. Leuconostoc mesenteroides subsp.dextranicum
MR-2026 pasteurized ca. Laâyoune Leuconostoc mesenteroides subsp.mesenteroides ATCC 8293 (T)
Cluster X (3 isolats)
MR-1931 raw camel milk Grotte d'herc. Leuconostoc mesenteroides subsp.mesenteroides ATCC 8293 (T)
MR-1920 raw camel milk Grotte d'herc. Leuconostoc mesenteroides subsp.mesenteroides ATCC 8293 (T)
Cluster XI (2 isolats)
MR-1894 raw camel milk Dakhla Leuconostoc mesenteroides subsp.mesenteroides ATCC 8293 (T)
MR-1826 raw camel milk Douar lagsabi Leuconostoc mesenteroides subsp.mesenteroides ATCC 8293 (T)
Cluster XII (2 isolats)
MR-1923 raw camel milk Grotte d'herc. Leuconostoc mesenteroides subsp.mesenteroides ATCC 8293 (T)
MR-1884 raw camel milk Assa-zag Leuconostoc mesenteroides subsp.mesenteroides ATCC 8293 (T)
MR-1929 raw camel milk Grotte d'herc. Leuconostoc mesenteroides subsp.mesenteroides ATCC 8293 (T)
MR-1925 raw camel milk Grotte d'herc. Leuconostoc mesenteroides subsp.mesenteroides ATCC 8293 (T)
MR-1849 raw camel milk Oued zem Leuconostoc mesenteroides subsp.mesenteroides ATCC 8293 (T)
MR-1810 raw camel milk Oued zem Leuconostoc mesenteroides subsp.mesenteroides ATCC 8293 (T)
MR-1847 raw camel milk Oued zem Leuconostoc mesenteroides subsp.mesenteroides ATCC 8293 (T)
Cluster XIII (8 isolats)
MR-1900 raw camel milk Dakhla Leuconostoc mesenteroides subsp.mesenteroides ATCC 8293 (T)
MR-1908 raw camel milk Dakhla Leuconostoc mesenteroides subsp.mesenteroides ATCC 8293 (T)
MR-1904 raw camel milk Dakhla Leuconostoc mesenteroides subsp.mesenteroides ATCC 8293 (T)
MR-1902 raw camel milk Dakhla Leuconostoc mesenteroides subsp.mesenteroides ATCC 8293 (T)
Cluster XIV (2 isolats)
MR-1928 raw camel milk Grotte d'herc. Leuconostoc mesenteroides subsp.dextranicum NRIC 1539 (T)
MR-1834 raw camel milk Oued Zem Leuconostoc mesenteroides subsp.mesenteroides ATCC 8293 (T)
MR-1896 raw camel milk Dakhla Leuconostoc mesenteroides subsp.mesenteroides ATCC 8293 (T)
MR-1848 raw camel milk Oued Zem Leuconostoc mesenteroides subsp.mesenteroides ATCC 8293 (T)
MR-1833 raw camel milk Leuconostoc mesenteroides subsp.mesenteroides ATCC 8293 (T)
MR-1853 raw camel milk Oued Zem Leuconostoc mesenteroides subsp.mesenteroides ATCC 8293 (T)
MR-1924 raw camel milk Grotte d'herc. Leuconostoc mesenteroides subsp.mesenteroides ATCC 8293 (T)
MR-1926 raw camel milk Grotte d'herc. Leuconostoc mesenteroides subsp.mesenteroides ATCC 8293 (T)
Cluster XV (5 isolats)
LMG-6908T Leuconostoc mesenteroides dextrani
MR-1809 raw camel milk Oued Zem Leuconostoc mesenteroides subsp.mesenteroides ATCC 8293 (T)
MR-1818 raw camel milk Oued Zem Leuconostoc mesenteroides subsp.mesenteroides ATCC 8293 (T)
MR-1828 raw camel milk Douar-Lagsa. Leuconostoc mesenteroides subsp.mesenteroides ATCC 8293 (T)
MR-1922 raw camel milk Grotte d'herc. Leuconostoc mesenteroides subsp.mesenteroides ATCC 8293 (T)
MR-1930 raw camel milk Grotte d'herc. Leuconostoc mesenteroides subsp.mesenteroides ATCC 8293 (T)
MR-1937 raw camel milk Grotte d'herc. Leuconostoc mesenteroides subsp.mesenteroides ATCC 8293 (T)
Cluster XVI (3 isolats)
LMG-6909 Leuconostoc mesenteroides cremoris
LMG-6987 Lactococcus lactis cremoris
LMG-7938 Enterococcus faecalis
MR-1933 raw camel milk Grotte d'herc. Streptococcus merionis WUE 3771(T)
LMG-8894 Leuconostoc lactis

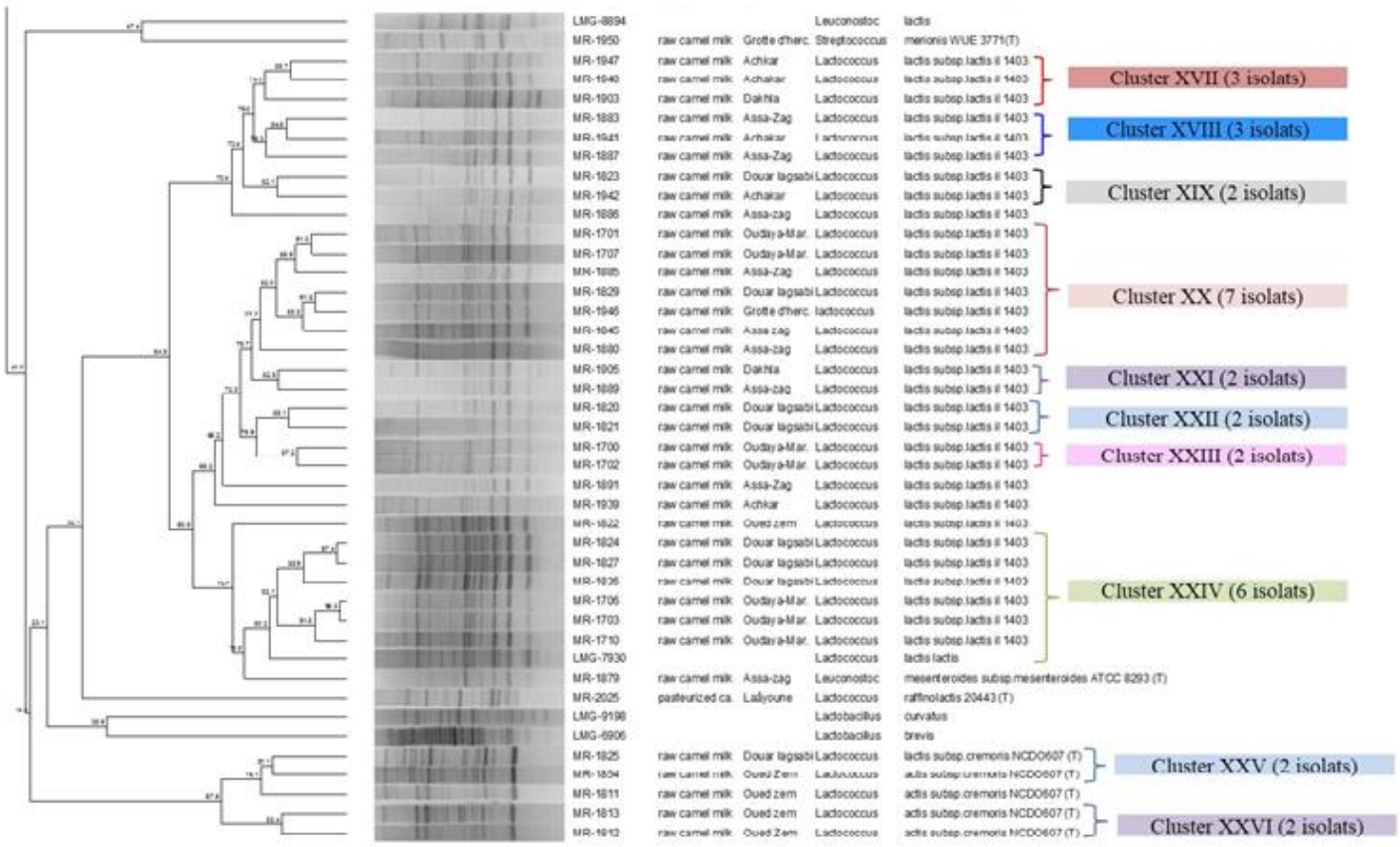


Fig 5. 3: Cluster analysis of $(GTG)_5$-PCR fingerprints and 16S gene sequencing of LAB isolats. The dendrogram was generated by the UPGMA method and Pearson correlation coefficient. MR numbers are referring to isolates references and LMG numbers are referring to reference strains used for identification**.**

The combination of the $(GTG)_5$-PCR and 16S rRNA gene sequencing allowed to tentatively identify all 125 isolates to the species level. The obtained results revealed the presence of seven species and four subspecies of LAB: *Leuc. mesenteroides* subsp. *mesenteroides* (41.1%), *L. lactis* subsp. *lactis* (31.8%), *Leuc. pseudomesenteroides* (6.9%), *L. lactis* subsp. *cremoris* (6.2 %), *Leuc. citreum* (5.4%), *Leuc. mesenteroides* subsp. *dextranicum* (2.3%), *E. faecium* (1.6%), *E. durans* (0.8%) and *L. raffinolactis* (0.8%) (Tab. 5.1).

Leuc. mesenteroides subsp. *mesenteroides* and *L. lactis* subsp. *lactis* were present in all camel milk samples analysed, except for samples from Marrakech and Oued Zem. The sample of Marrakech was dominated by a single species, *L. lactis* subsp. *lactis*, while those of Oued Zem and Dakhla were dominated by the species *Leuc. mesenteroides* subsp. *mesenteroides.* The samples of Assa-zag, Goulemime, and Tangier had no dominant species (Tab. 5.1). Both *E. durans* and *E. faecium* species were isolated from samples of Oued Zem, while *Leuc. citreum* was only recovered from one sample of

Assa-Zag. *L. raffinolactis* was isolated once from the pasteurized milk collected from Laayoune. Finally, the novel *Streptococcus species*, named *S. moroccensis, S. rifensis, S. cameli* and *S. tangierensis* were each recovered once from one sample of Tangier (Tab. 5.1).

Discussion

Physico-chemical analysis

Samples of camel milk collected from different areas of Morocco were analyzed to evaluate their physico-chemical composition and study the occurrence of lactic acid bacteria. The average pH value was 6.4, which is in agreement with the results of Tuteja et al. (2003) and Ismaili et al. (2015). The titratable acidity of the samples ranged from 18 to 22.5°D with an average value of 20.1°D. In a recent study of microbial quality of raw camel milk produced in south of Morocco, Ismaili et al. (2015) reported a mean value of titratable acidity of 18.5°Dornic, which correspond to the results obtained in the present study. The protein content of the camel milk samples ranged from 29.2 to 34.5 g l^{-1} with an average of 32.5 g l^{-1} while the fat content ranged from 23.7 to 37.4 g l^{-1} with an average of 31.7 g l^{-1}. These findings are similar to those reported by Konuspayeva et al. (2009) and Alwan et al. (2014).

Enumeration of LAB

LAB were present in 9 out of 12 samples analyzed. Counts on M17 and MRS agar ranged from 3.8 10^2 to 7 10^8 and 6 10^2 to 7.6 10^8 CFU/ml respectively, while counts of total bacteria ranged from 7.3 10^2 to 6.5 10^8 CFU/ml. LAB and total bacteria counts found in camel milk samples are similar to those reported by Benkerroum et al. (2003), Ismaili et al. (2015) and Khedid et al. (2009).

Distribution of LAB in camel milk

In this study, 129 isolates were obtained from nine camel milk samples and were identified using a stepwise approach based on $(GTG)_5$-PCR and 16S rRNA gene sequencing. The dominant LAB species recovered from raw camel milk samples were *Leuc. mesenteroides* subsp. *mesenteroides* and *L. lactis* subsp. *lactis* (Tab. 5.1, Fig 5.2). These findings are similar to those reported by Fatma and Benmechernene (2013) and Khedid et al. (2009). Several studies on the characterization of LAB isolated from raw camel milk also reported the presence of *Leuc. pseudomesenteroides* (Jans et al. 2012), *E.*

faecium and *E. durans* (Akhmetsadykova et al. 2015; Khay et al. 2011), *Leuc. mesenteroides* subsp. *dextranicum*, *L. raffinolactis*, and *L. lactis* subsp. *cremoris* (Khedid et al. 2009). As far as we know, this is the first report of *Leuc. citreum* isolated from camel milk. However, the isolation of *Leuc. citreum* was already reported in sheep and human milk (Quigley et al. 2013). In addition, the preponderance of cocci in lactic acid microbiota of camel milk has been already reported before (Akhmetsadykova et al. 2014; Benkerroum et al. 2003; Grillet, 2006; Kacem et al. 2002; Khedid et al. 2009; Rahman et al. 2010).

Occurrence of lactococci, leuconostocs, enterococci and streptococci (Tab. 5.1)

Leuc. mesenteroides subsp. *mesenteroides* was found in 75% of the raw camel milk samples analyzed and represent 41.1% of total LAB isolated. *Leuc. pseudomesenteroides* was recovered from three samples (Tab. 5.1). *Leuc. citreum* and *Leuc. mesenteroides* subsp. *dextranicum* were found in samples of Assa-Zag and Tangier, respectively. Repeat analyses of additional samples of these regions should reveal if this microbiota is region specific. Previous research has shown that LAB are the main group of bacteria that are considered technologically important in the fermentation and ripening of fermented dairy products worldwide (Akweya et al. 2012; Fernández et al. 2015; Garabal, 2007; Kongo, 2013; Wedajo, 2015; Widyastuti et al. 2014). Wouters et al. (2012) and Nguyen, (2012) reported that the predominance of LAB in milk and dairy products could restrict the growth of undesirable bacteria, thereby contributing to the safety and the shelf life of the product. Species belonging to the *Leuconostoc* genus are frequently used as starter cultures for butter-milk, butter, cream, and cheese (Chang and Chang, 2010; Laguerre, 2012; Leroy and De Vuyst, 2004; Tamime and Marshall, 1997). In addition, it has been shown that some *Leuconostoc* species have anti-fungal and antibacterial activities (Baek el al. 2012; Fatma and Benmechernene, 2013).

L. lactis subsp. *lactis* represented 31.8% of the LAB isolates and was the main *Lactococcus* species isolated. Wouters et al. (2012) reported that *Lactococcus* species could inhibit the growth of other endogenous bacteria. Also, many bacteriocin producing *Lactococcus* strains have been used successfully as starter cultures for cheese, sour cream and fermented milk production to improve the safety and quality of the product (Cogan and Accolas, 1996; Delves-Broughton et al. 1996; Ryan et al. 1996; Tamime and Marshall, 1997). Some authors assume that the use of these strains in starter cultures may result in a loss of vital survival functions outside a dairy habitat (Corroler et al. 1998).

In the present study, we obtained only one isolate of *L. raffinolactis* in the pasteurized milk. This species is not used in the dairy industry (Sharpe, 1979; Huggins, 1984), but is able to ferment α-galactosides, such as melibiose and raffinose that are not utilized by *L. lactis* strains (Boucher et al. 2003; Khedid et al. 2009). Both *E. durans* and *E. faecium* were isolated from raw camel milk at a similar frequency (Tab. 5.1) (Zamfir et al. 2006). The presence of enterococci in dairy products has long been considered an indication of inadequate sanitary conditions during milk production and processing and these species may carry virulence factors and antibiotic-resistance genes (Franz et al. 1999). However, enterococci play an important role in the aroma, flavour development of Mediterranean cheeses (Giraffa, 2003) and their technological and probiotic benefits are widely recognized (Giraffa, 1995).

Finally, the LAB diversity of camel milk includes several novel members of the genus *Streptococcus* as well. In general, some streptococci have been found to be responsible of mastitis in cows and camels (Bekele and Molla, 2001; Ebrahimi et al. 2008), while other streptococci have been implemented in fermented milks, in combination with other lactic acid bacteria and bifidobacteria, attributing thereby sensory and nutritional qualities (Collado and Hernández, 2007). However, the role of the four novel *Streptococcus* species (Tab. 5.1, chapters 5.2 and 5.3) is currently not known and additional studies eluciding their effect are needed. Lactobacilli were absent from all samples analyzed. As already reported by Benkerroum et al. (2003), this may suggest that camel milk is not an adequate medium for their growth due either to their sensitivity to the natural inhibitors of the milk or to the lack of essential growth factors. Zamfir et al. (2006) reported that the variability of the LAB species diversity isolated from the samples tested reflects their traditional production. LAB biodiversity of raw camel milk samples is limited to leuconostocs, lactococci, enterococci and streptococci with a dominating microbiota consisting of leuconostocs and lactococci. Consequently, according to the classification of Robinson and Tamime (1990), Moroccan camel milk could be assigned to the group of mesophilic milks.

Conclusions

This study provides, to the best of our knowledge, the first detailed investigation of the LAB microbiota of Moroccan raw camel milk, sampled from a large geographical area, using modern molecular biology techniques. The LAB diversity of Moroccan raw camel milk appears limited to lactococci, leuconostocs, enterococci and streptococci.

The latter represented four new *Streptococcus* species (chapters 5.2 and 5.3). Lactobacilli were not isolated. Isolates from the present Moroccan raw camel milk study could be a source of interesting LAB (Jans et al. 2012; Khedid et al. 2009) with specific functional properties, which might be used for the development of new starter cultures and innovative food products.

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CHAPTER 5: CLASSIFICATION AND IDENTIFICATION OF CAMEL MILK LAB

5.2 Description of two novel *streptococcus* species: *Streptococcus moroccensis* sp. nov. and *Streptococcus rifensis* sp. nov., isolated from raw camel milk in Morocco.

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***Streptococcus moroccensis* sp. nov. and *Streptococcus rifensis* sp. nov., isolated from raw camel milk in Morocco.**

International Journal of Systematic and Evolutionary Microbiology (2014), 64, 2480-2485.

PREAMBLE

In the frame of the camel milk LAB diversity study described in chapter 5.1 several LAB taxa were identified as putative novel *Streptococcus* species. In the present study two of these novel LAB taxa within the genus *Streptococcus* were fully characterised using polyphasic taxonomic studies. The $(GTG)_5$-PCR and 16S rRNA gene sequence data obtained were complemented with sequence analysis of the protein encoding genes *pheS*, *rpoA* and *atpA*, DNA-DNA hybridization experiments, determination of the DNA base composition (% G+C), MALDI-TOF MS analyses and a detailed phenotypic characterization. Together this allowed the formal description of two novel *Streptococcus* species for which the names *Streptococcus moroccensis* and *Streptococcus rifensis*, were proposed.

OUTLINE CONTRIBUTIONS

Experimental design: Prof. Peter VANDAMME and Prof. Mohamed AMAR.
Experimental work: Zaina KADRI, Margo CNOCKAERT.
Data analysis: KADRI Zaina, Prof. Peter VANDAMME and Prof. Mohamed AMAR.
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Abstract

Two catalase and oxidase negative *Streptococcus*-like strains, LMG 27682^T and LMG 27684^T, were isolated from raw camel milk in Morocco. Comparative 16S rRNA gene sequencing assigned these bacteria to the genus *Streptococcus* with *Streptococcus rupicaprae* 2777-2-07^T as their closest phylogenetic neighbor (95.9% and 95.7% similarity, respectively). The level of 16S rRNA gene sequence similarity between the two strains was 96.7%. Although strains LMG 27682^T and LMG 27684^T shared a DNA-DNA hybridization level that corresponded to the threshold level for species delineation (68%), both strains could be distinguished by multiple biochemical tests, sequence analysis of the phenylalanyl-tRNA synthase (*pheS*), RNA polymerase (*rpoA*) and ATP synthase (*atpA*) genes and by their MALDI-TOF MS profiles. On the basis of these considerable phenotypic and genotypic differences, we propose to classify both strains as novel *Streptococcus* species for which the names *Streptococcus moroccensis* sp. nov. (with LMG 27682^T = CCMM B831^T as the type strain) and *Streptococcus rifensis* sp. nov. (with LMG 27684^T = CCMM B833^T as the type strain) are proposed.

Genbank accession numbers

The GenBank accession numbers for the 16S rRNA gene sequences of strains LMG 27682^T and LMG 27684^T are KF999654 and KF999655. The EMBL accession numbers for the *pheS*, *rpoA* and *atpA* gene sequences of strains LMG 27682^T and LMG 27684^T are HG799789; HG799790; HG799788 and HG799792; HG799793; HG799791, respectively.

The genus *Streptococcus* embraces a broad range of Gram-positive, facultatively anaerobic, catalase negative, chain-forming and coccus-shaped organisms with a low G+C content (Hardie & Whiley, 1997). It has undergone significant expansion due to improved phenotypic and molecular identification methods, and includes 80 recognized species and 16 subspecies at the time of writing (Parte, 2014; http://www.bacterio.cict.fr/s/streptococcus.html). Animal-associated streptococci have been isolated from a wide range of environments (Kilian, 1998) and some of them have been associated with diseases such as endometritis, respiratory infections, endocarditis, meningitis, arthritis and mastitis (Chanter, 1997; Kohler, 2007).

In the course of a study of the microbiota of raw camel milk in Morocco, two catalase and oxidase negative *Streptococcus*-like strains (LMG 27682^T and LMG 27684^T) were isolated on M17 agar (Biokar Diagnostics). Total DNA was extracted as described by Pitcher et al. (1989). Rep-PCR fingerprints were determined as described previously (Gevers et al. 2001). PCR mixtures, cycle conditions and treatment of the rep-PCR profiles were the same as reported by Ouadghiri et al. (2009). Both isolates had clearly distinct profiles (data not shown) and thus represented different strains which remained unidentified after comparison with our *in house* database (Ouadghiri et al. 2009).

To assess the phylogenetic position of both strains, their 16S rRNA genes were amplified by PCR using the universal primers PA (5' AGA GTT TGA TCC TGC TC AG 3') and PH (5' AAG GAG GTG ATC CAG CCG CA 3') described previously (Edwards et al. 1989). PCR was performed on 150 ng DNA, with 0.5 U KAPA2G fast DNA polymerase (KAPA Biosystems), 1x KAPA 2G Buffer, 1.5 mM MgCl2, 0.2 mM of each dNTPs, and 300 nM of each primer in a 25µl reaction volume under the following conditions: preheating at 95°C for 2 min, then 35 cycles of denaturation at 95 °C for 15 s, annealing at 60 °C for 15 s and extension at 72 °C for 2 s, followed by final extension of 30 s at 72 °C. The amplified products were purified using EXOSAP-IT (Affymetrix) and sequence reaction was performed with the primers PDR (5'GTATTACCGCGGCTGCTG 3'), PCF (5'CTACGGGAGGCAGCAGTGGG 3') and PEF (5'CATGGCTGTCGTCAGCTCGT 3') (Edwards et al. 1989) by using a Big Dye terminator cycle sequencing kit version 3.1 (Applied Biosystems). Sequencing products were purified by using G50 gel filtration [sephadex G50 superfine (Sigma Aldrich)] and loaded onto an ABI3130XL capillary sequencer. Analysis of the produced electrophoregrams was done with the sequencing analysis software version 5.3.1 (Applied Biosystems). Sequence assembly was performed by Geneious software (Drummond et al.

2011). The consensus sequences were edited using MEGA (Molecular Evolutionary Genetics Analysis) version 5.2.2 software (Tamura et al. 2011) and compared with publicly available sequences. Analysis of both sequences using the EzTaxon-e database software (Kim et al. 2012) showed that strain LMG 27682^T and LMG 27684^T belonged to the genus *Streptococcus* with the *Streptococcus rupicaprae* type strain as nearest phylogenetic neighbor (95.9% and 95.7% similarity, respectively), although both strains shared similar similarity levels towards several additional streptococci. The similarity level between strains LMG 27682^T and LMG 27684^T was 96.7%. Phylogenetic analyses were performed using MEGA 5.2.2 software (Tamura et al. 2011). Pairwise alignment homologies were calculated and a phylogenetic tree showing the phylogenetic position of both strains was constructed by using the maximum-likelihood method (Fig 5.3). In this phylogenetic tree, both strains consistently clustered with the *Streptococcus merionis* type strain.

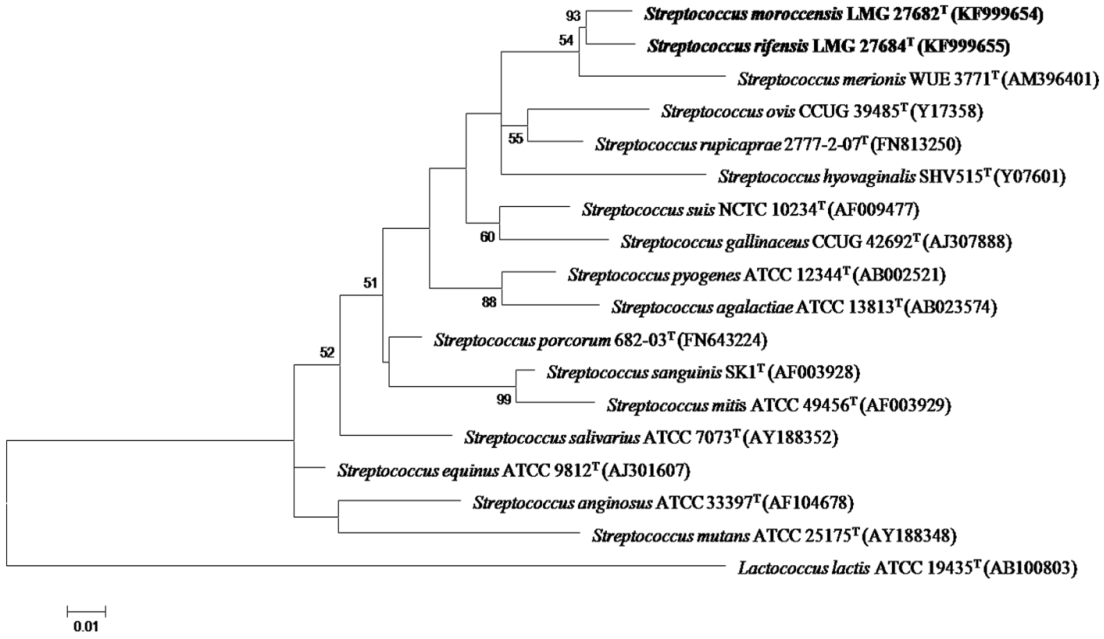

Fig 5. 4: Maximum-likelihood phylogenetic tree based on 16S rRNA gene sequences of strains LMG 27682^T and LMG 27684^T, their nearest phylogenetic neighbors (i.e. those *Streptococcus* species with type strains that share >95.5% of their 16S rRNA sequences with strains LMG 27682^T and LMG 27684^T [as determined using the EzTaxon-e database]), and some representatives of the main phylogenetic clades within this genus. *Lactococcus lactis* ATCC 19435^T was used as an outgroup. Bootstrap values based on 1000 replications are indicated at the nodes; values of less than 50% are not shown. The scale bar indicates the number of substitutions per site.

DNA–DNA hybridizations were subsequently performed between strains LMG 27682^T and LMG 27684^T. DNA was extracted from 0.75-1.25 g cell mass using the protocol described by Gevers et al. (2001). The microplate method was used as described by

Ezaki et al. (1989) as modified by Goris et al. (1998) using a HTS7000 Bio Assay Reader (Perkin Elmer) for fluorescence measurements. Biotinylated DNA was hybridized with unlabelled ssDNA which was bound non-covalently to microplate wells. Hybridizations were performed at 36 °C in hybridization mixture (2x SSC, 5 x Denhardt's solution, 2.5% dextran sulphate, 50% formamide, 100 mg denaturated salmon sperm DNA ml^{-1}, 1250 ng biotinylated probe DNA ml^{-1}). The average level of DNA-DNA hybridization between strains LMG 27682^T and LMG 27684^T was 68% (the reciprocal values were 67% and 69%) which is the threshold level for species delineation (Wayne et al. 1987).

The DNA base composition of strains LMG 27682^T and LMG 27684^T and *Streptococcus merionis* LMG 27712^T was determined by HPLC as described by (Mesbah et al., 1989) using a Waters Breeze HPLC system and XBridge Shield RP18 column. The solvent used was 0.02M $NH_4H_2PO_4$ (pH 4.0) 1.5% (v/v) acetonitrile. Non-methylated lambda phage (Sigma, Taufkirchen, Germany) and *Escherichia coli* DNA were used as calibration reference and control, respectively. The G+C content of strains LMG 27682^T, LMG 27684^T and LMG 27712^T was 41.9, 42.0, and 41.9 mol%, respectively.
Sequence analysis of protein encoding genes is increasingly used in taxonomic studies of various lactic acid bacteria including streptococci (e.g. Huch et al. 2013). In general these studies confirm that housekeeping genes, especially when applied in a multilocus approach, have a taxonomic resolution superior to that of 16S rRNA gene sequence analysis. In order to examine the phylogenetic relationships of strains LMG 27682^T and LMG 27684^T in more detail we sequenced the *pheS*, *rpoA* and *atpA* genes of both strains. DNA was extracted using an alkaline lysis approach (Niemann et al. 1997) and partial sequences of *pheS* (395-455 bp), *rpoA* (413-793bp), and *atpA* (862-963 bp) genes were amplified using the primer combinations pheS-21-F/pheS-23-R, rpoA-21-F/rpoA-23-R and atpA-20-F/atpA-26-R, respectively. Amplification conditions and sequencing reactions were as described by (Naser et al. 2005a, b). SeaView version 4 was used to concatenate the *pheS*, *rpoA,* and *atpA* gene sequences of both strains (Gouy et al. 2010). The software package MEGA (Molecular Evolutionary Genetics Analysis) version 5.2.2 (Tamura et al. 2011) was used to align the translated concatenated gene sequences and to analyse the nucleotide sequences. The pairwise sequence similarity values of the *pheS*, *rpoA,* and *atpA* gene sequences of strains LMG 27682^T and LMG 27684^T were 94.3%, 91.7% and 95.2%, respectively.

We subsequently evaluated the use of Matrix-Assisted Laser Desorption/Ionization Time-Of-Flight Mass Spectrometry (MALDI-TOF MS) for the differentiation of strains LMG 27682^T and LMG 27684^T. Bacterial cells were grown on Columbia agar base (BBL) supplemented with 5% sheep blood for 24h at 37°C under microaerobic conditions. Cell extraction for MALDI-TOF MS analysis was performed as described by Wieme et al. (2012). Bacterial fingerprints were acquired using the 4800 Plus MALDI TOF/TOF™ Analyzer (Applied Biosystems, Framingham, MA, USA) in linear positive ion mode as described before (Wieme et al. 2012). A mass range of 2000 to 20,000 Da was used for the analysis. The MALDI-TOF MS profiles of strains LMG 27682^T and LMG 27684^T were highly similar but could be distinguished by peptides with *m/z* values of about 6100 and 6350 (Fig 5.4).

Finally, strains LMG 27682^T and LMG 27684^T were analysed biochemically using the API 20 Strep and API 50 CHL microtest systems (bioMérieux) according to the manufacturer's instructions. In addition, the same tests were performed for *Streptococcus merionis* LMG 27712^T, *Streptococcus gallinaceus* LMG 21849^T, *Streptococcus ovis* LMG 19174^T to verify the reproducibility of published data. The data obtained from the type strain of *S. merionis* (WUE 3771^T) supported the results from (tappe et al. 2009). Only the result of acid production from amygdalin was different. As well as the type strain of *S. gallinaceus* (CCUG 42692^T), exhibited almost identical biochemical characteristics reported by collins et al. (2002), except for the acid production from amygdalin and melezitose. Howewer, β-Galactosidase activity was different in comparison with our parallel study for the type srain of *S. ovis* (CCUG 39485$^{T)}$ while the α-Galactosidase activity has been reported as variable (Collins et al. 2001). Also, acid production from D-tagatose and glycogen was different from published data (Collins et al. 2001). The results are listed below and demonstrate that each of these strains is readily distinguished. The proposed novel species could also be distinguished from their nearest phylogenetic neighbors by several phenotypic characteristics (Tab. 5.2). Moreover, differential biochemical tests, including eight characteristics that distinguish between strains LMG 27682^T and LMG 27684^T are shown in Tab. 5.2 which provided further proof that both isolates belonged to the different phylotypes.

In conclusion, results of the present study demonstrated that strains LMG 27682^T and LMG 27684^T share 96.7% of their 16S rRNA gene sequence and a rather high DNA-DNA hybridization level (68%) that corresponds to the threshold level for species delineation, but also that they can be distinguished by multiple biochemical tests (Tab.

5.2), sequence analysis of 16S rRNA (Fig 5.3) and protein encoding genes and by their MALDI-TOF MS profiles (Fig 5.4). Given these ample possibilities for distinguishing the taxa represented by these strains we propose to classify them as two distinct species within the genus *Streptococcus,* for which we propose the names *Streptococcus moroccensis* sp. nov., and *Streptococcus rifensis* sp. nov.

Description of *Streptococcus moroccensis* sp. nov.

Streptococcus moroccensis (mo.roc.cen'sis. N.L. adj. *moroccensis* pertaining to Morocco, from where the type strain was isolated).

Cells stain Gram-positive, non-spore-forming cocci and are arranged predominantly in small groups. No catalase or oxidase activity. Colonies on Brain heart infusion agar supplemented with 5% horse blood agar are about 0.5-0.8 mm in diameter, yellow-golden, round with raised margin, smooth, convex and non-hemolytic at 37°C. Cells grow well under aerobic and microaerobic conditions at 25, 30, 37 and 45°C but not at 10°C. In API 50 CHL tests and API 20 STREP, the strain was positive for leucine amino peptidase, α-galactosidase and esculin. Acid is produced from galactose, glucose, fructose, L-arabinose, melezitose, L-rhamnose, D-xylose, D-mannose, N-Acetyl-Glucosamine, salicin, cellobiose, arbutin, inulin, amygdalin, lactose, sucrose, D-trehalose and gentiobiose. The strain showed negative activities for hippurate, pyrrolidonyl arylamidase, β-glucuronidase, β-galactosidase, arginine dihydrolase, alkaline phosphatase. Also, acetoin and acid production from glycerol, erythritol, D-arabinose, D-ribose, L-xylose, adonitol, β-methyl-D-xyloside, D-mannitol, sorbitol, D- maltose, sorbose, dulcitol, inositol, α-methyl-D-mannoside, α-methyl-D-glucoside, melibiose, raffinose, starch, glycogen, xylitol, D-turanose, D-lyxose, D-tagatose, DL-fucose, DL-arabitol, gluconate and 2- and 5-ketogluconate were negative. DNA G+C content is 41.9 mol%. The type strain is LMG 27682^{T} (= CCMM B831^{T}), and was isolated from raw camel milk in the north of Morocco in 2012.

Description of *Streptococcus rifensis* sp. nov.

Streptococcus rifensis (rif. en'sis N.L. adj. *rifensis* of Rif, named after the northern region of Morocco, from where the type strain was isolated).

Cells stain Gram-positive, non-spore-forming cocci and are arranged predominantly in small groups. No catalase or oxidase activity. Colonies on brain heart infusion agar supplemented with 5% horse blood agar are about 0.5-0.8 mm in diameter, yellow-golden, round with raised margin, smooth, convex and non-hemolytic at 37°C. Cells grow well under aerobic and anaerobic conditions at 25, 30, 37°C but not at 10 and 45°C. In API 50 CHL tests and API 20 STREP, the strain was positive for leucine amino peptidase, β-glucuronidase and esculin. Acid is produced from galactose, glucose, fructose, L-arabinose, melezitose, D-mannitol, sorbitol, D-maltose, N-Acetyl-Glucosamine, salicin, cellobiose, arbutin, inulin, amygdalin, lactose, sucrose, D-trehalose and gentiobiose. The strain showed negative activities for hippurate, pyrrolidonyl arylamidase, α-galactosidase, β-galactosidase, arginine dihydrolase, alkaline phosphatase. Also, acetoin and acid production from glycerol, erythritol, D-arabinose, D-ribose, L-rhamnose, D-xylose, D-mannose, L-xylose, adonitol, β-methyl-D-xyloside, sorbose, dulcitol, inositol, α-methyl-D-mannoside, α-methyl-D-glucoside, melibiose, raffinose, starch, glycogen, xylitol, D-turanose, D-lyxose, D-tagatose, DL-fucose, DL-arabitol, gluconate and 2- and 5-ketogluconate were negative. DNA G+C content is 42 mol%. The type strain is LMG 27684^T (= CCMM B833^T), and was isolated from raw camel milk in the north of Morocco in 2012.

Acknowledgments

This work was supported by the Belgian General Administration for International Cooperation (project: FRAB / 2005) and the Centre National pour la Recherche Scientifique et Technique, Rabat, Morocco (Programme d'urgence 2009 - 2012).

We thank Prof. El Fahime Mostafa and Mr. Melloul Marouane for their help to sequence and analyze 16S rRNA gene.

We thank Mrs. Isabel Snauwaert and Mrs. Charlotte Peeters for helping us constructing phylogenetic trees.

Tab. 5. 2: Characteristics that differentiate strains LMG 21782^T and LMG 21784^T from closely related streptococcal species.

Characteristic	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**
Production of acid from :										
L-Arabinose	+	+	-/-	-	+	-/-	-	-/-	-	**ND**
D-Xylose	+	-	-/-	-	+	-/-	-	**-/ND**	**ND**	**ND**
L-Rhamnose	+	-	-/-	-	+	**-/ND**	-	**-/ND**	**ND**	-
Amygdalin	+	+	+/-	+	+	**-/ND**	-	**-/ND**	**ND**	-
D-Maltose	-	+	+/+	+	+	+/+	+	+/+	+	+
Melezitose	+	+	**-/ND**	-	-	-/-	-	-/-	-	-
Raffinose	-	-	+/+	+	+	+/+	-	+/+	**V**	-
D-Mannitol	-	+	-/-	-	-	+/+	+	+/+	-	-
Sorbitol	-	+	-/-	-	-	-/-	+	+/+	-	-
D-Mannose	+	-	**+/ND**	+	+	**+/ND**	+	**+/ND**	**ND**	+
Starch	-	-	+/+	-	+	**ND /ND**	-	**-/ND**	+	-
α-Methyl-D-Glucoside	-	-	**-/ND**	-	+	**-/ND**	-	**-/ND**	**ND**	**ND**
Inulin	+	+	+/+	-	+	**-/ND**	-	**-/ND**	+	+
Glycogen	-	-	-/-	-	+	-/-	-	-/+	+	**ND**
Melibiose	-	-	-/-	-	+	-/+	-	-/-	**V**	+
D-Ribose	-	-	-/-	-	-	+/+	V	-/-	-	**ND**
Gentiobiose	+	+	**+/ND**	+	+	**-/ND**	-	**-/ND**	**ND**	**ND**
D-Tagatose	-	-	**-/ND**	-	-	-/-	-	-/+	**ND**	**ND**
Arbutin	+	+	**+/ND**	+	+	**-/ND**	**ND**	**-/ND**	**ND**	-
Salicin	+	+	**+/ND**	+	+	**+/ND**	+	**+/ND**	+	-
Production of :										
α-Galactosidase	+	-	-/-	-	+	**ND/+**	-	**+/V**	+	-
β-Glucuronidase	-	+	+/+	-	+	**ND/-**	+	-/-	+	-
β-Galactosidase	-	-	+/+	-	+	**ND/+**	+	+/-	**V**	+
Acetoin	-	-	-/-	-	-	**ND/-**	+	-/-	-	-
Alkaline phosphatase	-	-	-/-	-	-	**ND/-**	+	-/-	-	-
Hydrolysis of :										
Hippurate	-	-	+/+	-	-	**ND/-**	+	-/-	-	-
Arginine Dihydrolase	-	-	+/+	-	+	**ND/+**	-	**+/V**	+	+
N-Acetyl-Glucosamine	+	+	**+/ND**	+	+	**+/ND**	+	**+/ND**	**ND**	+

Taxa 1, *Streptococcus moroccensis* LMG 21782^T; 2, *Streptococcus rifensis* LMG 21784^T; 3, *Streptococcus merionis* WUE 3771^T; 4, *Streptococcus porcorum* 682-03^T; 5, *Streptococcus rupicaprae* 2777-2-07^T ; 6, *Streptococcus gallinaceus* CCUG 42692^T; 7, *Streptococcus hyovaginalis* SHV515^T; 8, *Streptococcus ovis* CCUG 39485^T ; 9, *Streptococcus suis* NCTC 10234^T ; 10, *Streptococcus sanguinis* SK1^T. Data for taxa 3, taxa 4, taxa 5, taxa 6, taxa 7, taxa 8, taxa 9 and taxa 10 are from (Tappe et al. 2009),

(Vela et al. 2011b), (Vela et al. 2011a), (Collins et al. 2002), (Devriese et al. 1997), (Collins et al. 2001), (Kilpper-Balz et al. 1987) and (Kilian et al. 1989), respectively. +, positive reaction; -, negative reaction; ND: not determined; V: variable.

Supplementary information:

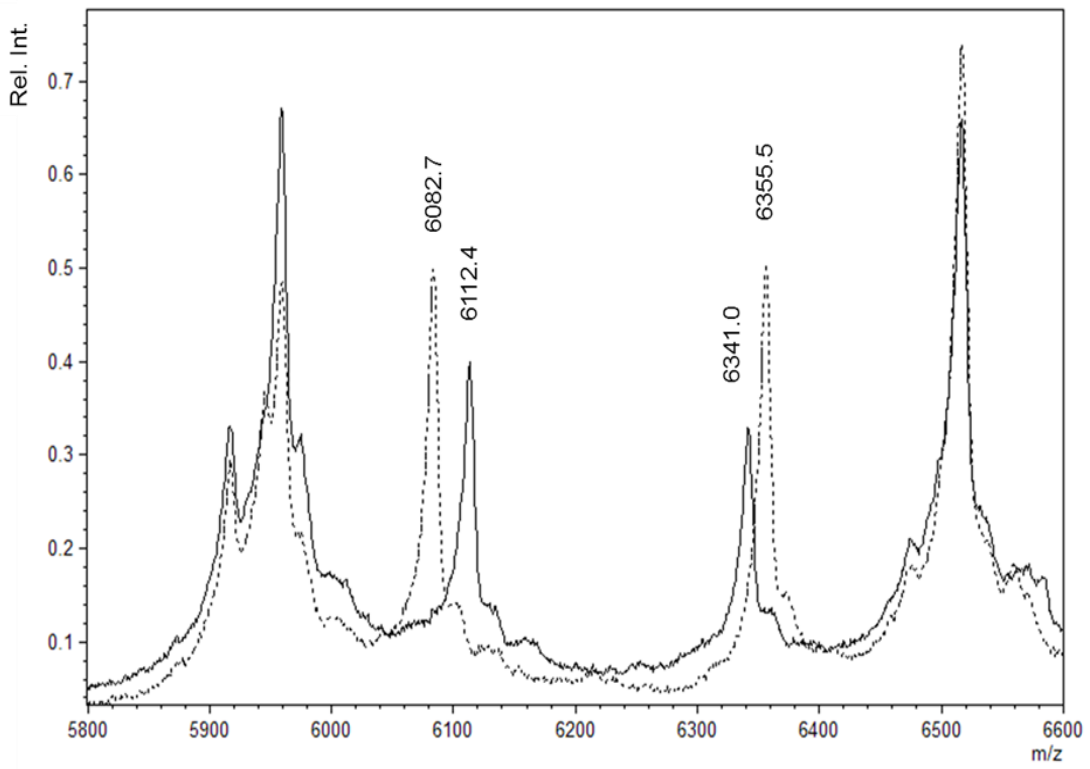

Fig 5. 5: Comparison of the MALDI-TOF MS profiles of strains LMG 27682^T (solid line) and LMG 27684^T (dotted line) using the mMass 5.1.0-software (Strohalm et al. 2010).

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CHAPTER 5: CLASSIFICATION AND IDENTIFICATION OF CAMEL MILK LAB

5.3 Description of two novel *streptococcus* species: *Streptococcus tangierensis* sp. nov. and *Streptococcus cameli* sp. nov., two novel *Streptococcus* species isolated from raw camel milk in Morocco.

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***Streptococcus tangierensis* sp. nov., and *Streptococcus cameli* sp. nov., two novel *Streptococcus* species isolated from raw camel milk in Morocco**

Antonie van Leeuwenhoek (2015) 107:503-510. doi 10.1007/s10482-014-0346-6

PREAMBLE

In the frame of the camel milk LAB diversity study described in chapter 5.1 several LAB taxa were identified as putative novel *Streptococcus* species. In the present study two of these novel LAB taxa within the genus *Streptococcus* were fully characterised using polyphasic taxonomic studies. The $(GTG)_5$-PCR and 16S rRNA gene sequence data obtained were complemented with sequence analysis of the protein encoding genes *pheS*, *rpoA* and *atpA*, DNA-DNA hybridization experiments, determination of the DNA base composition (% G+C), MALDI-TOF MS analyses and a detailed phenotypic characterization. Together this allowed the formal description of two novel *Streptococcus* species for which the names *Streptococcus tangierensis* and *Streptococcus cameli*, were proposed.

OUTLINE CONTRIBUTIONS

Experimental design: Prof. Mohamed AMAR and Prof. Peter VANDAMME.
Experimental work: Zaina KADRI, Margo CNOCKAERT.
Data analysis: KADRI Zaina, Prof. Mohamed AMAR and Prof. Peter VANDAMME.
Writing of manuscript: KADRI Zaina.
Critically reviewing of manuscript: Prof. Mohamed AMAR and Prof. Peter VANDAMME.

Abstract

Biochemical and molecular genetic studies were performed on two unidentified Gram-stain-positive, catalase and oxidase negative, non-hemolytic *Streptococcus*-like organisms recovered from raw camel milk in Morocco. Phenotypic characterization and comparative 16S rRNA gene sequencing demonstrated the two strains were highly different from each other and that they did not correspond to any recognized species of the genus *Streptococcus.* Phylogenetic analysis based on 16S rRNA gene sequences showed the unidentified organisms each formed a hitherto unknown sub-line within the *Streptococcus* genus, displaying a close affinity with *Streptococcus moroccensis, Streptococcus minor* and *Streptococcus ovis*. DNA-DNA hybridization experiments, DNA G+C content determination, MALDI-TOF mass spectrometry and biochemical tests demonstrated the bacterial isolates represented two novel species. Based on the phenotypic distinctiveness of the new bacterium and molecular genetic evidence, it is proposed to classify the two strains as *Streptococcus tangierensis* sp. nov., with CCMM B832^T (= LMG 27683^T) as the type strain, and *Streptococcus cameli* sp. nov., with CCMM B834^T (= LMG 27685^T) as the type strain.

Key words: *Streptococcus tangierensis* sp. nov., *Streptococcus cameli* sp. nov., raw camel milk, Morocco, taxonomy, identification

Introduction

Camel milk is one of the main food available in arid and sub-arid regions where it covers a substantial part of the quantitative and qualitative nutritional needs. The indigenous populations have long believed that raw camel milk is safe and even has therapeutic virtues as compared to that of other animal species (Barbour et al. 1984; Benkerroum et al. 2003). Moreover, raw camel milk could be an additional source of typical dairy lactic acid bacteria (LAB) species (Janoskova and Kmet, 2004). Within the LAB, streptococci are coccoid bacteria that belong to the phylum *Firmicutes*, class *Bacilli,* order *Lactobacillales* and the family *Streptococcaceae* (Clavel et al. 2013). In recent studies, *Streptococcus salivarius* subsp. *thermophilus*, *Streptococcus agalactiae*, *Streptococcus moroccensis* and *Streptococcus rifensis* have been isolated from raw camel milk (Khedid et al. 2009; Jans et al. 2012; Kadri et al. 2014). Despite the large number of described streptococcal species, there is a growing awareness that many others remain to be discovered (Benkerroum et al. 2003). In fact, molecular identification methods have driven the description of a considerable number of novel streptococcal species from human and animal sources in recent years (Facklam, 2002; Thompson et al. 2013). In the present paper, we report the characterization of two strains isolated from raw camel milk (Tangier, Morocco), using phenotypic and molecular genetics methods and their description as two new species within the genus *Streptococcus*.

Materials and methods

Isolation and bacterial strains

In an ongoing study of LAB diversity in raw camel milk samples, two streptococcal-like strains were isolated on M17 agar incubated at 30°C under aerobic conditions. Cultures were then purified by subculturing and preserved in MicroBank™ vials at -80°C. Both strains have been deposited in The Moroccan Coordinated Collections of Micro-organisms (CCMM) and Global Catalogue of Microorganisms (www.gcm.wfcc.info) under the accession numbers CCMM B832^T and CCMM B834^T, and in the Belgian Coordinated Collections of Micro-organisms (BCCM/LMG) under the accession numbers LMG 27683^T and LMG 27685^T, respectively. *Streptococcus* reference strains used in this study were *Streptococcus moroccensis* LMG 27682^T, *Streptococcus minor* LMG 21734^T, *Streptococcus ovis* LMG 19174^T and *Streptococcus gallinaceus* LMG 21849^T, which were obtained from BCCM/LMG and cultured under the same conditions for comparative analyses.

Morphology, Growth, physiological and biochemical characteristics and antibiotic sensitivity

Cell morphology of the isolates was first phenotypically described based on Gram staining followed by microscopy. Catalase and oxidase activities were determined as described previously (Albuquerque et al. 2005). The growth temperature range of the cells was tested at 10, 25, 30, 37 and 45°C under aerobic and microaerobic conditions. Hemolytic activity was examined on Brain Heart Infusion agar supplemented with 5% horse blood at 37°C. API 50 CHL and API 20 STREP microtest systems were performed to determine physiological and biochemical characteristics according to the recommendations of the manufacturer (API bioMérieux, France). In addition, the same tests were performed for the type strains of neighbour species, i.e. *Streptococcus minor* LMG 21734^T, *Streptococcus ovis* LMG 19174^T, *Streptococcus gallinaceus* LMG 21849^T, to verify the reproducibility of published data. Acid production from glucose was determined for strain CCMM B834^T using a mineral salt solution as described by Mckenna et al. (2002) containing peptone 20 g l^{-1} and phenol red 0.005 g l^{-1}. This minimal media was tested alone and supplemented with glucose 10 g l^{-1} as negative and positive controls, respectively, to determine the acid production from glucose. Antibiotic sensitivity was determined using disk diffusion method following the CLSI guidelines. Ten different antibiotics were tested: kanamycin (30µg), gentamicin (10µg), streptomycin (10µg), erythromycin (15µg), ampicillin (10µg), penicillinG (10UI), teicoplanin (30µg), chloramphenicol (30µg), vancomycin (30µg) and tetracycline (30µg).

16S rRNA gene sequences and phylogenetic analysis

DNA for performing 16S rRNA gene amplifications was prepared using a protocol described by Pitcher et al. (1989). The whole fragment (1,508 bp) of the 16S rRNA gene was amplified using universal primers PA (AGA GTT TGA TCC TGG CTC AG) and PH (AAG GAG GTG ATC CAG CCG CA) and sequenced using the following primers; PDR (GTATTACCGCGGCTGCTG), PCF (CTACGGGAGGCAGCAGTGGG) and PEF (CATGGCTGTCGTCAGCTCGT) described by Edwards et al. (1989) using a Big Dye terminator cycle sequencing kit version 3.1 (Applied Biosystems). Analysis of the produced electrophoregram was performed by the sequencing analysis software version 5.3.1 (Applied Biosystems) and the sequence assembly was performed by Geneious software (Drummond et al. 2011). The consensus sequences were edited using MEGA (Molecular Evolutionary Genetics Analysis) software version 5.2.2 (Tamura et al.

2011) and compared with publicly available sequences using the EzTaxon-e database software (Kim et al. 2012). Phylogenetic analyses were performed using MEGA 5.2.2 software (Tamura et al. 2011). Pairwise alignment homologies were calculated and a phylogenetic tree showing the phylogenetic position of both strains was constructed by using the maximum-likelihood method (Fig 5.5), neighbor-joining and minimum-evolution methods (Fig 5.7 and Fig 5.8 respectively).

DNA G+C content analysis

High-molecular weight DNA was prepared as described by Gevers et al. (2001). Analysis of the DNA base ratio was determined by HPLC according to Mesbah et al. (1989).

MALDI-TOF mass spectrometry

After 24 h incubation period at 37°C on Columbia agar base (BBL) supplemented with 5% sheep blood for 24h at 37°C under microaerobic conditions, a loopful of cells was harvested and cell extraction for matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) analysis was performed according to Wieme et al. (2012). Bacterial fingerprints were acquired using the 4800 Plus MALDI TOF/TOF™ Analyzer (Applied Biosystems, Framingham, MA, USA) as previously described (Wieme et al. 2012). A mass range of 2,000-20,000 Da was used for the analysis. The MALDI-TOF MS profiles of strains CCMM B832^T and CCMM B834^T were compared using the mMass 5.5.0 software (Strohalm et al. 2010).

Vancomycin resistance gene detection by PCR

DNA extracted using the protocol described by Pitcher et al. (1989) was used as template for detecting the vancomycin resistance genes *vanA* and *vanB* as described previously (Janoskova and Kmet, 2004). *Enterococcus faecium* CCMM B736 (=LMG 17173) and *Enterococcus faecalis* CCMM B735 (=LMG 16216) were used as positive controls for detection of vancomycin resistance genes *vanA* and *vanB* respectively and *Enterococcus faecium* CCMM B733 (=LMG 16200) was used as negative control.

Results and discussion

While studying the bacterial diversity of raw camel milk produced in North Morocco in 2012 (the coordinates of the sampling site are 35° 46′ 01″ N 5 ° 48′ 00″ W), two isolates (CCMM B832^T and CCMM B834^T) that could not be assigned to species with a validly published name were obtained. The isolates were observed to be non-motile, Gram-positive, non-spore-forming cocci that occurred predominantly in small groups,

and to be catalase and oxidase negative. Cells grow well under aerobic and microaerobic conditions at 25, 30, 37 °C but not at 10 °C and 45°C. Hemolytic activity was not detected for either strain. The two strains were sensitive to kanamycin, gentamicin, streptomycin, erythromycin, ampicillin, penicillin G, teicoplanin, chloramphenicol, vancomycin and tetracycline. Consistent with the vancomycin sensitivity, the *vanA* or *vanB* resistance genes were not detected in either strain (data not shown).

16S rRNA gene sequence analyses using the EzTaxon-e database software (Kim et al. 2012) revealed that both novel isolates belonged to the genus *Streptococcus* and demonstrated that the closest established relatives for strains CCMM B832^T and CCMM B834^T were *S. moroccensis* LMG 27682^T and *S. minor* LMG 21734^T, respectively. The similarity value between strain CCMM B832^T and *S. moroccensis* LMG 27682^T was found to be 97.67% and the similarity value between CCMM B834^T and *S. minor* strain LMG 21734^T was found to be 96.86%. The similarity level between strains CCMM B832^T and CCMM B834^T was found to be 97.41%.

Their 16S rRNA gene sequence similarity levels towards other *Streptococcus* strains were below 97.5%. Notably, these 16S rRNA similarity levels towards their nearest neighbours are below the currently accepted threshold necessitating DNA–DNA hybridization studies (Kim et al. 2014). These results suggest that both isolates represent distinct taxa within the genus *Streptococcus* (Drancourt et al. 2000).

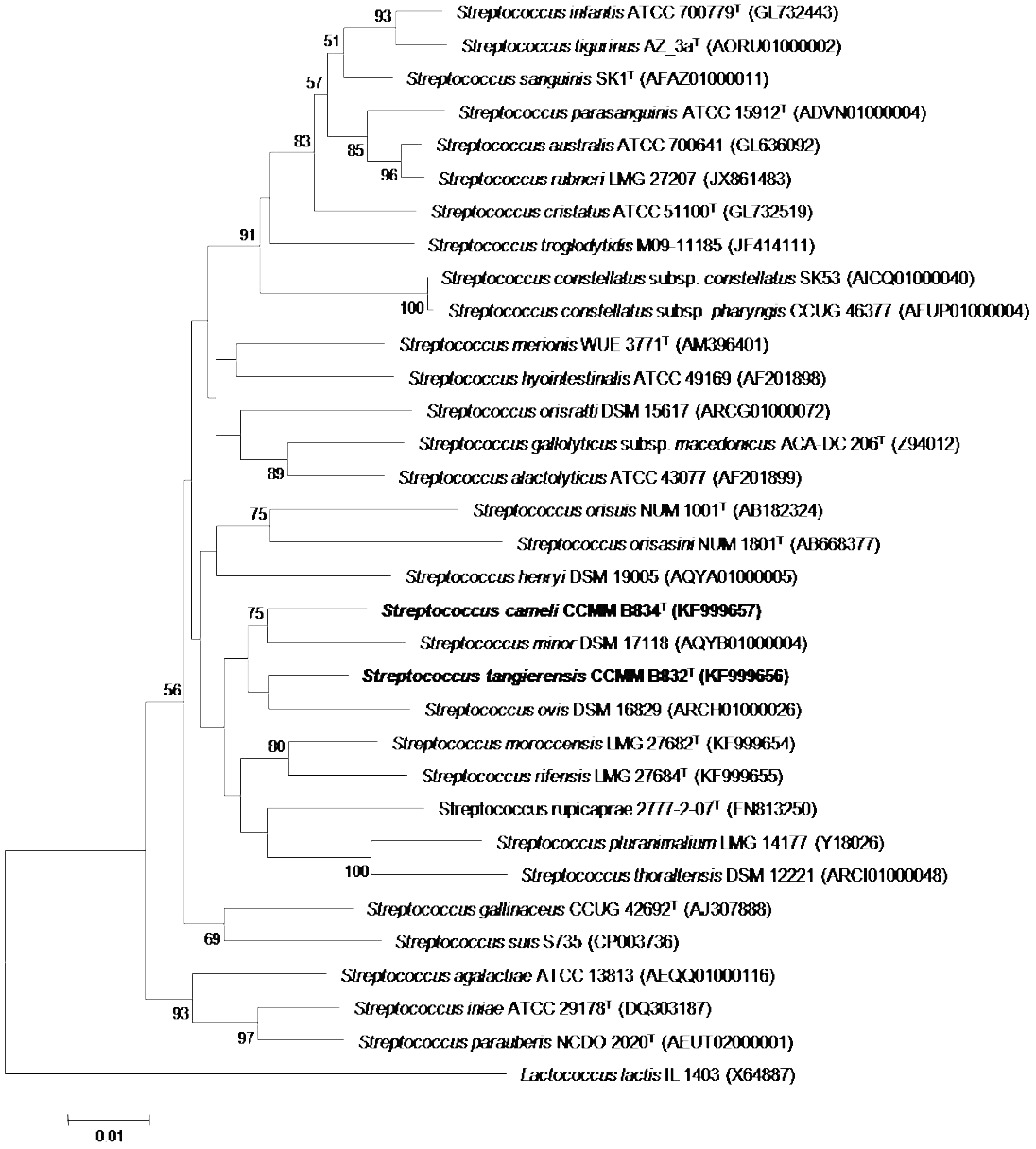

Fig 5. 6: Maximum-likelihood phylogenetic tree based on 16S rRNA gene sequences (1504 bp alignment) of strains CCMM B832^T and CCMM B834^T, and their nearest phylogenetic neighbors (i.e. those *Streptococcus* species with type strains that share >95% of their 16S rRNA sequences with strains CCMM B832^T and CCMM B834^T [as determined using the EzTaxon-e database]). *Lactococcus lactis* ATCC 19435^T was used as an outgroup. Bootstrap values based on 1000 replications are indicated at the nodes; values of less than 50% are not shown. The scale bar indicates the number of substitutions per site.

In the maximum-likelihood (Fig 5.6), the neighbor-joining (Fig 5.8) and the minimum-evolution (Fig 5.9) phylogenetic dendrograms based on 16S rRNA gene sequences, strains CCMM B832^{T}and CCMM B834^T were placed in a cluster in the genus *Streptococcus.* The strains were shown to be closely related to *S. ovis* LMG 19174^T and *S. minor* LMG 21734^T, although this cluster has low bootstrap support. The mol% G+C

content of strains CCMM B832^T and CCMM B834^T were found to be 41.6 and 40.8 % respectively.

Subsequently, MALDI-TOF MS experiments revealed that *S. moroccensis* LMG 27682^T, *S. minor* LMG 21734^T, *S. ovis* LMG 19174^T and the strains CCMM B832^T and CCMM B834^T had clearly different mass spectra (Fig 5.6). The CCMM B832^T MALDI-TOF mass spectrum shared many peaks with the profiles of the *S. moroccensis*, *S. ovis* and *S. minor* type strains, but nevertheless several discriminating spectral features were present. For strain CCMM B834^T, the mass spectrum was dominated by a doublet peak at m/z 3005.7 and 3023.9. A detailed analysis of the higher m/z region within this profile (not shown), revealed that several of the high m/z peaks are shared between the CCMM B834^T profile and the spectra of the related species, as for example the high m/z peak around m/z 15,000. Yet, additional discriminative peaks are present as well, for example the second highest peak at m/z 9419.4 which is uniquely present in the profile acquired from CCMM B834^T.

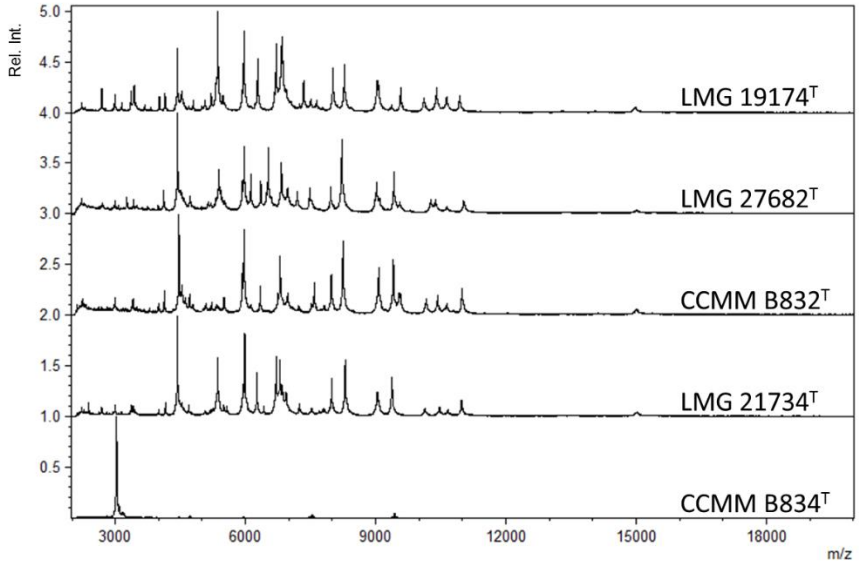

Fig 5. 7: MALDI-TOF MS profiles ranging from m/z 2000 to 20000 from crude cell extracts prepared from *S. ovis* LMG 19174^T, *S. moroccensis* LMG 27682^T, CCMM B832^T, *S. minor* LMG 21734^T and CCMM B834^T. The figure was created with the mMass v5.5.0 software package (Strohalm et al. 2010).

We finally demonstrated that both strains could be distinguished from each other by multiple biochemical tests, including acid production from galactose or melezitose and hydrolysis of *N*-acetyl glucosamine (Tab. 5.3), and from closely related species by several biochemical characteristics such as acid production from cellobiose or gentiobiose or arginine hydrolysis by arginine dihydrolase (Tab. 5.3). Test results obtained for the type strain of *S. minor* supported results obtained by Vancanneyt et al. (2004; Tab. 5.3),

except for the production of acid from sucrose. Similarly, except for the production of acid from melibiose, all results obtained for the type strain of *S. gallinaceus* supported results obtained by Collins et al. (2002; Tab. 5.3). Finally, for *S. ovis* LMG 19174^T, we observed three divergent test results: acid production from glycogen and D-tagatose was absent and β-galactosidase activity was present in the present study while the opposite test results were obtained by Collins et al. (2001; Tab. 5.3). These aberrant test results may be explained through the use of different microtest galleries.

In conclusion, we have demonstrated that the two strains isolated from raw camel milk represent two novel species within the *Streptococcus* genus which can be distinguished from their nearest phylogenetic neighbours and from each other by both phenotypic and genotypic criteria. We therefore propose to formally classify these two strains in two novel species for which the names *Streptococcus tangierensis* sp. nov., and *Streptococcus cameli* sp. nov., are proposed, with strains CCMM B832^T and CCMM B834^T as the type strains, respectively.

Description of *Streptococcus tangierensis* sp. nov.

Streptococcus tangierensis (Tangier. en'sis N.L. adj. *tangierensis* of Tangier, named after the town in Morocco, from where the bacterial species was isolated).

Cells were very small (<1µm in diameter), Gram-positive, non-spore-forming, cocci arranged predominantly in small groups, catalase and oxidase negative. Colonies on Brain Heart Infusion agar supplemented with 5% horse blood are about 0.8 mm in diameter, yellow-golden, round with raised margins, smooth, convex and not hemolytic at 37°C. Cells grow well under aerobic and microaerobic conditions at 25, 30, 37°C but not at 10°C and 45°C. In API 50 CHL tests and API 20 STREP tests, leucine aminopeptidase, α-galactosidase and esculin hydrolase activity are present. Acid is produced from galactose, glucose, fructose, D-raffinose, D-mannose, D-mannitol, D-sorbitol, lactose, D-trehalose, salicin, D-maltose, sucrose, melezitose, D-turanose, *N*-acetylglucosamine and D-arabitol.

Acid are not produced from methyl D-glucoside, glycerol, erythritol, D-arabinose, ribose, L-xylose, adonitol, methyl D-xyloside, sorbose, dulcitol, inositol, methyl D-mannoside, acetoin, melibiose, starch, glycogen, xylitol, D-lyxose, D-tagatose, DL-fucose, L-arabitol, gluconate and 2- and 5-ketogluconate, L-arabinose, cellobiose, arbutin, inulin, amygdalin, L-rhamnose, D-xylose and gentiobiose. No activity of the following enzymes: hippuricase, β-galactosidase, arginine dihydrolase, β-glucuronidase and alkaline phosphatase. DNA G+C content of the type strain is 41.6 mol%.

The type strain is CCMM B832^T (=LMG 27683^T) was isolated from raw camel milk in the north of Morocco. The GenBank accession number for the 16S rRNA sequence of strain CCMM B832^T is KF999656.

Description of *Streptococcus cameli* sp. nov.

Streptococcus cameli (ca.me.'li.N.L. adj. *cameli* of a camel, the *Camelus dromedarius* referring to the isolation of the type strain from camel milk).

Cells are small (<1 µm in diameter), Gram-positive, non-spore-forming, cocci arranged predominantly in small groups, catalase and oxidase negative. Colonies on Brain Heart Infusion agar supplemented with 5% horse blood were about 0.8 mm in diameter, yellow-golden, round with raised margins, smooth, convex and not hemolytic at 37°C. Cells grow well under aerobic and microaerobic conditions at 25, 30, 37°C but not at 10°C and 45°C. In API 50 CHL tests and API 20 STREP, acid is produced from lactose only. Leucine aminopeptidase and esculin hydrolase activity are present.

Acid are not produced from glycerol, erythritol, D-arabinose, ribose, L-xylose, adonitol, methyl D-xyloside, sorbose, dulcitol, inositol, methyl D-mannoside, methyl D-glucoside, melibiose, starch, acetoin, glycogen, xylitol, D-lyxose, D-tagatose, DL-fucose, L-arabitol, gluconate, 2- and 5-ketogluconate, L-arabinose, *N*-acetylglucosamine, cellobiose, arbutin, inulin, amygdalin, L-rhamnose, D-xylose, galactose, fructose, D-raffinose, D-mannose, D-mannitol, D-sorbitol, D-trehalose, salicin, D-maltose, sucrose, melezitose, D-turanose, D-arabitol and gentiobiose. No activity of the following enzymes: hippuricase, β-galactosidase, arginine dihydrolase, β-glucuronidase, α-galactosidase and alkaline phosphatase. The DNA G+C content is 40.8 mol%. The type strain is CCMM B834^T (=LMG 27685^T) was isolated from raw camel milk produced in the north of Morocco. The GenBank accession number for the 16S rRNA sequence of strain CCMM B834^T is KF999657.

Acknowledgments

This work was supported by National Center for Scientific and Technical Research, Rabat-Morocco and Belgian Cooperation for development (project: FRAB / 2005). We thank Mr. Melloul Marouane for his assistance to sequence 16S rRNA gene. We thank Mrs. Isabel Snauwaert and Mrs. Charlotte Peeters for helping us constructing phylogenetic trees.

TABLES:

Tab. 5. 3: Characteristics that differentiate strains CCMM B834^T and CCMM B832^T from type strains of closest phylogenetic relatives (i. e., those *Streptococcus* species with type strains that share >95.8% of their 16S rRNA sequences with strains CCMM B832^T and CCMM B834^T [as determined using the EzTaxon-e database]).

Taxa / **Characteristic**	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**
Acid production from :												
Galactose	-	+	+/+	+/ND	+/ND	+	ND	+	ND	+	+	+
Glucose	-	+	+/+	+/+	+/+	+	ND	+	ND	+	+	+
Fructose	-	+	+/+	+/ND	+/ND	+	ND	+	ND	+	+	+
Amygdalin	-	-	+/+	-/ND	-/ND	-	ND	-	-	+	+	+
D- Maltose	-	+	+/+	+/+	+/+	+	+	+	+	+	-	+
Gentiobiose	-	-	+/+	-/ND	-/ND	-	ND	ND	ND	+	+	+
D-Raffinose	-	+	-/V	+/+	+/+	-	-	-	+	+	-	-
D-Mannitol	-	+	+/+	+/+	+/+	+	+	-	-	-	-	+
D-Sorbitol	-	+	+/V	+/+	-/-	+	-	-	-	-	-	+
D-Mannose	-	+	+/+	+/ND	+/ND	+	ND	+	ND	+	+	-
Starch	-	-	+/V	+/ND	-/ND	-	+	-	ND	+	-	-
Trehalose	-	+	+/+	+/+	+/+	+	+	+	-	+	+	+
Glycogen	-	-	+/+	-/+	-/-	-	+	ND	-	+	-	-
Sucrose	-	+	-/+	+/+	+/+	ND	+	+	+	+	+	+
Salicin	-	+	+/+	+/ND	+/ND	+	ND	-	ND	+	+	+
Cellobiose	-	-	+/+	-/ND	-/ND	+	ND	+	ND	+	+	+
D-Ribose	-	-	-/-	-/-	+/+	V	-	ND	-	-	-	-
D-Turanose	-	+	-/-	-/ND	-/ND	-	ND	ND	ND	-	-	-
D-Arabitol	-	+	-/-	-/-	-/-	-	ND	ND	-	-	-	-
Melezitose	-	+	-/-	-/-	-/-	-	-	-	-	-	+	+
D-Tagatose	-	-	-/V	-/+	-/-	-	-	ND	+	-	-	-

D-Xylose	-	-	-/-	-/ND	-/-	-	ND	ND	ND	+	+	-
L-Rhamnose	-	-	-/-	-/ND	-/ND	-	ND	-	ND	+	+	-
Inulin	-	-	-/-	-/ND	-/ND	-	+	+	-	+	+	+
Arbutin	-	-	-/-	-/ND	-/ND	ND	ND	-	-	+	+	+
Lactose	+	+	+/+	+/+	+/+	+	-	+	+	+	+	+
Methyl α-D-glucoside	-	-	-/-	-/ND	-/ND	-	ND	ND	ND	+	-	-
L-Arabinose	-	-	-/-	-/-	-/-	-	-	ND	-	+	+	+
Melibiose	-	-	-/-	-/-	-/+	-	+	+	-	+	-	-
Production of :												
α-Galactosidase	-	+	+/V	+/V	ND/+	-	+	-	-	+	+	-
β-Galactosidase	-	-	-/-	+/-	ND/+	+	+	+	+	+	-	-
Acetoin	-	-	-/ND	-/-	ND/-	+	-	-	ND	-	-	-
Alkaline phosphatase	-	-	-/-	-/-	ND/-	+	-	-	-	-	-	-
β-Glucuronidase	-	-	-/-	-/-	ND/-	+	-	-	-	+	-	+
Hydrolysis of :												
Arginine Dihydrolase	-	-	+/+	+/V	ND/+	-	-	+	-	+	-	-
N-Acetyl glucosamine	-	+	+/+	+/ND	+/ND	+	ND	+	ND	+	+	+
Hippurate	-	-	-/-	-/-	ND/-	+	-	-	-	-	-	-
Esculin	+	+	+/+	+/+	+/+	ND	+	+	-	+	+	+

Taxa: 1 *Streptococcus cameli* CCMM B834^T; **2** *Streptococcus tangierensis* CCMM B832^T; **3** *Streptococcus minor* LMG 21734^T (data from the present study)/CCUG 47487^T (data from (Vancanneyt et al. (2004)); **4** *Streptococcus ovis* LMG 19174^T (data from the present study)/CCUG 39485^T (data from Collins et al. (2001)); **5** *Streptococcus gallinaceus* LMG 21849^T (data from the present study)/CCUG 42692^T (data from Collins et al. (2002)); **6** *Streptococcus hyovaginalis* SHV515^T (Devriese et al. 1997); **7** *Streptococcus henryi* 126^T (Milinovich et al. 2008); **8** *Streptococcus sanguinis* SK1^T (Kilian et al. 1989) ; **9** *Streptococcus infantis* 0-122 ^{T} (Kawamura et al. 1998) ; **10** *Streptococcus rupicaprae* 2777-2-07^T (Vela et al. 2011) ; **11** *Streptococcus moroccensis* LMG 27682^T (Kadri et al. 2014) ; **12** *Streptococcus rifensis* LMG 27684^T (Kadri et al. 2014). +, positive reaction; -, negative reaction; ND: not determined; V: variable.

Supplementary information

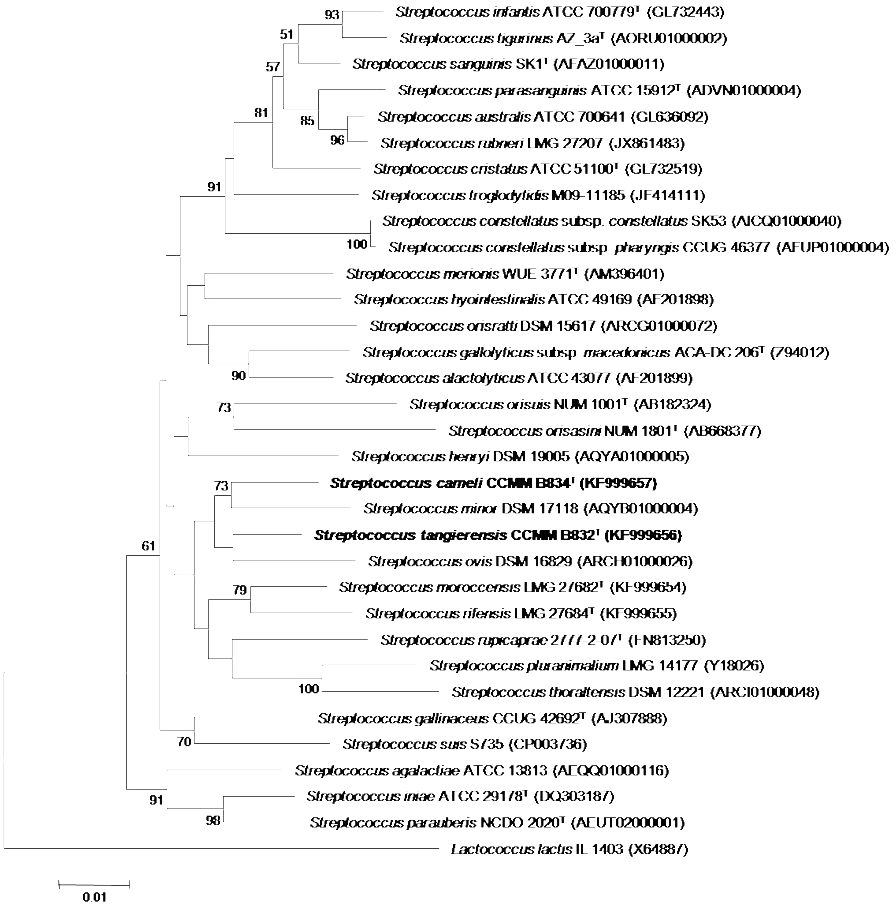

Fig 5. 8: Neighbor-joining phylogenetic tree based on 16S rRNA gene sequences (1504 bp alignment) of strains CCMM B832^T and CCMM B834^T, and their nearest phylogenetic neighbors (i.e. those *Streptococcus* species with type strains that share >95% of their 16S rRNA sequences with strains CCMM B832^T and CCMM B834^T [as determined using the EzTaxon-e database]). *Lactococcus lactis* ATCC 19435^T was used as an outgroup. Bootstrap values based on 1000 replications are indicated at the nodes; values of less than 50% are not shown. The scale bar indicates the number of substitutions per site

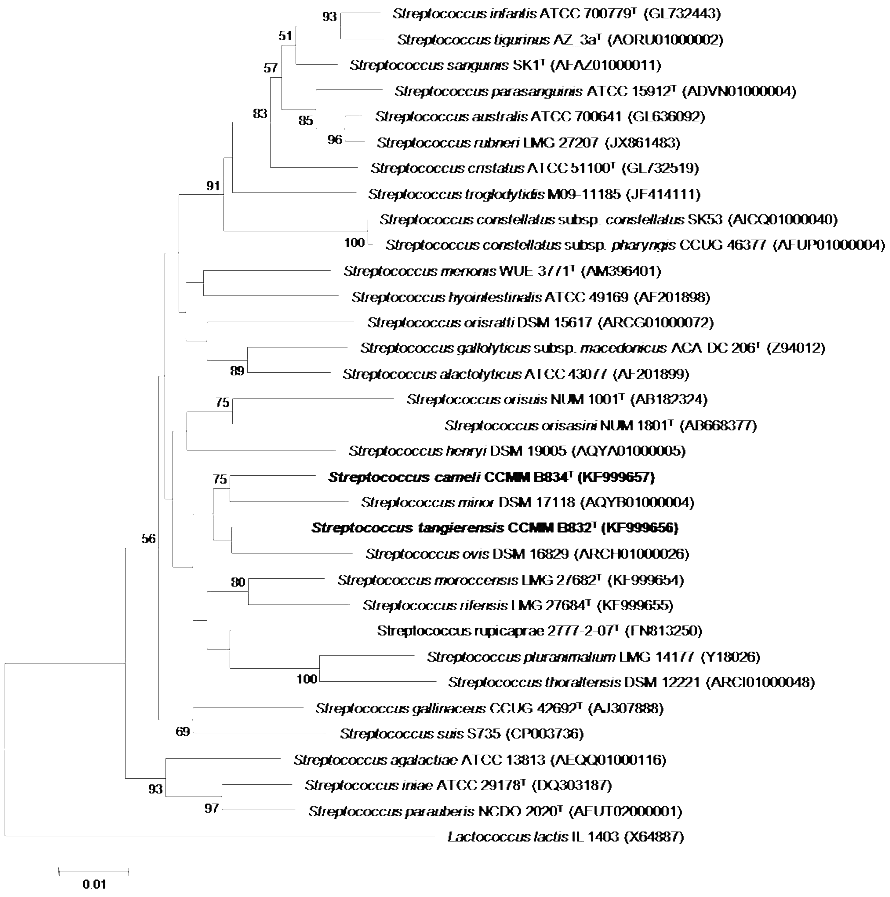

Fig 5. 9: Minimum evolution phylogenetic tree based on 16S rRNA gene sequences (1504 bp alignment) of strains CCMM B832^T and CCMM B834^T, and their nearest phylogenetic neighbors (i.e. those *Streptococcus* species with type strains that share >95% of their 16S rRNA sequences with strains CCMM B832^T and CCMM B834^T [as determined using the EzTaxon-e database]). *Lactococcus lactis* ATCC 19435^T was used as an outgroup. Bootstrap values based on 1000 replications are indicated at the nodes; values of less than 50% are not shown. The scale bar indicates the number of substitutions per site.

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CHAPTER 6: THE BACTERIAL DIVERSITY OF CAMEL MILK.

6.1 The bacterial diversity of camel milk: a contemporary update.

Zaina Kadri, Freek Spitaels, Margo Cnockaert, Mohamed Amar and Peter Vandamme.

In preparation for submission

PREAMBLE

Culturomics is a term used in microbial diversity studies in which unusally large numbers of isolates are picked and analyzed to characterize the total cultivable bacterial community of a given sample, and therefore it requires a fast processing technique such as MALDI-TOF MS (Lagier et al. 2012; Pfleiderer et al. 2013). The aim of the present study was to perform a modest culturomics study in order to characterize the total cultivable bacterial camel milk microbiota using a variety of culture conditions and MALDI-TOF MS as a dereplication tool. A final identification of the microbiota was obtained through sequence analysis of 16S rRNA and of protein encoding genes.

OUTLINE CONTRIBUTIONS

Experimental design: Prof. VANDAMME Peter and Dr. SPITAELS Freek.
Experimental work: KADRI Zaina, Dr. SPITAELS Freek and CNOCKAERT Margo.
Data analysis: KADRI Zaina, Dr. SPITAELS Freek and Prof. VANDAMME Peter.
Writing of manuscript: KADRI Zaina.
Critically reviewing of manuscript: Dr. SPITAELS Freek, Prof. AMAR Mohamed, Prof. VANDAMME Peter.

Abstract

Camel milk is a valuable source of food for people living in arid areas of Morocco. As it is usually consumed in its raw state, the presence of pathogenic bacteria is of public health importance (Adugna et al. 2013; Eberlein, 2007; Saad & Thabet, 1993; Younan, 2004). The present study aimed to investigate the overall microbiota of raw camel milk collected from four different locations in Morocco. From four different samples, 808 isolates were obtained using ten combinations of growth media and incubation conditions. Subsequent dereplication using MALDI-TOF MS and identification of selected isolates through sequence analysis of 16S rRNA gene and protein encoding genes revealed a considerable species diversity. Thirty-four bacterial species including two novel lactic acid bacteria (LAB) and belonging to four distinct phyla, i.e. *Firmicutes* (64.7% of the isolates), *Proteobacteria* (30.0%), *Actinobacteria* (4.9%), and *Bacteroidetes* (0.4%), were isolated.

Keywords: microbiota of raw camel milk; culturomics approach; MALDI-TOF MS; sequence analysis; 16S rRNA; protein encoding genes.

Introduction

The dromedary camel is able to produce milk in a valuable quantity, despite it is living in the harsh environments of semi-arid and arid climate zones (Faye, 2005; Schwartz and Dioli, 1992). Dromedary camel milk has been used in different parts of the world including India, Russia, Sudan, Libya, in the treatment of diseases such as dropsy, jaundice, tuberculosis, asthma and leishmaniasis (Abdelgadir et al. 1998; Shalash, 1984). Recently, camel milk was reported to have other potential applications like its anti-carcinogenic (Magjeed, 2005), anti-diabetic (Agrawal et al. 2007a), anti-hypertensive (Quan et al. 2008) and hypo-allergic (Shabo et al. 2005) properties and effects in the treatment of immunity deficiencies (Alwan et al. 2014). Camel milk is highly nutritious, since it contains high contents of minerals such as sodium, potassium, iron, copper, zinc and magnesium (Yagil, 1982), and it is a rich source of unsaturated fatty acids which contributes to its overall dietary quality (Ereifaj et al. 2011; Karray et al. 2005; Konuspayeva et al. 2008). It is also reported to be rich (24-36 mg/l) in vitamin C compared to cow milk (3-23 mg/l) (Field et al. 1997; Jassim & Naji, 2002; Kappeler, 1998), making it a good source of this vitamin for the people of the desert where fruits and vegetables are scarce (Alwan and Igwegbe, 2014). Camel milk possesses superior keeping quality compared to cow milk due to its high contents of proteins (lactoperoxidase, lactoferrin, immunoglobulins, lysozyme) that have inhibitory properties against bacterial contaminants (Konuspayeva et al. 2007; Younan, 2004). This makes raw camel milk a marketable commodity, even under conditions of high temperatures (Younan, 2004). In Morocco, a small number of studies addressing the diversity and dynamics of LAB solely and/or the microbiological quality of raw camel milk have been carried out using phenotypic characterization of isolates only (Benkerroum et al. 2003; Ismaili et al. 2015; Khedid et al. 2009). Therefore, the hygienic status of raw camel milk produced in Morocco is currently not known.

Microbial culturomics is a term used in microbial diversity studies in which unusally large numbers of isolates are picked and analyzed to characterize the total cultivable bacterial community of a given sample (Goodman et al. 2011; Lagier et al. 2012; Vartoukian et al. 2010). This recent approach can be applied because of the availability of matrix-assisted laser desorption ionization time-of-flight mass spectrometry, which can accurately and rapidly identify such large number of microorganisms in a cost-effective manner (Lagier et al. 2012; Seng et al. 2009). MALDI-TOF mass spectrometry has

already been introduced as a high-throughput tool for species level identification in clinical, environmental and food-related studies (Andrés-Barrao et al. 2013; Benagli et al. 2011; Carbonnelle et al. 2011; Clark et al. 2013; Dieckmann et al. 2005; Dušková et al. 2012). In the present study, we used MALDI-TOF MS as a high-throughput dereplication tool to assess the microbial composition of raw camel milk produced collected in different areas of Morocco. Representative isolates were further identified using sequence analysis of 16S rRNA gene and/or protein encoding genes. The latter gene sequences have a superior taxonomic resolution to distinguish between closely related species (Cleenwerck et al. 2010; Naser et al. 2007; Wieme et al. 2014).

Materials and methods

Collection of raw camel milk samples

Samples of fresh dromedary milk were collected from four different locations in Morocco (Tab. 6.1). Five percent DMSO solution was added to the samples which were homogenized and stored at -80°C until further use (Kerckhof et al. 2014). Samples were serially diluted in 0.85 % NaCl and 50 µL of each dilution was plated in triplicate on multiple agar isolation media. A total of sixteen combinations of different growth media and incubation conditions [28°C, 37°C or 45°C and aerobic, anaerobic or 5 % CO_2 enriched atmosphere] were tested to evaluated their redundancy for the isolation of general and specific bacterial fractions. This resulted in a set of ten isolation conditions listed below. All isolation media were supplemented with 5 ppm amphotericin B (Sigma-Aldrich, Bornem, Belgium) to inhibit fungal growth. Samples were incubated on De Man-Rogosa Sharpe (MRS) agar (Oxoid, Erembodegem, Belgium) (De Man et al. 1960) at 45°C anaerobically, on M17 agar (Oxoid) (Terzaghi and Sandine, 1975) at 28°C aerobically and 37°C anaerobically, on Columbia Blood Agar (CBA) (Oxoid) at 37°C anaerobically, on Violet Red Bile Glucose (VRBG) agar (Mossel et al. 1962, 1978), on Trypticase Soy Agar (TSA) (Oxoid) supplemented with 4.5% NaCl (TSA+4.5% NaCl), on All Culture agar (ACA) defined as follows (g/liter): proteose peptone (Oxoid), 20.0; beef extract (Oxoid), 3.0; yeast extract (Oxoid), 3.0; malt extract (Oxoid), 3.0; dextrose (Oxoid), 5.0; ascorbic acid (Oxoid), 0.2; bacteriological agar, 15.0), on ACA supplemented with 2% milk powder (ACA+ 2% milk powder), on Plate Count Agar supplemented with 2% milk powder (PCA+ 2% milk powder) (Jackman et al. 1983) and on Mannitol Salt Agar (MSA) (Alonso-Calleja et al. 2002; Papamanoli et

al. 2003). When not specified, inoculated agar plates were incubated at 28°C under aerobic conditions. Colonies on plates comprising 25 to 250 colony forming units (CFU) were counted after 1 to 7 days of incubation and for each of the ten isolation conditions about 20 colonies, or all colonies if the counts were lower, were randomly picked up.

Tab. 6. 1: Coordinates of sampling.

Sites of sampling	Coordinates
Tazarin	31°17'41"N, 8°35'13"W
Dakhla	23°43′N, 15°57′W
Laayoune	27.1565° N, 13. 2036°W
Zagora	30.3306° N, 5.8381°W

Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI TOF MS) dereplication and identification

Isolates were subcultured twice using their respective isolation conditions and MALDI-TOF MS was performed using the third generation of pure cultures by means of a 4800 Plus MALDI TOF/TOF TM Analyzer (AB SCIEX, Framingham, MA, USA), as previously described (Wieme et al. 2012). In short, Data Explorer 4.0 software (AB SCIEX) was used to convert the mass spectra into .txt-files to import them into a BioNumerics 5.1 (Applied Maths Sint-Martens-Latem, Belgium) database. The spectral profiles were compared using the Pearson Product-Moment Correlation Coefficient (PPMCC) and a dendrogram was built using the Unweighted Pair Group Method with Arithmetic Mean (UPGMA) cluster algorithm. Homogeneous clusters consisting of isolates with visually identical or virtually identical mass spectra were delineated. From each cluster, depending on the cluster size and the number of samples the isolates were retrieved from, one up to six isolates were randomly selected for further identification through sequence analysis of 16S rRNA genes and/or protein encoding genes. Sequence analysis of *rpoB* gene was performed to confirm the identity of members of the *Enterobacteriaceae* family (Mollet al. 1997; Nhung et al. 2007, Spitaels et al. 2014), of the *dnaJ* gene for the genus *Staphylococcus* (Shah et al. 2007), of the *gyrB* gene for the *Pseudomonadaceae* family (Kupfer et al. 2006; Yanez et al. 2003), and of the *pheS* gene to identify LAB (De Bruyne et al. 2007, and 2008; Naser et al. 2005, and 2007; Spitaels et al. 2014). All

PCR assays were performed as described by Snauwaert et al. (2013). Bacterial DNA was obtained via the protocol described by Niemann et al. (1997). EzTaxon-e (Kim et al. 2012) and NCBI BLAST (www.ncbi.nlm.nih.gov/ BLAST) (Altschul et al. 1997) analyses were performed to determine the most similar sequences in the public sequence databases.

Results

Enumeration of bacteria

Tab. 6.2 presents an overview of the enumeration analyses. In general, the viable counts were high on all media tested, except for the MRS and MSA of the Tazarin sample (Tab. 6.2).

Tab. 6. 2: Results of plate counts on different agar isolation media

Samples	**VRBG 28°C AE**	**MRS 45°C AN**	**M17 28°C AE**	**M17 37°C AN**	**ACA 28°C AE**	**ACA+2% milk powder 28°C AE**	**PCA+2% milk powder 28°C AE**	**MSA 28°C AE**	**CBA 37°C AE**	**TSA+4.5 % NaCl 28°C AE**
Tazarin	6.5	3.3	8.6	8.5	8.7	8.6	8.4	3. 9	9.6	6.6
Laayoune	8.7	5.6	7.7	7.9	7.8	7.6	7.7	6.4	7.9	6.8
Dakhla	5.6	5.4	7.8	7.1	8.3	8.4	8.5	6.4	8.5	6.5
Zagora	4.7	6.6	7.1	6.6	7.5	7.5	7.5	6.4	7.6	7.4

*Values represent log CFU/ml. AE: aerobic conditions. AN: anaerobic conditions.

Identification of bacteria

Tab. 6.3 presents the identifications of the MALDI-TOF MS clusters. A total of 808 isolates were obtained from the four camel milk samples analyzed. First, MALDI-TOF MS was performed to dereplicate all 808 bacteria isolates from raw camel milk. MS profiles of good quality were generated for all 808 isolates and cluster analysis was performed, resulting in 108 clusters. A total of 175 representative isolates were subjected to 16S rRNA gene sequencing and/or sequence analysis of protein encoding genes. Results of 16S rRNA gene sequences and sequence analysis of the genes *dnaJ, rpoB, gyrB and pheS* were then extrapolated to all other isolates in the respective clusters of the MALDI-TOF MS dendrogram. However, some representative isolates of

distinct MALDI-TOF MS clusters remained unidentified after comparative sequence analysis, indicating that they represented novel species which were subsequently characterized as the novel species *Enterococcus bulliens* (chapter 6.2) and *Lactococcus multifermentans* (chapter 6.3).

The 808 isolates obtained represented 34 bacterial species belonging to four phyla. This considerable species diversity was dominated by LAB and *Enterobacteriaceae* species, which represented almost 84% of the total isolates.

Tab. 6. 3: Identification of raw camel milk microbiota.

Identification, isolate and cluster	**Accession number highest hit**	**Gene and similarity level (%)**	**Species distribution among isolates (%)**	**Family**
Escherichia/Shigella R-54789 / T17 R-54806 / T11 R-54814 / T15 R-54819 / T14 R-54822 / T18 R-54794 / T16 R-54824 / T13	CP007442/CP011511	*rpoB* / 100	8.4	*Enterobacteriaceae*
Enterobacter xiangfangensis R-54924 / L19 R-54886 / L5 R-54927 / L7a R-54930 / L7a R-54915 / L8 R-54893 / L9 R-54935 / L10 R-54909 / L11 R-54897 / L13 R-54900 / L13 R-54917 / L17b R-54933 / L14a R-54988 / D3 R-55033 / D4	HF679054	*rpoB* / 99	5.5	*Enterobacteriaceae*
Enterobacter hormaechei R-54942 / L14b	AJ543724	*rpoB* / 99	4.6	*Enterobacteriaceae*

R-54925 / L7b R-54989 / D5 R-54990 / D6 R-54991 / D6 R-54992 / D6 R-54993 / D7 R-54994 / D7 R-54995 / D8 R-54996 / D8				
Aeromonas caviae R-54887; R-54899; R-54936 / L1 R-54888; R-54895 / L3	AJ868400	*gyrB* / 97-98	2.0	*Enterobacteriaceae*
Pantoea agglomerans R-55121 / Z17 R-55123 / Z15	KF482728	*rpoB* / 99	1.9	*Enterobacteriaceae*
Serratia marcescens subsp. *marcescens* R-54787 / T4 R54802 / T3 R54803 / T5 R-54880 / T1 R-54818 / T2	JX425316	*rpoB* / 98-99	1.4	*Enterobacteriaceae*
Raoultella ornithinolytica R-54934; R-54885; R-54932 / L16	AF129447	*rpoB* / 100	0.7	*Enterobacteriaceae*
Citrobacter werkmanii R-55114 / Z13	AF025373	16S rRNA / 99.98	0.6	*Enterobacteriaceae*
Citrobacter freundii R-55122 / Z19	HG798908	*rpoB* / 99	0.4	*Enterobacteriaceae*
Klebsiella oxytoca R-54798; R-54804 / T12 R-54937 / L21	EU010109	*rpoB* / 99	0.4	*Enterobacteriaceae*
Enterobacter asburiae R-54923 / L17a	AJ543727	*rpoB* / 97	0.3	*Enterobacteriaceae*
Enterobacter cloacae subsp. *clocae* R-54931 / L20 R-54926 / L6	EU643264	*rpoB* / 99	0.3	*Enterobacteriaceae*

Pseudomonas moraviensis R-55119 / Z16 R-55120 / Z13	FN554743	*rpoB* / 98	2.5	*Pseudomonaceae*
Acinetobacter bouvetti R-55102 / Z20 R-55118 / Z21	APQD01000004	16S rRNA / 100	1.1	*Pseudomonaceae*
Moraxella osloensis 54920 / L38	EU499677	16S rRNA / 99.7	0.1	*Pseudomonaceae*
Lactococcus lactis subsp. *lactis*[a] R-54788; R-54808; R-54801 / T9 R-54795 / T8 R-54800 / T7 R-54805 / T6 R-54807 / T10 R-54790 / T19 R-54892; R-54905 / L37 R-55012 / D21 R-55013 / D23 R-55014 / D22 R-55015 / D24 R-55016 / D25 R-55017 / D26 R-55018; R-55019; R-55020; R-55021; R-55022; R-55023/ D27 R-55024; R-55025; R-55026; R-55027; R-55028 ; R-55029 / D28 R-55030 / D29 R-55031 / D30 R-55032 / D31 R-55056; R-55107 / Z10 R-55095 / Z18	AE005176	*pheS* / 99-100	32.6	*Streptococcaceae*
Enterococcus faecium R-54911; R-54912; R-54941; R-54913 / L26 R-54914 / L28	AJ843428	*pheS* / 100	13.7	*Enterococcaceae*

R-54997; R-54998; R-54999 / D9 R-55052 / Z4 R-55053 / Z7 R-55055 / Z9				
Streptococcus suis R-54903; R-54898; R-54921 / L31 R-54906 / L32 R-54907 / L35 R-54908 / L36 R-55011 / D20	AM269555	*pheS* / 95-96	3.6	*Streptococcaceae*
Enterococcus bulliens R-55001 / D11 R-55002 / D12 R-55003 / D13 R-55004; R-55005 / D14 R-55006; R-55007 / D15 R-55008 / D16 R-55009 / D17	KR827650	*pheS* / 100	2.5	*Enterococcaceae*
Enterococcus italicus R-54890; R-54904; R-54901 / L29 R-55000 / D10	AJ843426	*pheS* / 99-100	2.5	*Enterococcaceae*
Lactococcus multifermentans R-54894; R-54896 / L23 R-54902 / L24	KR827654	*pheS* / 100	1.2	*Streptococcaceae*
Leuconostoc pseudomesente-roides [a] R-54985; R-54986 / D1 R-55096; R-55113 / Z22	AM711197	*pheS* / 98	0.7	*Leuconostocaceae*
Streptococcus salivarius R-55034 / D19	AM269544	*pheS* / 96	0.3	*Streptococcaceae*
Enterococcus faecalis R-55116 / Z12	AJ843387	*pheS* / 100	0.1	*Enterococcaceae*
Lactobacillus rhamnosus R-55032 / D31	AM087716	*pheS* / 100	0.1	*Lactobacillaceae*
Staphylococcus aureus subsp. *aureus* R-54883; R-54884 / T22	EU652831	*dnaJ* / 100	2.6	*Staphylococcaceae*

Staphylococcus simulans R-54811; R-54812; R-54813 / T20	EU652837	*dnaJ* / 99	2.0	*Staphylococcaceae*
Macrococcus caseolyticus R-55047; R-55048 / Z2 R-55049; R-55051 / Z3	Y15711	16S rRNA / 100	2.1	*Staphylococcaceae*
Staphylococcus capitis subsp. *capitis* R-54797 / T21	AB234060	*dnaJ* / 100	0.4	*Staphylococcaceae*
Rothia endophytica R-55044; R-55045; R-55046 / Z1 R-55098; R-55100 / Z26 R-55104 / Z27	KC806052	16S rRNA / 100	3.8	*Micrococcaceae*
Rothia marina R-55117 / Z1	FJ425908	16S rRNA / 100	1.0	*Micrococcaceae*
Kocuria salsicia R-54918 / L22	GQ352404	16S rRNA / 99.72	0.1	*Micrococcaceae*
Chryseobacterium arthro-sphaerae R-54916; R-54922 / L25	FN398101	16S rRNA / 99.77	0.3	*Flavobacteriaceae*
Empedobacter falsenii R-54919 / L4	AM084341	16S rRNA / 98.69	0.1	*Flavobacteriaceae*

Where: T, L, D and **Z** are referering to Moroccan area where samples were collected: Tazarin, Laayoune, Dakhla and Zagora. **T, L, D** and **Z** with numbers correspond to cluster numbers.

Selectivity of the media used

All media used supported the growth of LAB except for MSA and VRBG, but the relative species distribution was different. Members of the *Enterobacteriaceae* were isolated from all media used, except for MRS agar, with the highest number of isolates obtained from VRBG. Members of the *Staphylococcaceae* were isolated mostly from MSA while those belonging to the *Micrococcaceae* were recovered from various media used, yet the highest numbers of isolates were obtained from PCA+2% milk powder. A detailed species distribution across the different culture conditions used is shown in Tab. 6.4.

Tab. 6. 4: Distribution of the different species across the media used.

Species identification	Culture conditions	Number of isolates	% of isolates per species
L. lactis subsp. *lactis*	ACA	48	17.9
	ACA+2% milk powder	40	14.9
	CBA	22	8.2
	PCA+2% milk powder	44	16.4
	M17 AN	84	31.4
	M17 AE	30	11.2
E. faecium	MRS 45 AN	64	57.1
	M17 AN	8	7.1
	M17 AE	8	7.1
	ACA	13	11.6
	PCA+2% milk powder	4	3.6
	ACA+2% milk powder	4	3.6
	TSA+4.5% NaCl	4	3.6
	CBA	7	6.3
Escherichia/Shigella	TSA+4.5% NaCl	24	35.3
	PCA+2% milk powder	9	13.2
	VRBG	15	22.1
	ACA	18	26.5
	M17 AN	1	1.5
	CBA	1	1.5
Ent. xiangfangensis	TSA+4.5% NaCl	11	25.0
	ACA	6	13.6
	VRBG	11	25.0
	ACA+2% milk powder	5	11.4
	MSA	1	2.3
	PCA+2% milk powder	5	11.4
	CBA	5	11.4
Ent. hormaechei	TSA+4.5% NaCl	11	29.7
	ACA	2	5.4
	VRBG	16	43.2
	M17 AN	2	5.4
	CBA	6	16.2
Rot. endophytica	TSA+4.5% NaCl	6	19.4
	ACA+2% milk powder	4	12.9
	ACA	6	19.4
	PCA+2% milk powder	3	9.7
	M17 AE	8	25.8
	CBA	4	12.9
S. suis	ACA+2% milk powder	5	17.2
	M17 AE	7	24.2
	M17 AN	11	37.9
	CBA	3	10.3
	PCA+2% milk powder	3	10.3
St. aureus subsp. *aureus*	MSA	21	100.0
	M17 AN	8	40.0
	ACA	1	5.0
	ACA+2% milk powder	2	10.0

E. bulliens	PCA+2% milk powder	3	15.0
	CBA	2	10.0
	TSA+4.5% NaCl	4	20.0
E. italicus	ACA	1	5.6
	ACA+2% milk powder	2	11.1
	TSA+4.5% NaCl	4	22.2
	M17 AN	1	5.6
	M17 AE	4	22.2
	CBA	5	27.8
	PCA+2% milk powder	1	5.6
Mac. caseolyticus	TSA+4.5% NaCl	6	31.6
	M17 AN	3	15.8
	ACA+2% milk powder	4	21.1
	CBA	4	21.1
St. simulans	MSA	16	100.0
Ps. moraviensis	VRBG	4	23.5
	ACA+2% milk powder	2	11.8
	PCA+2% milk powder	5	29.4
	M17 AE	3	17.7
	CBA	3	17.7
Aer. caviae	ACA	5	31.3
	ACA+2% milk powder	3	18.8
	CBA	3	18.8
	VRBG	2	12.5
	PCA+2% milk powder	1	6.3
	M17 AE	2	12.5
Pan. agglomerans	ACA	3	20.0
	VRBG	12	80.0
Ser. marcescens subsp. *marcescens*	ACA	1	9.1
	M17	3	27.3
	VRBG	7	63.6
L. multifermentans	CBA	1	10.0
	ACA+2% milk powder	5	50.0
	PCA+2% milk powder	4	40.0
Aci. bouvetti	VRBG	4	44.4
	ACA+2% milk powder	5	55.6
Rot. marina	TSA+4.5% NaCl	3	37.5
	CBA	2	25.0
	M17	3	37.5
Leuc. pseudomesen-teroides	ACA	2	33.3
	PCA+2% milk powder	4	66.7
Raoult. ornithinolytica	VRBG	2	33.3
	ACA	1	16.7
	TSA+4.5% NaCl	3	50.0
Cit. werkmanii	M17 AE	1	20.0
	VRBG	3	60.0
	PCA+2% milk powder	1	20.0
Cit. freundii	ACA	1	33.3
	VRBG	2	66.7
Klebsiella oxytoca	M17 AE	2	66.7
	VRBG	1	33.3

St. capitis subsp. *capitis*	CBA	3	100.0
Ent. asburiae	PCA+2% milk powder	1	50.0
	MSA	1	50.0
Ent. cloacae subsp. *clocae*	VRBG	1	50.0
	TSA+4.5% NaCl	1	50.0
S. salivarius	M17 AN	2	100.0
Chr. arthrosphaerae	PCA+2% milk powder	2	100.0
E. faecalis	PCA+2% milk powder	1	100.0
Lb. rhamnosus	MRS	1	100.0
Koc. salsicia	PCA+2% milk powder	1	100.0
Mor. osloensis	PCA+2% milk powder	1	100.0
Emp. falsenii	PCA+2% milk powder	1	100.0

Distribution of the species across the four samples

Overall, the microbiota isolated from the four camel milk samples analyzed belonged to the phyla of *Firmicutes* (64.7%), *Proteobacteria* (30.0%), *Actinobacteria* (4.9%), and *Bacteroidetes* (0.4%). The *Firmicutes* were dominated by members of *Streptococcaceae* (37.7%), *Enterococcaceae* (18.8%), *Staphylococcaceae* (7.1%), *Leuconostocaceae* (0.7%), and *Lactobacillaceae* (0.1%). The LAB species identified were assigned to the genus *Lactococcus*, *Enterococcus*, *Streptococcus* and *Leuconostoc*, with *Lactococcus* (*L.*) *lactis* subsp. *lactis* (32.6%) as dominant organism in each of the samples. Also *Enterococcus* (*E.*) *faecium* (13.7%) was dominantly isolated although it was not isolated from the Tazarin sample. Species belonging to the *Staphylococcaceae* family were retrieved in the sample collected from Tazarin only and belonged to the genera *Staphylococcus* and *Macrococcus*.

Also members of the *Enterobacteriaceae* (26.5%) family which belong to the phylum of *Proteobacteria* (30.0%) were dominantly present in all four samples. Members of the *Actinobacteria* (4.9%) and *Bacteroidetes* (0.4%) phyla were isolated only sporadically from some of the samples.

The distribution of the species across the four samples is shown in Tab. 6.5. The number of the species retrieved varied between seven and eighteen. The highest diversity was observed for the sample collected from Laayoune with eighteen different species, identified as members of the *Enterobacteriaceae*, LAB, *Flavobacteriaceae* and *Pseudomonaceae*. Isolates of samples from Zagora and Dakhla represented twelve and ten species, respectively. Those of the sample from Zagora belonged to the the *Actinobacteria*, LAB, *Staphylococcaceae* and *Enterobacteriaceae*, while those of the sample from

Dakhla were restricted to members of the *Enterobacteriaceae* and LAB. A lower diversity was observed in the sample from Tazarin with seven species only, identified as members of LAB, *Enterobacteriaceae* and *Staphylococcaceae.*

Tab. 6. 5: Distribution of species and corresponding numbers of isolates across the different samples.

Sampling sites	N° of clusters	Total N° of isolates	Species retrieved	N° of isolates	% per sample
Tazarin	22	220	*Lactococcus lactis* subsp. *lactis*	119	54.1
			Escherichia/Shigella	48	21.8
			Staphylococcus aureus subsp. *aureus*	21	9.5
			Staphylococcus simulans	16	7.3
			Serratia marcescens subsp. *marcescens*	11	5.0
			Staphylococcus capitis	3	1.4
			Klebsiella oxytoca	2	0.9
Laayoune	38	204	*Enterobacter xiangfangensis*	37	18.2
			Lactococcus lactis subsp. *lactis*	29	14.2
			Streptococcus suis	27	13.2
			Enterococcus faecium	27	13.2
			Escherichia/Shigella	20	9.8
			Enterococcus italicus	18	8.8
			Aeromonas caviae	16	7.8
			Lactococcus multifermentans	10	4.9
			Raoultella ornithinolytica	6	2.9
			Enterobacter hormaechei	3	1.5
			Enterobacter cloacae	2	1.0
			Enterobacter asburiae	2	1.0
			Chryseobacterium arthrosphorae	2	1.0
			Empedobacter falsennii	1	0.5
			Citrobacter freundii	1	0.5
			Klebsiella oxytoca	1	0.5
			Kocuria salsicia	1	0.5
			Moraxella osloensis	1	0.5
Dakhla	31	200	*Lactococcus lactis* subsp. *lactis*	111	56.0
			Enterobacter hormaechei	34	17.0
			Enterococcus bulliens	20	10.0
			Enterococcus faecium	18	9.0
			Enterobacter xiangfangensis	7	3.5
			Leuconostoc pseudomesenteroides	4	2.0
			Streptococcus suis	2	1.0
			Streptococcus salivarius	2	1.0
			Lactobacillus rhamnosus	1	0.5
Zagora	27	184	*Enterococcus faecium*	65	36.4

			Rothia endophytica	31	16.8
			Pseudomonas moraviensis	20	10.9
			Macrococcus caseolyticus	17	9.2
			Pantoea agglomerans	15	8.1
			Acinetobacter bouvettii	9	4.9
			Lactococcus lactis subsp. *lactis*	9	4.9
			Rothia marina	8	4.3
			Citrobacter werkmanii	5	2.7
			Citrobacter freundii	2	1.0
			Leuconostoc pseudomesenteroides	2	1.0
			Enterococcus faecalis	1	0.5

Discussion

At present, the overall microbiota of raw camel milk produced in Morocco is not known. The few studies thus far examined rather low numbers of LAB isolates solely which were identified using biochemical methods only (Benkerroum et al. 2003; Khedid et al. 2009). The latter methods are known to have an inadequate taxonomical resolution for accurate species level identification of these microorganisms (Cleenwerck & De Vos, 2008; De Bruyne et al. 2008; Nguyen et al. 2012; Nhung et al. 2007; Vandamme et al. 1996). The present study represents a first detailed investigation of the bacterial microbiota of raw camel milk produced in Morocco. The use of MALDI-TOF MS as a high-throughput dereplication technique allowed to compare fingerprints of hundreds of isolates grown in multiple culture conditions and to reduce these isolates to a non-redundant set of species that were further identified using sequence analysis of various genes (Dieckmann et al. 2005; Spitaels et al. 2014; Vandamme et al. 1996). This approach facilitated an in depth analysis of the cultivable microbiota of this ecosystem and resulted in the isolation and description of two novel LAB species, i.e., *E. bulliens* sp. nov., and *L. multifermentans* sp. nov. (see chapters 6.2 and 6.3, respectively).

Generally, the bacterial species recovered from camel milk samples belonged to four phyla, which corresponded to results of previous studies in bacterial community composition in bulk tank raw cow milk (McInnis et al. 2015) and raw goat milk (Weber et al. 2014).

The analyzed samples contained high numbers of LAB and were dominated by cocci, which confirmed results of Benkerroum et al. (2003). No lactobacilli were isolated except for a single *Lb. rhamnosus* strain. In contrast, Khedid et al. (2009) analysed 120 LAB isolates associated with the production of raw camel milk using phenotypic characterization only. The LAB isolates were dominated by members of the *Lactobacillus* genus. The discrepancy between both studies may be explained by the different nutritional and environmental conditions of the dromedary camels, different sampling areas, different culture conditions and the lack of specificity of the phenotypic techniques used for the identification.

L. lactis subsp. *lactis* was the main LAB species recovered from all four samples. *L. lactis* subsp. *lactis* strains are important in food technology (Garvie, 1984; Salama et

al. 1991) and many bacteriocin producing *Lactococcus* strains have been used successfully as starter cultures for cheese to improve the safety and quality of the product (Delves-Broughton et al. 1996; Ryan et al. 1996). Enterococci too were dominantly present in three of the samples. Their presence may be an indicator of inadequate sanitary conditions during milk production and processing and some enterococci are considered apportunistic pathogens (Heikens et al. 2011; Protonotariou et al. 2010). Yet, enterococci produce a variety of products such as aromatic compounds (Centeno et al. 1999), enzymes (Ghrairi et al. 2008) and bacteriocins (enterocins) (Achemchem et al. 2005). They contribute to the texture, flavor, aroma and safety of different foods as in the case of handmade cheeses (Andrighetto et al. 2001) and additional fermented products (Giraffa, 2000; Gardini et al. 2001; Gomes et al. 2008). *Leuconostoc* strains too, although isolated in small numbers in the present study, are frequently used as starter cultures for butter, cream and cheese (Leroy and De Vuyst, 2004; Tamime and Marshall, 1997).

Also *Enterobacteriaceae* were dominantly present in the raw camel milk samples examined. Some of these species were previously reported in camel milk (Abdelgadir et al. 2005; Adugna et al. 2013; Barbour et al. 1985; Eberlein, 2007; Omer and Eltinay, 2008; Saad and Thabet, 1993; Semereab & Molla, 2001;Yam et al. 2014) and like enterococci they are considered indicators of poor sanitary methods of food processing (Castillo et al. 2006; Prema et al. 2005). The growth and metabolic activity of *Enterobacteriaceae* in milk and dairy products can result in off-flavours, odours, colour defects and other organoleptic deviations (Baylis et al. 2011; Robinson, 1990; Schmidt-Lorenz & Spillmann, 1988) and, again like enterococci, they are opportunistic pathogens, which can be responsible for urinary tract and intestinal infections but also cerebral abscesses, pneumonia, meningitis, septicemia and bacteremia (Farmer et al. 2007; Russo & Johnson, 2008). However, *Enterobacteriaceae* are not particularly heat resistant and thus all are easily eliminated from milk by pasteurization or equivalent heat treatments (Joklik et al. 1992; Robinson, 1990).

Staphylococci too were detected in some of the samples examined confirming earlier studies (Abdelgadir et al. 2005; Benkerroum et al. 2003; Elgadi et al. 2008; El-Ziney and Al-Turki, 2007; Jans et al. 2012; Khedid et al. 2009; Semereab and Molla, 2001; Shuiep et al. 2009; Tuteja et al. 2003; Yam et al. 2014). They can be transferred to milk through the teat canal, equipment, the environment or human handling (Rosengren et al. 2010) and cause illness through the production of heat-stable enterotoxins, which

can withstand pasteurisation (Balaban & Rasooly, 2000). Genera belonging to the *Pseudomonaceae* were present such as *Pseudomonas* and *Acinetobacter* spp. (Raats et al. 2011). They have already been reported in camel milk (Adugna et al. 2013; Elhaj et al. 2014; Zahran and Al-Saleh, 1997) and are a common cause of milk spoilage (Adugna et al. 2013; Ercolini et al. 2009; Hafez et al. 1987; Raats et al. 2011; Quigley et al. 2013). Other organisms like *Actinobacteria* and members of the *Flavobacteriaceae* family were isolated in low numbers. As far as we know, this is the first report of *Rothia* and *Kocuria* strains and of members of the *Flavobacteriaceae* family isolated from camel milk. Dairy flavobacteria are considered problematic as they are psychrotolerant and produce proteinase in refrigerated milk (Jooste et al. 1986).

Conclusion

In the present study we present a first detailed description of the bacterial microbiota of raw camel milk. To this end we collected 808 isolates from 10 combinations of selective and general growth media and incubation conditions and used MALDI-TOF MS for dereplication, thus performing a modest culturomics approach (Lagier et al. 2012). The dereplicated clusters of isolates were further identified through sequence analysis of 16S rRNA and protein encoding genes to achieve a state-of-the-art identification of all isolates. Our study revealed a considerable bacterial diversity consisting of members of the phyla *Firmicutes*, *Proteobacteria*, *Actinobacteria* and *Bacteroidetes.* We demonstrated the presence of typical dairy LAB, as well as a range of bacteria that are considered indicators of poor hygienic conditions like enterococci and members of the *Enterobacteriaceae* family. In addition, psychrotrophic spoilage organisms like pseudomonads and members of the *Flavobacteriaceae* were detected as well.

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CHAPTER 6: BACTERIAL DIVERSITY OF CAMEL MILK.

6.2 Description of a novel *Enterococcus* species: *Enterococcus bulliens* sp. nov., a novel lactic acid bacterium isolated from camel milk

Redrafted from: Zaina Kadri, Freek Spitaels, Margo Cnockaert, Jessy Praet, Omar El Farricha, Jean Swings and Peter Vandamme. *Enterococcus bulliens* sp. nov., a novel lactic acid bacterium isolated from camel milk.

Antonie van Leeuwenhoek. doi 10.1007/s10482-015-0579-z

PREAMBLE

In the course of the camel milk bacterial diversity study, four LAB isolates remained unidentified after MALDI-TOF MS fingerprinting, 16S rRNA gene sequencing and sequence analysis of the protein encoding genes *pheS*, *rpoA* and *atpA*. These isolates were further studied using a polyphasic identification approach combining RAPD analysis, DNA-DNA hybridization experiments, determination of the DNA base composition (% G+C) and a detailed phenotypic characterisation which allowed the description of novel *Enterococcus* species for which we proposed the name *Enterococcus bulliens* sp. nov.

OUTLINE CONTRIBUTIONS

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Abstract

Four lactic acid bacteria isolates obtained from fresh dromedary camel milk produced in Dakhla, a city in southern Morocco, were characterised in order to determine their taxonomic position. The four isolates had highly similar MALDI-TOF MS and RAPD fingerprints and identical 16S rRNA gene sequences. Comparative sequence analysis revealed that the 16S rRNA gene sequence of the four isolates was most similar to that of *Enterococcus sulfureus* ATCC 49903^T and *Enterococcus italicus* DSM 15952^T (99.33% and 98.59% similarity, respectively). However, sequence analysis of the phenylalanyl-tRNA synthase (*pheS*), RNA polymerase (*rpoA*) and ATP synthase (*atpA*) genes revealed that the taxon represented by strain LMG 28766^T was well separated from *E. sulfureus* LMG 13084^T and *E. italicus* LMG 22039^T, which was further confirmed by DNA-DNA hybridization values that were clearly below the species demarcation threshold. The novel taxon was easily differentiated from its nearest neighbour species through sequence analysis of protein encoding genes, MALDI-TOF mass spectrometry and multiple biochemical tests, but had a similar percentage G+C content of about 39%. We therefore propose to formally classify these isolates as *Enterococcus bulliens* sp. nov., with LMG 28766^T (=CCMM B1177^T) as the type strain.

Key words: *Enterococcus bulliens* sp. nov.; lactic acid bacteria; camel milk microbiota; fresh dromedary milk; taxonomy.

Introduction

The genus *Enterococcus*, belonging to the family *Enterococcaceae*, was first described by Schleifer and Kilpper-Balz, (1984) and consists at present of 49 species with validly published names (Parte, 2014; http://www.bacterio.net/*enterococcus*.html). Species of the genus *Enterococcus* are Gram-positive, facultatively anaerobic micro-organisms, with a fermentative metabolism resulting in L (+)-lactic acid as the major product of glucose fermentation. They are generally catalase-negative cocci, although some strains exhibit catalase or pseudo-catalase activity (Hardie & Wiley, 1997; Rurangirwa et al. 2000; Svec et al. 2001; Svec & Devriese, 2009). Enterococci represent an important group of lactic acid bacteria found ubiquitously in the environment, the gastrointestinal tract, traditional fermented foods and in dairy products, where they may predominate with respect to lactobacilli and lactococci (Fortina et al. 2004; Franz et al. 1999; Giraffa, 2002). The presence of enterococci in dairy products has long been considered an indicator of inadequate sanitary conditions during the production and processing of milk. However, enterococci may have a desirable role in some cheeses, because of their proteolytic and lipolytic activities, in the development of typical flavours, and for the production of enterocins with anti-*Listeria* activity (Ennahar & Deschamps, 2000; Fortina et al. 2004; Giraffa et al. 1997). The present study describes the taxonomic characterisation of four *Enterococcus* strains isolated from raw camel milk.

Materials and methods

Collection of raw camel milk samples and isolation of strains

Samples of fresh dromedary milk were collected in Dakhla, a city in southern Morocco (23°43′N 15°57′W). Five percent DMSO solution was added to the samples which were homogenized and stored at -80°C until further use (Kerckhof et al. 2014). Tenfold dilutions in physiological saline (0.85 % NaCl) were plated on M17 agar, Plate Count Agar supplemented with 2% of milk powder (PCA+2% milk powder), All Culture (AC) agar and Tryptone Soy Agar supplemented with 4.5% NaCl (TSA+4.5% NaCl), each containing 5 ppm amphotericin B to prevent fungal growth. Inoculated plates were incubated anaerobically (M17) at 37°C, or aerobically (PCA+2% milk powder, AC agar, and TSA+4.5% NaCl) at 28°C.

After 24h of incubation, colonies were picked from the agar plates and were dereplicated using matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) as described previously (Nguyen et al. 2013; Spitaels et al.

2014). Species level identification of most isolates was achieved through comparison of the obtained spectra with those of an in-house database of the LM-UGent research group. Isolates which remained unidentified were further examined using sequence analysis of 16S rRNA and protein encoding genes as described below.

Representative isolates of four MALDI-TOF MS clusters that remained unidentified after comparative sequence analysis, i.e. LMG 28766^T, LMG 28912, R-55003 and R-55006, were chosen for further analyses. These representatives were recovered from the same milk sample but were isolated from the four different culture media described above. Isolates were routinely incubated aerobically on BHI agar at 37°C together with the following reference strains: *Enterococcus sulfureus* LMG 13084^T, *Enterococcus italicus* LMG 22039^T and *Enterococcus camelliae* LMG 24745^T.

RAPD-PCR

Random amplified polymorphic DNA (RAPD) analysis was performed on the isolates LMG 28766^T, LMG 28912, R-55003 and R-55006, and the *E. sulfureus* and *E. italicus* type strains using primer RAPD-272 (5'-AGCGGGCCAA-3') as previously described (Williams et al. 1990).

Phylogenetic analyses

The 16S rRNA gene sequences of all four isolates were determined as previously described (Snauwaert et al. 2013) and analysed by means of the EzTaxon-e database software (Kim et al. 2012). The obtained sequences and those of the reference strains (which were publicly available) were aligned using the SILVA Incremental Aligner (SINA v1.2.11) (http://www.arb-silva.de/aligner/) (Pruesse et al. 2012), with the corresponding SILVA SSURef 111 database (Pruesse et al. 2007). Partial *pheS*, *rpoA* and *atpA* sequences were generated for all four isolates, according to the method described by Naser et al. (2005a,b); the corresponding sequences of their nearest phylogenetic neighbours were again publicly available. The pairwise sequence similarity values of the *pheS*, *rpoA* and *atpA* sequences of the four isolates and the type strains of *E. sulfureus* and *E. italicus* were then calculated using the MEGA 5.2.2 software package. In order to construct phylogenetic trees, the sequences of the novel isolates and of selected enterococcal type strains were aligned by MUSCLE using the MEGA 5.2.2 software (Tamura et al. 2011). The MEGA 5.2.2 software package was used to obtain phylogenetic trees by using the maximum-likelihood method. A discrete Gamma distribution

was used to model evolutionary rate differences among sites. Tree topologies were analysed statistically using 1000 bootstrapping replication.

DNA–DNA hybridization and DNA % G+C content analysis

High-molecular weight DNA was prepared as described by Vancanneyt et al. (2004). DNA-DNA hybridization studies between strain LMG 28766^T and the *E. sulfureus* and *E. italicus* type strains were performed with biotin-labeled probes in microplate wells (Ezaki et al. 1989) by using an HTS7000 Bio Assay Reader (Perkin Elmer) for the fluorescence measurements. The hybridization temperature was 37°C in the presence of 50% formamide. Analysis of the DNA base ratio was determined by HPLC according to Mesbah et al. (1989).

Genbank accession numbers

The GenBank accession numbers for the 16S rRNA, *pheS*, *rpoA* and *atpA* sequences of strains LMG 28766^T, LMG 28912, R-55003 and R-55006 are: KR827627, KR827650, KR827665, KR827638; KR827628, KR827651, KR827666, KR827639; KR827629, KR827652, KR827667, KR827640; and KR827630, KR827653, KR827668, KR827641, respectively.

Phenotypic characterisation

Cell shape, size and arrangement and colony appearance were examined using cells grown on BHI agar for 24h. Motility was checked with light microscopy. Gram-stain behavior, endospore staining and presence of oxidase and catalase activities were performed using standard microbiological procedures (Mac Faddin, 1980). Hemolytic activity was tested on Columbia agar base (BBL) supplemented with 5% sheep blood for 24 h at 37°C in a 5% CO_2 enriched atmosphere (Oxoid CD0025A gas pack). Growth was determined in BHI agar at 10, 28, 37 and 45°C after 24-48h of incubation. Tolerance of NaCl was determined in BHI broth containing 2.0, 4.0, 6.0, 6.5, 8.0 and 10.0 % (w/v) NaCl at 37°C after 24-48h of incubation. Growth at pH 4, 5, 6, 7, 8 and 9 was determined at 37°C after 24-48h of incubation using M17 broth without di-sodium-glycerophosphate. Production of short chain fatty acids was determined after growth in BHI broth for 24h as described by De Baere et al. (2013). The D-/L-lactic acid (Rapid) Assay Kit (Megazyme) was used to determine D- or L-lactic acid production. The API 50 CHL, API 20 STREP and APY ZYM microtest systems were used according to the recommendations of the manufacturer (API bioMérieux, France) to characterise enzymatic activities and substrate utilisation of the four novel isolates and of the type strain

of their close phylogenetic neighbours, *E. sulfureus* LMG 13084^T, *E. italicus* LMG 22039^T and *E. camelliae* LMG 24745^T.

Results and discussion

In the course of a study of dromedary camel milk microbiota, four isolates (LMG 28766^T, LMG 28912, R-55003 and R-55006) were obtained from a single raw milk sample produced in southern Morocco. The four isolates had highly similar RAPD (Fig 6.1) and MALDI-TOF MS (Fig 6.2) fingerprints, and identical 16S rRNA gene sequences.

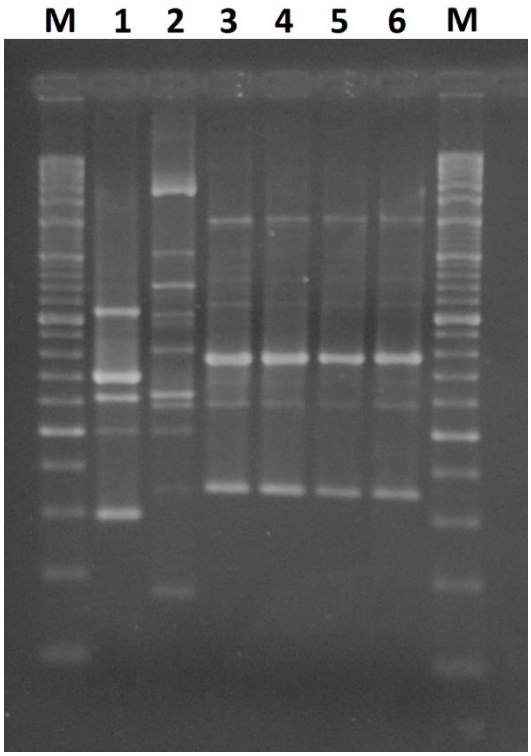

Fig 6. 1: RAPD fingerprints of *E. italicus* LMG 22039^T (lane 1), *E. sulfureus* LMG 13084^T (lane 2), *E. bulliens* sp. nov., isolates LMG 28766^T (lane 3), LMG 28912 (lane 4), R-55003 (lane 5) and R-55006 (lane 6). Lane M corresponds to the reference marker.

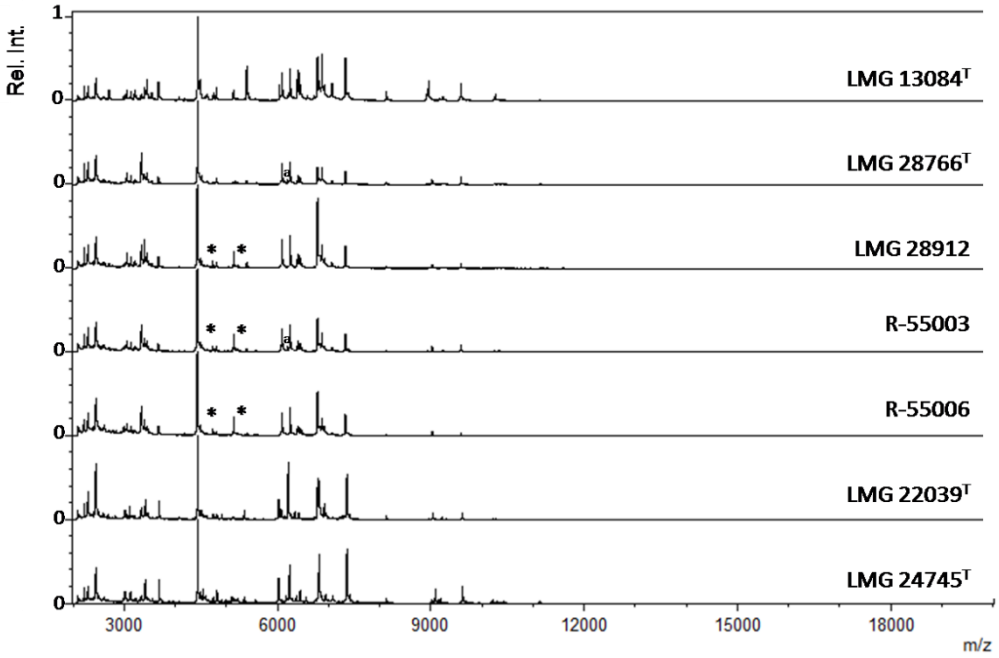

Fig 6. 2: MALDI-TOF MS profiles ranging from m/z 2000 to 20000 from crude cell extracts prepared from *E. sulfureus* LMG 13084^T, *E. bulliens* sp. nov., strains LMG 28766^T, LMG 28912, R-55003 and R-55006, *E. italicus* LMG 22039^T and *E. camelliae* LMG 24745^T. The figure was created with the mMass v5.5.0 software package (Strohalm et al. 2010). * indicates the peaks at m/z 4724 and m/z 5135 that are present in all isolates except for LMG 28766^T. [a] indicates the minor peak at m/z 6190 that is uniquely present in LMG 28766^T and R-55003.

Comparative 16S rRNA gene sequence analysis revealed that they belong to the genus *Enterococcus* with *E. sulfureus* and *E. italicus* as nearest neighbour species (99.33% and 98.59% sequence similarity towards the corresponding type strains, respectively). A 16S rRNA gene sequence based phylogenetic tree demonstrated that the four novel isolates and the *E. sulfureus* type strain represented a coherent lineage supported by a bootstrap value of 99% (Fig 6.3). Protein encoding genes such as *pheS*, *rpoA* and *atpA*, which are commonly used in taxonomic and identification studies of the genus *Enterococcus*, evolve at a higher pace and have a taxonomic discriminatory power superior to that of the more conserved 16S rRNA gene (Li et al. 2014; Naser et al. 2005b; Niemi et al. 2012). The *rpoA* gene sequences of the four isolates were identical. In addition, the *pheS* and *atpA* gene sequences of the isolates LMG 28766^T and R-55003 were identical as well; similarly, the *pheS* and *atpA* genes sequences of the isolates LMG 28912 and R-55006 were also identical, and were 98% and 99.9% similar to those of the former two isolates. The pairwise sequence similarity values of the *pheS*, *rpoA* and *atpA* sequences of strain LMG 28766^T towards *E. sulfureus* LMG 13084^T were 81.6 %, 98.9% and 86.0%, respectively, and the corresponding pairwise sequence similarity values towards *E. italicus* LMG 22039^T were 74.0%, 88.0% and 75.6%, respectively.

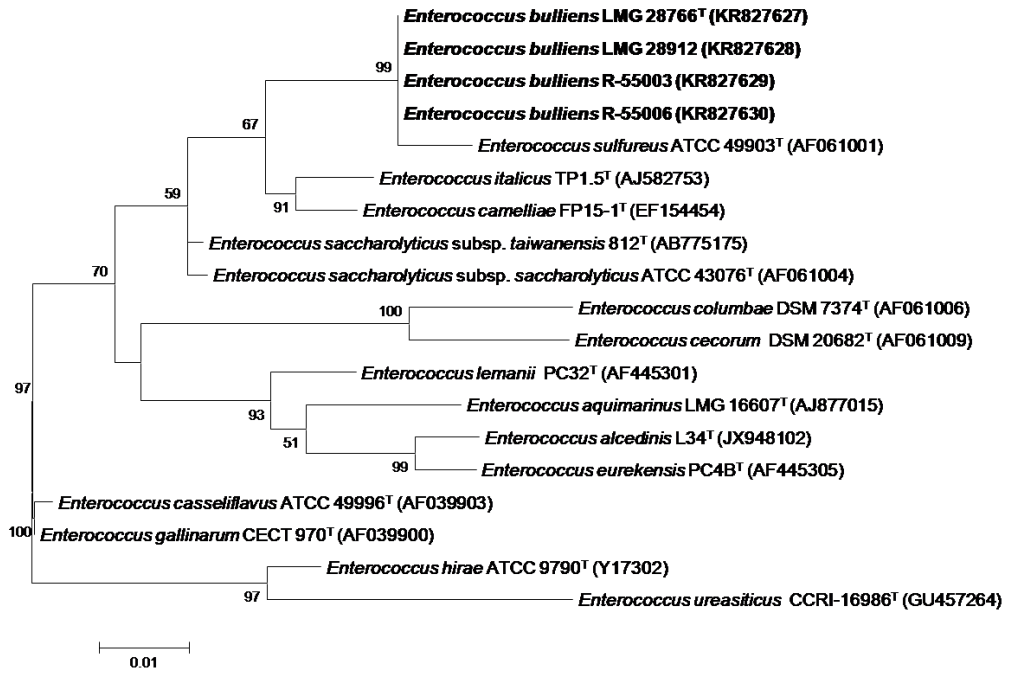

Fig 6. 3: Maximum-likelihood phylogenetic tree based on 16S rRNA gene sequences of isolates LMG 28766^T, LMG 28912, R-55003, R-55006 and selected type strains of the genus *Enterococcus.* Bootstrap values based on 1000 replications are indicated at the nodes. GenBank accession numbers are given in parentheses. Values <50% are not shown. Bar, 0.01 substitutions per site.

The higher taxonomic resolution of these protein encoding genes is illustrated in Fig 6.4 which shows a maximum-likelihood phylogenetic tree based on *pheS* gene sequences (maximum-likelihood phylogenetic trees based on *rpoA* and *atpA* gene sequences are shown in Fig 6.5 and 6.6). The obtained similarity values suggested that the four isolates represent a single genospecies that is different from its near phylogenetic neighbours. This was confirmed by DNA-DNA hybridization experiments which revealed hybridization values of 34 ± 4 % and 22 ± 11 % between strain LMG 28766^T and the type strains of *E. sulfureus* and *E. italicus,* respectively. The DNA G+C content of strain LMG 28766^T was determined to be 38.7 mol%, which is within the range (35.1-44.9 mol%) reported for the members of the genus *Enterococcus* (Švec & Devriese, 2009) and which is similar to that of *E. sulfureus* LMG 13084^T (38 mol% [Martinez-Murcia & Collins, 1991]) and *E. italicus* LMG 22039^T (39.9-41.1 mol% [Fortina et al. 2004]).

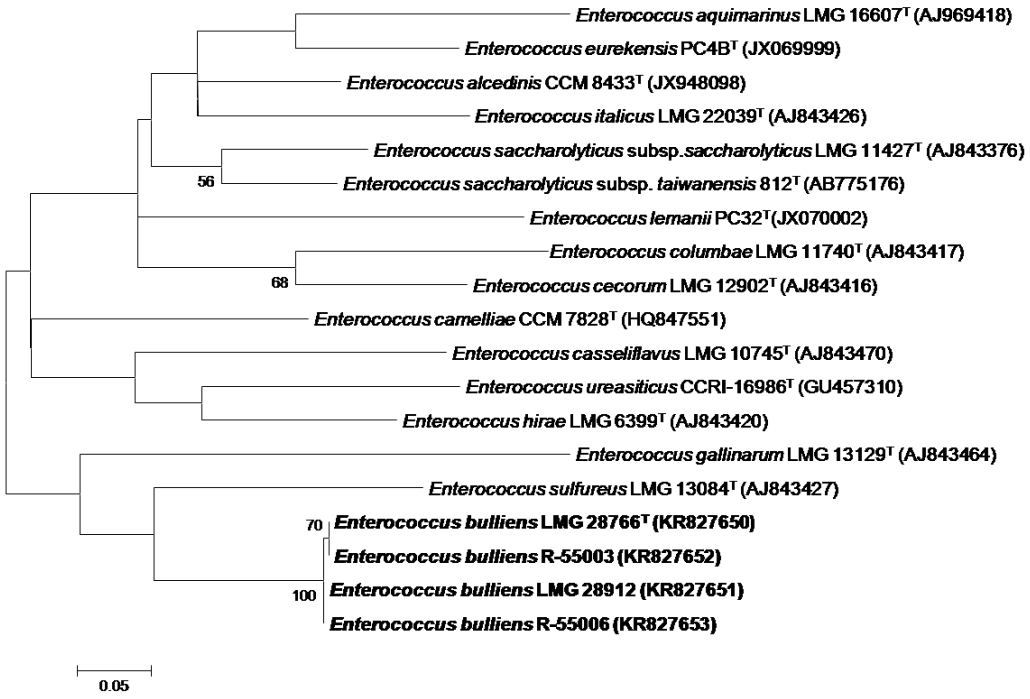

Fig 6. 4: Maximum-likelihood phylogenetic tree based on *pheS* gene sequences of strains LMG 28766^T, LMG 28912, R-55003, R-55006, and the selected type strains of the genus *Enterococcus.* Bootstrap values based on 1000 replications are indicated at the nodes. GenBank accession numbers are given in parentheses. Values <50% are not shown. Bar, 0.05 substitutions per site.

The MALDI-TOF MS experiments allowed a very straightforward separation of the four isolates from the type strains of their near phylogenetic neighbour species (Fig 6.2) and revealed additional differences among the four isolates, for example discriminative peaks that are uniquely absent (m/z 5135 [Fig 6.2 and 6.7]) in the profile acquired from the type strain LMG 28766^T. Also, the type strain LMG 28766^T shared two peaks at m/z 6190 (Fig 6.2) and m/z 6449 (Fig 6.8) with strain R-55003 which are absent in the profiles of the remaining isolates.

Biochemical characteristics were determined for all four isolates and for *E. sulfureus* LMG 13084^T, *E. italicus* LMG 22039^T and *E. camelliae* LMG 24745^T. Tests that yielded strain dependent results and differential tests are shown in Tab. 6.6. Characteristics shared by all seven isolates are as follows: growth at 28 and 37°C, at pH 4, 5, 6, 7, 8 and 9, and in the presence of 2.0, 4.0 and 6.0 % NaCl; production of acid from D-galactose, D-glucose, D-fructose, D-mannose, N-acetyl glucosamine, arbutin, salicin, cellobiose, D-maltose, lactose, sucrose, trehalose, gentiobiose, acetoin; and activity of hippuricase, arginine dihydrolase, acid phosphatase and aesculin hydrolysis. In addition, the following characteristics were uniformly absent: growth at 45 °C; acid production from glycerol, erythritol, D-arabinose, L- arabinose, D-xylose, L-xylose, adonitol,

methyl D-xyloside, L-sorbose, dulcitol, L-rhamnose, inositol, methyl-D-mannoside, inulin, starch, D-sorbitol, glycogen, xylitol, D-turanose, D-lyxose, D-tagatose, D-fucose, L-fucose, L-arabitol, 2- and 5-ketogluconate; and activity of lipase (C14), trypsin, α-chymotrypsin, α-mannosidase, β-glucuronidase, α-fucosidase, valine arylamidase, cystine arylamidase and N-acetyl-β-glucosaminidase.

In conclusion, the data reported here demonstrate that the four isolates from dromedary camel milk represent a novel species belonging to the genus *Enterococcus*. Overall, they conform to the general phenotypic characteristics of this genus. Indeed, the cells are ovoid, Gram-positive and occur in pairs or in short chains (Hardie & Whiley, 1997; Fisher & Phillips, 2009). They are facultatively anaerobic with optimum growth at 37 °C, the ability to grow at 10°C, in 6.5% NaCl broth and at pH 4 and pH 9 (Hardie & Whiley, 1997). They produce L-lactic acid as the major product of glucose fermentation, hydrolyse esculin and have a similar percentage G+C content of about 39% (Hardie & Whiley, 1997). This novel species is closely related to *E. sulfureus* and can easily be differentiated from its near neighbour species through sequence analysis of protein encoding genes (Fig 6.4, 6.5 and 6.6), MALDI-TOF mass spectrometry (Fig 6.2, 6.7 and 6.8), and multiple biochemical tests (Tab. 6.6). The four isolates had identical 16S rRNA and *rpoA* gene sequences but revealed differences in their *pheS* and *atpA* gene sequences, MALDI-TOF MS profiles and biochemical characteristics, suggesting they represent multiple different strains. We therefore propose to formally classify these four isolates as a novel *Enterococcus* species for which we propose the name *Enterococcus bulliens* sp. nov., with LMG 28766^T (=CCMM B1177^T) as the type strain.

Description of *Enterococcus bulliens* sp. nov.

Enterococcus bulliens (bul'li.ens L. part. adj. *bulliens*, boiling, bubbling; referring to the gas formation in the catalase test after cultivation on blood agar media).

Cells are Gram-stain positive, oxidase negative, non-spore-forming, non-motile and ovoid shaped. They are 1.2-1.5 µm in diameter and arranged in pairs or in short chains. Colonies are yellow pigmented, round with raised margins, smooth and convex. Non-hemolytic on blood agar at 37°C. Catalase activity is clearly evident when cultivated on blood agar but cells grown on blood-free medium are catalase-negative. Produces L- lactic acid and acetic acid in the ratio of 9.6:1. Grows on BHI agar and M17 agar at 37°C and is facultative anaerobic. Grows at 10, 28 and 37°C but not at 45°C. Growth is observed at pH 4, 5, 6, 7, 8 and 9 and in the presence of 2, 4, 6 and 6.5 %

NaCl, but not at 8 and 10% NaCl. In API 50 CHL, API 20 Strep and API ZYM tests, acid is produced from ribose, raffinose, galactose, D-glucose, D-fructose, D-mannose, N-acetyl glucosamine, amygdalin, arbutin, salicin, cellobiose, D-maltose, lactose, trehalose, sucrose and gentiobiose but not from glycerol, erythritol, D-arabinose, L-arabinose, D-xylose, L-xylose, adonitol, methyl D-xyloside, L-sorbose, L-rhamnose, dulcitol, inositol, D-sorbitol, methyl-D-mannoside, inulin, starch, glycogen, xylitol, D-turanose, D-lyxose, D-tagatose, D-fucose, L-fucose, gluconate, L-arabitol, 2-5-ketogluconate or acetoin. Activity of α-galactosidase, hippuricase, arginine dihydrolase, acid phosphatase and aesculin hydrolysis is present but no leucine arylamidase, trypsin, α-chymotrypsin, α-mannosidase, α-fucosidase, valine arylamidase, cystine arylamidase, N-acetyl-β-glucosaminidase, lipase (C14) and β-glucuronidase activity. Acid production from D-mannitol, methyl-D-glucoside, melezitose, melibiose, and D-arabitol, and activity of esterase lipase (C8), esterase (C4), phosphoamidase, alkaline phosphatase, pyrrolidonyl arylamidase, α-glucosidase, β-glucosidase, β-galactosidase and leucine aminopeptidase are strain dependent. The type strain produces acid from D-mannitol, methyl-D-glucoside, melezitose, melibiose, and D-arabitol; and exhibits leucine aminopeptidase and β-galactosidase activities, weak activity of esterase lipase (C8), esterase (C4) and alkaline phosphatase, and no activity of phosphoamidase or pyrrolidonyl arylamidase. The DNA % G+C content of the type strain is 38.7 mol%.

The type strain is LMG 28766^{T} (=CCMM B1177^{T}) was isolated from a sample of raw dromedary camel milk sample produced in southern Morocco. The GenBank accession numbers for the 16S rRNA, *pheS, rpoA* and *atpA* sequences of strain LMG 28766^{T} are KR827627, KR827650, KR827665, KR827638.

TABLES

Tab. 6. 6: Characteristics that differentiate *Enterococcus bulliens* sp. nov. strains LMG 28766^T, LMG 28912, R-55003 and R-55006 from type strains of closely related phylogenetic relatives.

Taxa / Characteristic	1	2	3	4	5	6	7
Acid production from:							
Ribose	+	+	+	+	+	-	-
Amygdalin	+	+	+	+	+	-	+¶
Melezitose	+	-	-	-	- *	+°	-
D-Raffinose	+	+	+	+	+	-	-
D-Mannitol	+	-	-	-	-	+	+
Melibiose	+	+	-	+	+	-	-
Gluconate	-	-	-	-	+	-	- ¶
D-arabitol	+	-	-	-	-	-	-
Methyl D-glucoside	+	+	+	-	+	+	-
Production of:							
α-Galactosidase	+	+	+	+	+	-	+
α-Glucosidase	+	+	-	+	+	-	W
β- Glucosidase	+	+	-	+	+	-	W
Pyrrolidonyl arylami-	-	-	+	-	+	+	+
dase	-	+	+	-	+	W	W
Phosphoamidase	W	W	+	W	+	-	W
Esterase lipase (C8)	W	W	+	W	+	-	-
Alkaline phosphatase	W	W	+	W	+	-	W
Esterase (C4)	-	-	-	-	W	+	+
Leucine arylamidase	+[a]/-[b]	+[a]/-[b]	+[a]/-[b]	+[a]/-[b]	+[a]/-[b]	-	-
Catalase	+	-	-	-	-	+	+
Leucine amino pepti-	+	+	-	+	+	-	-
dase	+	+	+	+	+	-	+
β-Galactosidase	-	-	-	-	+	-	-
Growth at :							
6.5% NaCl	+	+	+	+	+	-	+
8% NaCl	-	-	-	-	+	-	-
10% NaCl	-	-	-	-	+	-	-
10°C	+	+	+	+	+	+	-
Ratio LA: AA	6.9:1	ND	ND	ND	4.8:1	2.5:1	2.6:1

Taxa: 1, *E. bulliens* LMG 28766^T; **2**, *E. bulliens* LMG 28912; **3**, *E. bulliens* R-55003; **4**, *E. bulliens* R-55006; **5**, *E. sulfureus* LMG 13084^T; **6**, *E. italicus* LMG 22039^T; **7**, *E. camelliae* LMG 24745^T. +, positive reaction; -, negative reaction; WR, weak reaction; ND, not determined.

[a] Catalase reaction when the strain is cultivated on blood medium
[b] Catalase reaction when the strain is cultivated on blood-free medium
* This result does not correspond with published data (Martinez-Murcia & Collins 1991)
° This result does not correspond with published data (Fortina et al. 2004)
¶ This result does not correspond with published data (Sukontasing et al. 2007)

Supplementary information

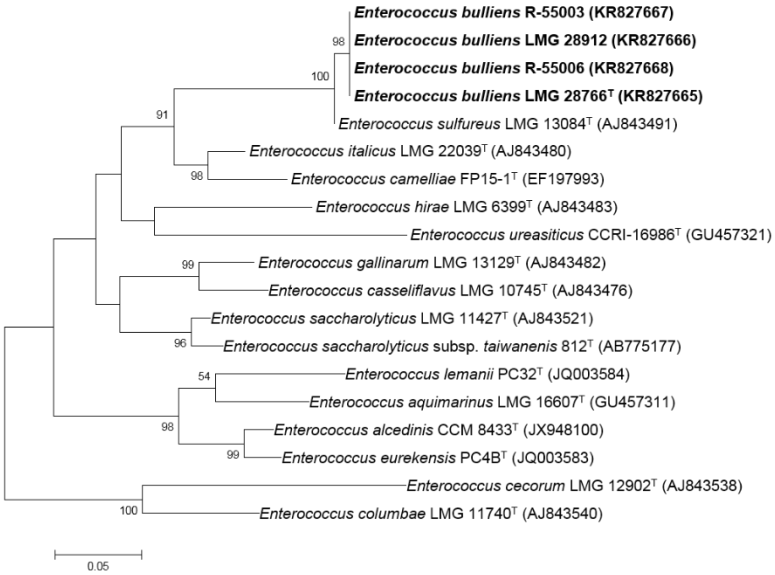

Fig 6. 5: Maximum-likelihood phylogenetic tree based on *rpoA* gene sequences of strains LMG 28766^T, LMG 28912, R-55003, R-55006, and the selected type strains of the genus *Enterococcus.* Bootstrap values based on 1000 replications are indicated at the nodes. GenBank accession numbers are given in parentheses. Values <50% are not shown. Bar, 0.05 substitutions per site.

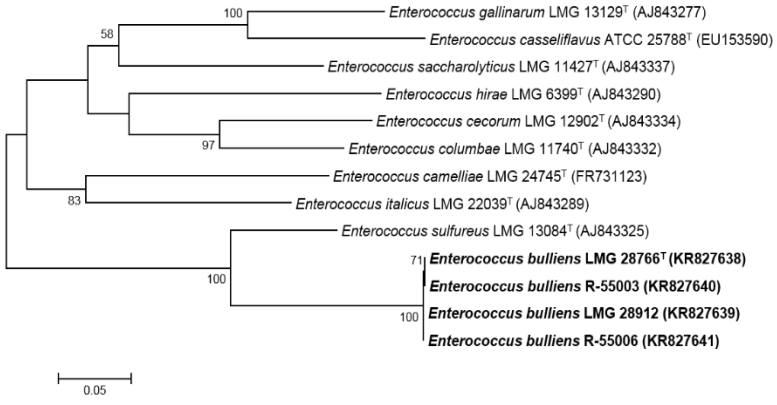

Fig 6. 6: Maximum-likelihood phylogenetic tree based on *atpA* gene sequences of strains LMG 28766^T, LMG 28912, R-55003, R-55006, and the selected type strains of the genus *Enterococcus.* Bootstrap values based on 1000 replications are indicated at the nodes. GenBank accession numbers are given in parentheses. Values <50% are not shown. Bar, 0.05 substitutions per site.

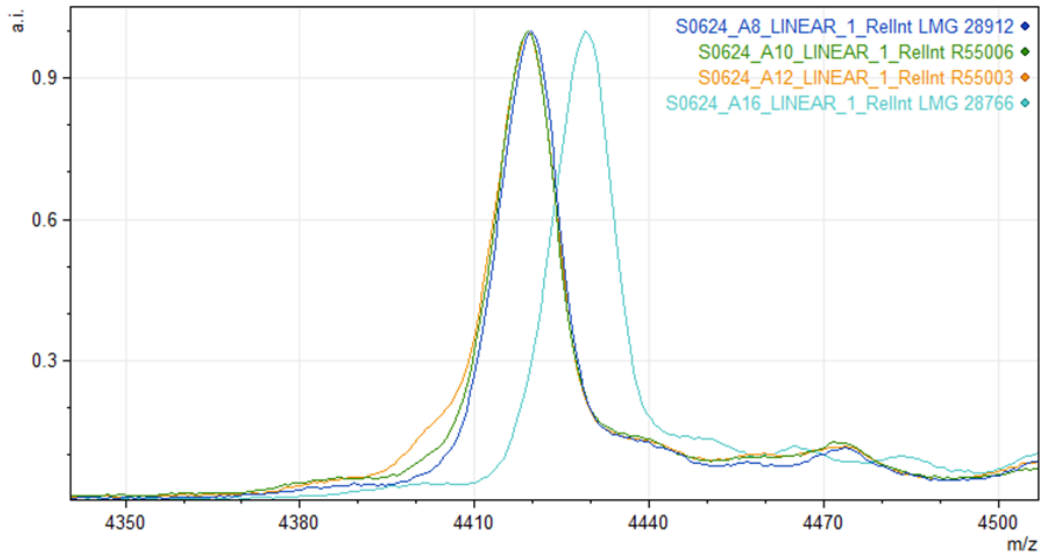

Fig 6. 7: MALDI-TOF MS profiles ranging from m/z 4350 to 4500 from crude cell extracts prepared from *E. bulliens* sp. nov., strains LMG 28766^{T}, LMG 28912, R-55003 and R-55006 showing the presence of an unique peak at m/z 4429 in the profile of LMG 28766^{T}. The figure was created with the mMass v5.5.0 software package (Strohalm et al. 2010).

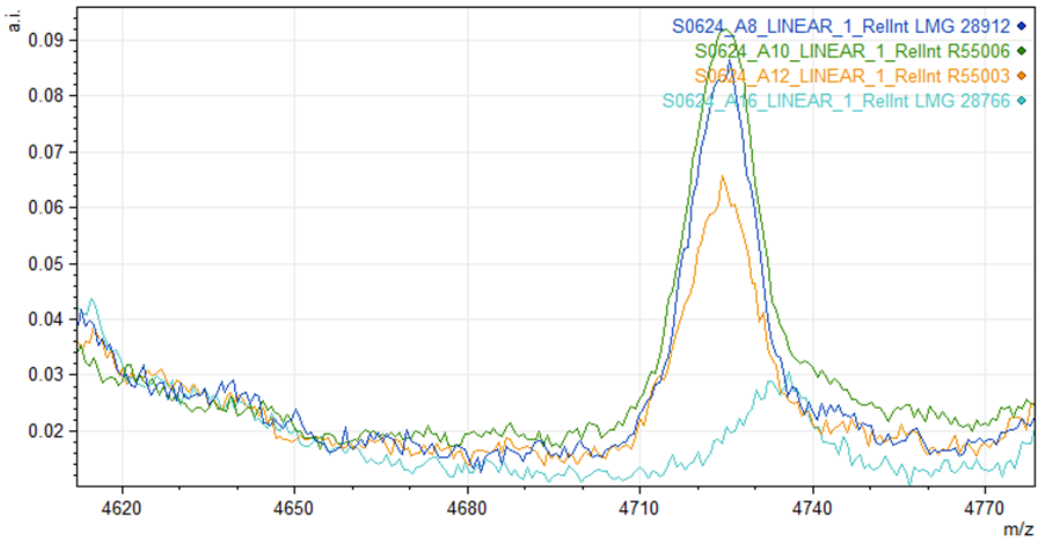

Fig 6. 8: MALDI-TOF MS profiles ranging from m/z 4620 to 4770 from crude cell extracts prepared from *E. bulliens* sp. nov., strains LMG 28766^{T}, LMG 28912, R-55003 and R-55006 showing the absence of a peak at m/z 4724 in the profile acquired for LMG 28766^{T}. The figure was created with the mMass v5.5.0 software package (Strohalm et al. 2010).

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CHAPTER 6: BACTERIAL DIVERSITY OF CAMEL MILK.

6.3 Description of a novel *Lactococcus* species: *Lactococcus multifermentans* sp. nov., a novel lactic acid bacterium isolated from camel milk.

Redrafted from: Zaina Kadri, Freek Spitaels, Margo Cnockaert, Omar El Farricha, Jean Swings, Mohamed Amar and Peter Vandamme

***Lactococcus multifermentans* sp. nov., a novel lactic acid bacterium isolated from camel milk.**

PREAMBLE

The study of microbiota occurring in raw camel milk produced in Southern Moroccco yielded two isolates that could not be assigned to a validly named species using MALDI-TOF mass spectrometry and sequence analysis of the 16S rRNA and *pheS* genes. A polyphasic taxonomic approach including RAPD analysis, DNA-DNA hybridization experiments, DNA % G+C content determination and a detailed physiological and biochemical characterization demonstrated that these two isolates represented a novel species for which we aimed to propose the name *Lactococcus multifermentans* sp. nov.

However, while finishing our PhD manuscript, a manuscript reporting two novel *Lactococcus* species, including *Lactococcus laudensis* sp. nov., was accepted for publication and appeared online as doi: 10.1099/ijs.0.000225 in July 2015 (Meucci et al. Int J Syst Evol Microbiol 65, 2091-2096). The *L. laudensis* type strain was isolated from cow milk in Italy, by using M17 agar incubated at 30°C for 18h under aerobic conditions. A comparison of the reported and our data demonstrated that *L. laudensis* 4195^T and *L. multifermentans* LMG 28767^T share many phenotypic characteristics. They are both non-spore-forming, non-motile and coccoid shaped and are about 1 µm in diameter. They both produce acid from amygdalin, galactose, lactose, sucrose, mannitol, gentiobiose and D-xylose. Their reaction was positive for aesculin hydrolysis, production of leucine aminopeptidase and acid phosphatase. However, they can be differentiated by acid production from ribose, L-arabinose and D-arabinose. In addition, *L. multifermentans* LMG 28767^T grows well at 45°C and at pH5, whereas *L. laudensis* did not grow at 40°C and growth at pH5 is weak. The DNA G+C content of *L. laudensis* was 41.1 mol%, which was quite distinct from that of *L. multifermentans* (36.95 mol%). Most compellingly however, the 16S rRNA sequence of the *L. laudensis* type strain (accession no: KJ394457) is 100% identical to that of the *L. multifermentans* type strain reported in the present study. Altogether these data demonstrate that the taxonomic relationships of both organisms should be verified before the study presented below can be submitted for publication.

OUTLINE CONTRIBUTIONS

Experimental design: Prof. Peter VANDAMME and Dr. Freek SPITAELS.
Experimental work: Zaina KADRI, Margo CNOCKAERT.

Data analysis: KADRI Zaina, Prof. Peter VANDAMME.

Writing of manuscript: KADRI Zaina.

Critically reviewing of manuscript: Prof. Peter VANDAMME.

Abstract

Two coccus-shaped isolates, designated LMG 28767^T and R-54896, were obtained from raw camel milk produced in Laâyoune, a city in southern Morocco, and characterised taxonomically. They were Gram-strain-positive, catalase and oxidase negative, facultatively anaerobic and formed L-lactic acid. Both isolates had highly similar MALDI-TOF MS and RAPD fingerprints and identical 16S rRNA and *pheS* gene sequences. Comparative 16S rRNA gene sequencing revealed that the novel taxon exhibited 98.60 % sequence similarity to that of the type strain *Lactococcus chungangensis* LMG 28725^T. However, the MALDI-TOF MS profiles of the novel isolates and *L. chungangensis* LMG 28725^T comprised several differential peaks, suggesting they represented different species. This was confirmed by a DNA–DNA hybridization value of 54% between strain LMG 28767^T and *L. chungangensis* LMG 28725^T. Both organisms could be distinguished through DNA–DNA hybridization, 16S rRNA and *pheS* gene sequence analyses, MALDI TOF MS profiles and multiple phenotypic characteristics. Based on the combined genotypic and phenotypic evidence, we propose to classify the isolates LMG 28767^T and R-54896 as a novel species of the genus *Lactococcus*, for which the name *Lactococcus multifermentans* sp. nov., is proposed. The type strain is LMG 28767^T (=CCMM B 1176^T).

Key words: *Lactococcus multifermentans* sp. nov.; lactic acid bacterium; camel milk; taxonomy.

Introduction

The genus *Lactococcus* was proposed by Schleifer and colleagues in 1985 to reclassify some species of the genera *Streptococcus* (Lancefield group N lactic streptococci) and *Lactobacillus* on the basis of chemotaxonomic studies and comparative 16S rRNA sequencing (Casalta et al. 2008; Collins et al. 1989; Schleifer et al. 1985; Schleifer and Killper-Bälz 1987). Currently, the genus *Lactococcus* comprises nine validly named species and four subspecies (Parte 2014, http://www.bacterio.net/lactococcus.html). All members of this genus are facultatively anaerobic, catalase-negative, gram-positive cocci that occur singly, in pairs, or in chains (Facklam and Elliott, 1995). Species of the genus *Lactococcus* have been isolated from various food, plant and animal sources (Cai et al. 2011; Chen et al. 2013; Pérez et al. 2011; Williams et al. 1990). They are lactic acid bacteria (LAB) that contribute significantly to the properties of fermented dairy products and are generally regarded as safe organisms (Salminen et al. 1998). Lactococci have also been used as probiotics that may contribute to the maintenance of human intestinal health (Steidler et al. 2000; Tanigawa et al. 2010). The aim of the present study was to formally classify two *Lactococcus* isolates from raw camel milk collected in southern Morocco by using a polyphasic characterisation.

Materials and methods

Isolation, culture conditions and MALDI-TOF MS dereplication

Two bacterial isolates designed LMG 28767^T and R-54896 were recovered from a single raw camel milk sample collected in Laâyoune, a city in southern Morocco (27.1565° N, 13.2036° W). They were isolated by standard dilution plating under aerobic conditions on Columbia agar base (BBL) supplemented with 5% sheep blood and All Culture (AC) agar, each containing 5 ppm amphotericin B to prevent fungal growth. Inoculated plates were incubated at 37 and 28°C, respectively. All bacterial isolates were dereplicated by matrix-assisted laser desorption/ionisation-time-of-flight mass spectrometry (MALDI-TOF MS) (Nguyen et al. 2013; Spitaels et al. 2014), followed by curve-based data analysis (Ghyselinck et al. 2011) using the BioNumerics 7 software (Applied Maths, Sint-Martens-Latem, Belgium). Species level identification of most isolates was achieved through comparison of the obtained spectra with those of an in-house database of the LM-UGent research group. Isolates which remained unidentified were further examined using sequence analysis of 16S rRNA and *pheS* genes as described below.

Representative isolates of two MALDI-TOF MS clusters with similar profiles that remained unidentified after comparative sequence analysis, i.e. LMG 28767^T and R-54896, were chosen for further analyses. These isolates were recovered from the same milk sample but were collected from the two different culture media described above. Both isolates and *L. chungangensis* LMG 28725^T which was used as reference strain were routinely incubated under aerobic conditions on M17 agar at 28°C unless otherwise indicated.

RAPD-PCR fingerprinting

Random amplified polymorphic DNA (RAPD) analysis was performed by using the primer RAPD-272 (5'-AGCGGGCCAA-3') as previously described (Williams et al. 1990).

Phylogenetic analyses

Amplification and sequencing of the 16S rRNA gene of both isolates was carried out as described by Snauwaert et al. (2013). Sequence similarities of the 16S rRNA gene were assessed by comparing the obtained sequences with those present in the EzTaxon-e database using EzBioCloud analysis (Kim et al. 2012). The obtained sequences and those of publicly available *Lactococcus* reference strains were aligned using the SILVA Incremental Aligner (SINA v1.2.11) (http://www.arb-silva.de/aligner/) (Pruesse et al. 2012) with the corresponding SILVA SSURef 111 database (Pruesse et al. 2007). Partial *pheS* gene sequences were generated for both isolates and for the type strain of their phylogenetic neighbour species, *L. chungangensis* (as determined through comparative 16S rRNA sequence analysis) as previously described by Naser et al. (2005a). The MEGA 5.2.2 software package (Tamura et al. 2011) was used to calculate pairwise sequence similarity values of the *pheS* sequences of both isolates and of the type strain of *L. chungangensis*, to align the *pheS* sequences of both isolates and of selected *Lactococcus* type strains by using MUSCLE and to construct a phylogenetic tree by using the maximum-likelihood method. A discrete Gamma distribution was used to model evolutionary rate differences among sites. Tree topologies were analysed statistically using 1000 bootstrapping replications.

DNA-DNA hybridization and DNA % G+C content analysis

High-molecular weight DNA was prepared as described by Vancanneyt et al. (2004). The DNA % G+C content was determined for strain LMG 28767^T and *L. chungangensis* LMG 28725^T using reversed-phase HPLC as described by Mesbah et al. (1989). DNA–DNA relatedness values were determined using the fluorometric hybridization

method in microdilution wells as described previously (Ezaki et al. 1989; Goris et al. 1998). The hybridization temperature was 37°C in the presence of 50% formamide. DNA-DNA hybridization values are presented as averages of reciprocal experiments performed in quadruplicate hybridization reactions.

Genbank accession numbers

The GenBank accession numbers for the 16S rRNA and *pheS* gene sequences of isolates LMG 28767^T and R-54896 are KR827631, KR827654; and KR827632, KR827655, respectively. The GenBank accession number for the *pheS* gene sequence of *L. chungangensis* LMG 28725^T is KR827656.

Phenotypic characterization

Cell shape, size and arrangement and colony appearance were examined using cells grown on M17 agar for 24h. Motility was checked with light microscopy. Gram-stain behavior, endospore staining and presence of catalase and oxidase activities were performed using standard microbiological procedures (Mac Faddin, 1980). Hemolytic activity was tested on Columbia agar base (BBL) supplemented with 5% sheep blood for 24 h at 37 °C in a 5% CO_2 enriched atmosphere (Oxoid CD0025A gas pack). Growth was determined in M17 agar at 10, 28, 37 and 45°C after 24-48h of incubation. Tolerance of NaCl was determined in M17 broth containing 2.0, 4.0, 6.0, 6.5 and 8.0 % (w/v) at 28°C after 24-48h of incubation. Growth at pH 4, 5, 6, 7, 8 and 9 was determined at 28°C after 24-48h of incubation using M17 broth without di-sodium-glycerophosphate. Production of short chain fatty acids was determined after growth in M17 broth for 24h as previously described by De Baere et al. (2013). The D-/L-Lactic acid (Rapid) Assay Kit (Megazyme) was used to determine D- or L-lactic acid production. Biochemical characteristics present in the API 20 Strep, API 50 CHL and API ZYM (API bioMérieux, France) microtest systems were determined for both novel isolates and for *L. chungangensis* LMG 28725^T according to the recommendations of the manufacturer (API bioMérieux, France).

Results and discussion

In a study of the microbiota of raw camel milk collected in southern Morocco, two Gram-strain-positive, catalase and oxidase negative isolates with highly similar RAPD (not shown) and MALDI-TOF MS (Fig 6.9) fingerprints remained unidentified after comparison with an in-house database. The two isolates were obtained from different isolation media but had identical 16S rRNA and *pheS* gene sequences. Identification

using the EzTaxon-e server revealed that the novel taxon belongs to the genus *Lactococcus*, with the highest 16S rRNA gene sequence similarities to *L. chungangensis* LMG 28725^{T} (98.60 %).

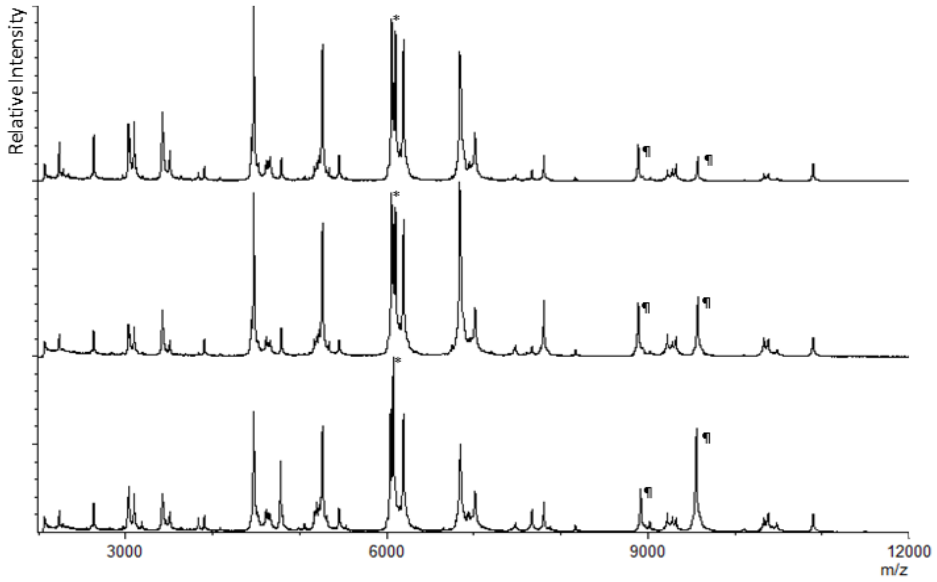

Fig 6. 9: MALDI-TOF MS profiles ranging from m/z 2000 to 12000 of *L. multifermentans* sp. nov., isolates R-54896 (upper), LMG 28767^{T} (middle) and *L. chungangensis* LMG 28725^{T} (lower). The figure was created with the mMass v5.5.0 software package (Strohalm et al. 2010). *Asterisk* and ¶ indicates the subtle spectral differences between the novel isolates and the type strain of *L. chungangensis* in the m/z regions around 6000 and 9000, respectively.

A 16S rRNA gene sequence based phylogenetic tree demonstrated that both novel isolates and the *L. chungangensis* type strain represented a coherent lineage supported by a bootstrap value of 92% (Fig 6.10).

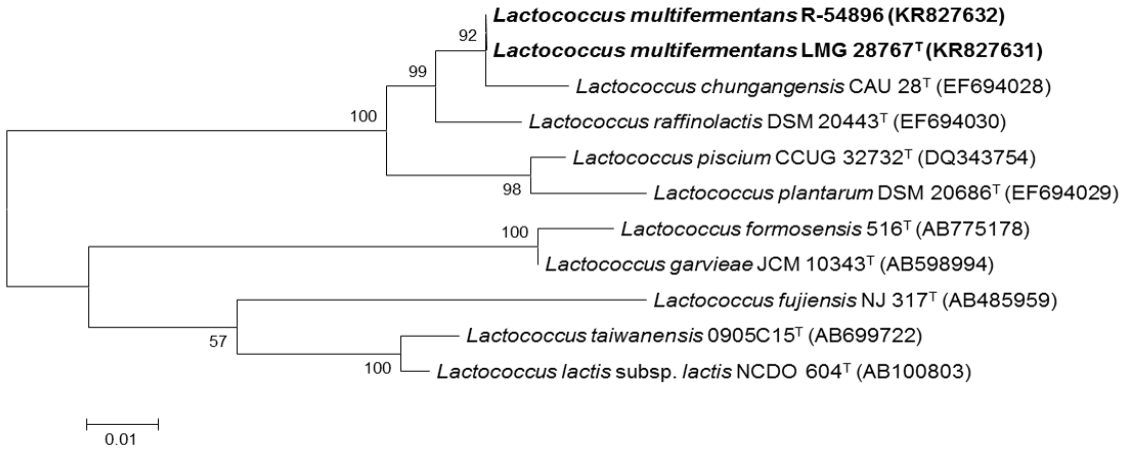

Fig 6. 10: Maximum-likelihood phylogenetic tree based on 16S rRNA gene sequences of isolates LMG 28767^{T}, R-54896, and selected type strains of the genus *Lactococcus*. Bootstrap values based on 1000 replications are indicated at the nodes. GenBank accession number are given in parentheses. Values of less than 50% are not shown. Bar, 0.01 substitutions per site.

Protein encoding genes such as *pheS*, which is commonly used in taxonomic and identification studies of the genus *Lactococcus* (Chen et al. 2014), evolve at a higher pace and have a taxonomic discriminatory power superior to that of the more conserved 16S rRNA gene (Kadri et al. 2014; Kadri et al. 2015b; Vandamme et al. 2014). The MEGA 5.2.2 analyses of the *pheS* gene of both novel isolates confirmed *L. chungangensis* LMG 28725^T as nearest neighbour taxon with 92.3% sequence similarity. A maximum-likelihood phylogenetic tree based on *pheS* gene sequences is shown in Fig 6.11. DNA-DNA hybridization experiments revealed hybridization values of 94 ± 5 % and 54 ± 4 % between LMG 28767^T and R-54896; and between LMG 28767^T and *L. chungangensis* LMG 28725^T, respectively, confirming that both isolates represent a single genospecies that is different from its near phylogenetic neighbour, *L. chungangensis* LMG 28725^T. The DNA % G+C content of LMG 28767^T was determined to be 36.95 mol%, which is within the range (34-43 mol%) reported for the members of the genus *Lactococcus* (Schleifer et al. 1985) and which is similar to that of *L. chungangensis* LMG 28725^T (38.5 mol%).

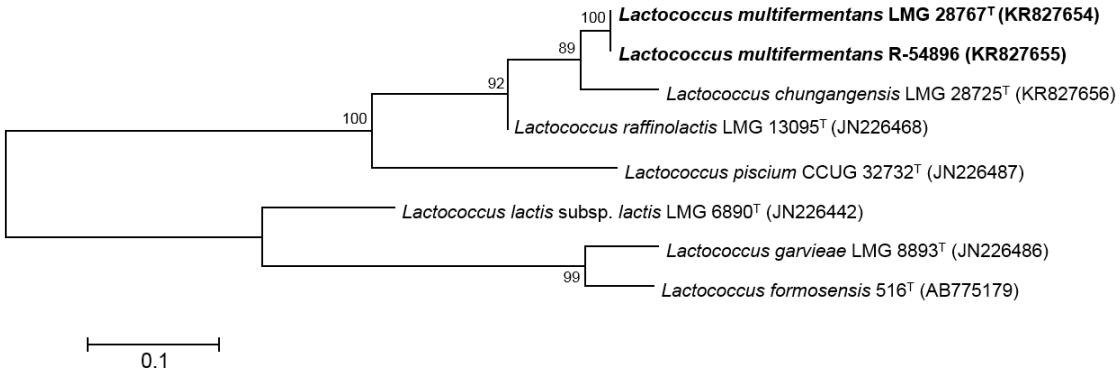

Fig 6. 11: Maximum-likelihood phylogenetic tree based on *pheS* gene sequences of isolates LMG 28767^T, R-54896, and selected type strains of the genus *Lactococcus*. Bootstrap values based on 1000 replications are indicated at the nodes. GenBank accession numbers are given in parentheses. Values of less than 50% are not shown. Bar, 0.1 substitutions per site.

Analysis of the MALDI-TOF MS profile of *L. chungangensis* LMG 28725^T revealed that the *L. chungangensis* LMG 28725^T MALDI TOF mass spectrum shared some peaks with the profiles of both novel isolates LMG 28767^T and R-54896. Nevertheless, additional discriminating spectral features were present among these isolates and strain LMG 28725^T in the m/z regions around 6000 (Fig 6.9 and 6.12) and 9000 (Fig 6.9 and 6.13).

Biochemical characteristics were determined for both isolates and for *L. chungangensis* LMG 28725^T. Both isolates had identical biochemical characteristics. Tests differentiating both isolates from *L. chungangensis* LMG 28725^T are shown in Tab.6.7. Characteristics shared by all three isolates are as follows: growth at 10 and 28°C, at pH 6, 7, 8 and 9, and in the presence of 2.0% NaCl; production of L-lactic acid; production of acid from D-glucose, D-fructose, D-mannose, D-mannitol, N-acetyl-glucosamine, amygdalin, arbutin, salicin, cellobiose, D-maltose, D-trehalose, gentiobiose, sucrose and acetoin; and activity of leucine amino peptidase, leucine arylamidase, acid phosphatase (weakly positive) and aesculin hydrolysis. In addition, the following characteristics were uniformly absent: acid production from erythritol, D-arabinose, L-arabinose, L-xylose, adonitol, methyl-D-xyloside, L-sorbose, rhamnose, dulcitol, inositol, methyl-D-mannoside, methyl-D-glucoside, melibiose, inulin, raffinose, starch, glycogen, xylitol, D-lyxose, D-fucose, L-fucose, D-arabitol, L-arabitol, 2- and 5-keto-gluconate; and activity of hippuricase, pyrrolidonyl arylamidase, α-galactosidase, β-galactosidase, arginine dihydrolase, alkaline phosphatase, esterase lipase (C8), lipase (C14), valine arylamidase, cystine arylamidase, trypsin, α-chymotrypsin, β-glucuronidase, α-glucosidase, N-acetyl-β-glucosaminidase, α-mannosidase and α-fucosidase.

In conclusion, the data reported here demonstrate that the isolates LMG 28767^T and R-54896 represent a single novel *Lactococcus* species that is closely related to *L. chungangensis.* These two species could be differentiated through DNA–DNA hybridization, 16S rRNA and *pheS* gene sequence analysis, MALDI-TOF MS profiles and multiple phenotypic characteristics. Although obtained on different isolation media and initially dereplicated in different MALDI-TOF MS clusters, both isolates had indistinguishable genotypic and phenotypic characteristics suggesting they represented isolates of the same strain. We consequently propose to formally classify these two isolates as a novel *Lactococcus* species for which we propose the name *Lactococcus multifermentans* sp. nov., with LMG 28767^T (=CCMM B1176^T) as the type strain.

Description of *Lactococcus multifermentans* sp. nov.

Lactococcus multifermentans (mul.ti.fer.men'tans. L. adj. *multus*, many; L. part. adj. *fermentans*, fermenting; N.L. part. adj. *multifermentans*, fermenting many substrates).

Cells are Gram-stain-positive, catalase and oxidase negative, non-spore-forming, non-motile and coccoid shaped. They are 0.8-1 µm in diameter and occur singly. Colonies on AC agar plates supplemented with 2% milk powder are white and opaque, small with a diameter of less than 0.5 mm. Grows well on Columbia blood agar, M17

agar and MRS agar, with an optimum at 28°C and is facultative anaerobic. Non-hemolytic on blood agar at 37°C. Produces L- lactic acid. Grows at 10, 28, 37 and 45°C. Growth was observed at pH of 4, 5, 6, 7, 8, and 9 and in the presence of 2.0, 4.0, 6.0, 6.5 and 8.0 % NaCl. Characteristics revealed by several API microtest systems are listed above and in Tab. 6.7. The DNA % G+C content of the type strain is 36.95 mol%. The type strain is LMG 28767^T (=CCMM B1176^T) and was isolated from a raw camel milk sample produced in southern Morocco. The GenBank accession numbers for the 16S rRNA and *pheS* sequences of strain LMG 28767^T are KR827631 and KR827654, respectively.

Acknowledgments

We thank Mr. Ouchari Lahcen (Laboratory of Microbiology and Molecular Biology-National Center for Scientific and Technical Research, Rabat, Morocco) for helping us collecting camel milk samples. We thank Dr. Maarten Aerts and Ms. Jessy Praet (Laboratory of Microbiology, Gent University, Belgium) for support with MALDI-TOF MS and short chain fatty acid data analyses, respectively.

TABLES

Tab. 6. 7: Characteristics that differentiate *Lactococcus multifermentans* sp. nov. type strain LMG 28767^T from closely related phylogenetic relatives.

Taxa / Characteristic	1	2*	3°	4¶	5°
Acid production from :					
Glycerol	+	-	-	-	-
Ribose	+	-	-®	+	-
D-xylose	+	-	+	+	-
Galactose	+	-	+	+	-
D-Lactose	+	-	+	+	-
D-Sorbitol	+	-	-	-	+
Melezitose	+	-	-[b]	+	+
D-Tagatose	+	-	-	-	-
Gluconate	+	-	-	+	-
D-Turanose	-	+[a]	V	+	V

Amygdalin	+	+	-®	+	+
Melibiose	-	-	+	+	-
Raffinose	-	-	+	+	-
Sucrose	+	+	-	+	+
Starch	-	-[a]	+	-®	-
L-arabinose	-	-	V	-	-
Production of:					
β- Glucosidase	+	-	ND	ND	ND
Esterase (C_4)	W	+	ND	ND	ND
Phosphoamidase	W	-	ND	ND	ND
α-galactosidase	-	-	+	ND	-
Growth at :					
4% NaCl	+	-	-	-	+
6% NaCl	+	-	-[b]	-[b]	-[b]
6.5% NaCl	+	-	ND	ND	ND
8% NaCl	+	-	ND	ND	ND
Growth at :					
37°C	+	-	ND	ND	ND
45°C	+	-	-	-	-
Growth at :					
PH=4	+	-	-®	-®	-®
PH=5	+	-	-®	-®	-®
DNA % G+C content (mol %)	36.95	38.5	41.5®	38.5	36.9-38.1

Taxa: 1, *L. multifermentans* LMG 28767^T; **2,** *L. chungangensis* LMG 28725^T; **3,** *L. raffinolactis* DSM 20443^T; **4,** *L. piscium* NCFB 2778^T; **5,** *L. plantarum* NCDO 1869^T.

+, positive reaction; -, negative reaction; WR, weak reaction; V, variable.

*Data are from the present study
°Data are from Schleifer et al. (1985)
¶ Data are from Williams et al. (1990)
[b] Data are from Chen et al. (2014)
® Data are from Cai et al. (2011)
[a] This result does not correspond with published data (Cho et al. 2008)

Supplementary information:

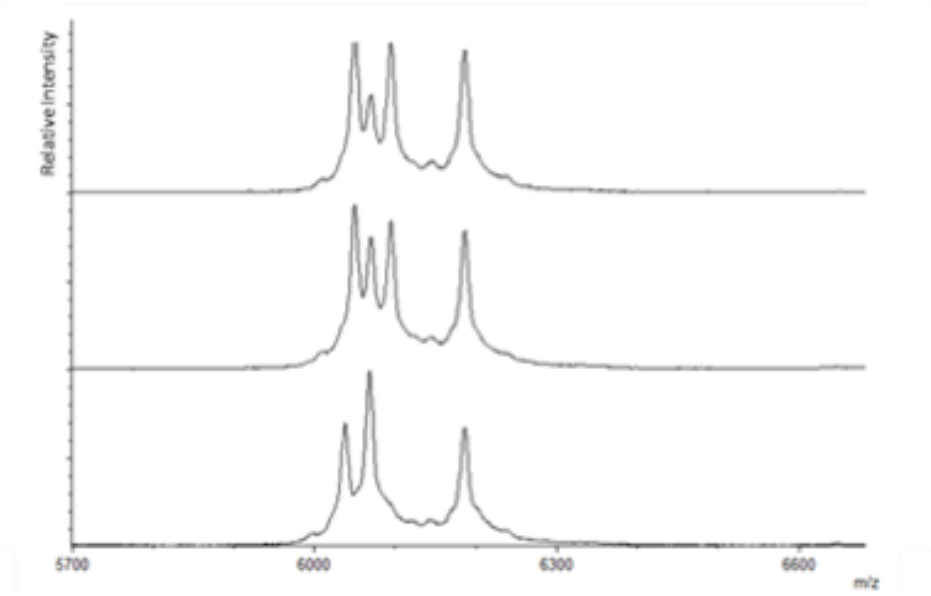

Fig 6. 12: MALDI-TOF MS profiles ranging from m/z 5700 to 6600 of *L. multifermentans* sp. nov., isolates R-54896 (upper), LMG 28767^T (middle) and *L. chungangensis* LMG 28725^T (lower). The figure was created with the mMass v5.5.0 software package (Strohalm et al. 2010) showing the subtle spectral differences between the isolates and the type strain of *L. chungangensis* in the m/z regions around 6000 (peak loss and mass shift).

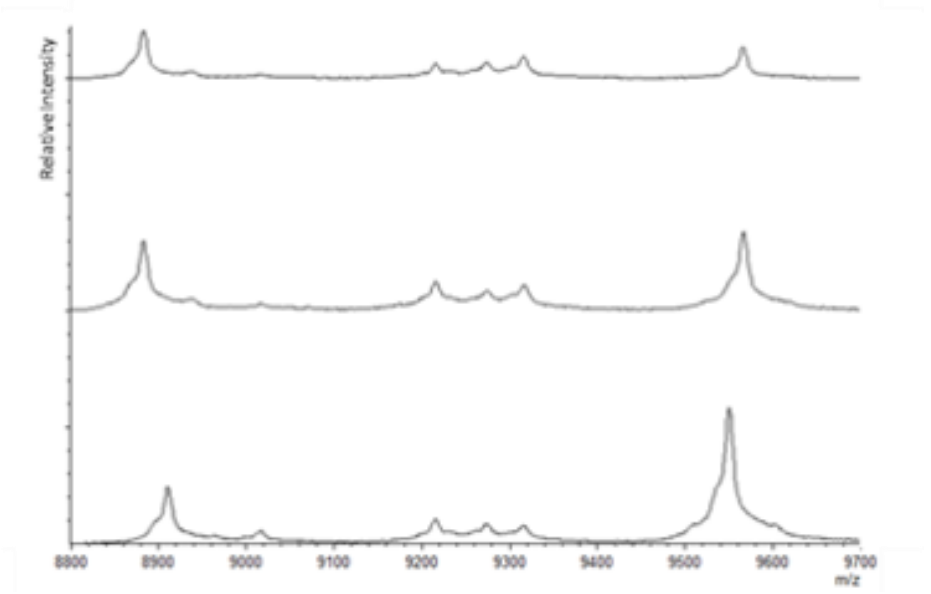

Fig 6. 13: MALDI-TOF MS profiles ranging from m/z 8800 to 9700 of *L. multifermentans* sp. nov., isolates R-54896 (upper), LMG 28767^T (middle) and *L. chungangensis* LMG 28725^T (lower). The figure was created with the mMass v5.5.0 software package (Strohalm et al. 2010) showing the subtle spectral differences between the isolates and the type strain of *L. chungangensis* in the m/z regions around 9000 (2 mass shifts).

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PART IV

GENERAL DISCUSSION AND FUTURE PERSPECTIVES

CHAPTER 7: DISCUSSION AND PERSPECTIVES

7.1. General discussion

7.1.1 Diversity of LAB and overall microbiota of raw camel milk produced in Morocco

Notwithstanding the increasing popularity of camel milk, only a few papers addressed its microbiological aspects (Benkerroum et al. 2003; Khedid et al. 2009). These studies were performed using biochemical methods solely and were limited in the number of isolates dealt with and the taxonomical information that was obtained (Benkerroum et al. 2003; Khedid et al. 2009). Since the publication of these early studies, the taxonomy of the microbiota, especially of the lactic acid bacteria (LAB) identified in raw camel milk underwent many changes and biochemical identification methods were shown inadequate to reliably identify these microorganisms (Corsetti et al. 2001; Hammes & Vogel, 1995; Kämpfer & Glaeser, 2012; Nguyen et al. 2012; Pot et al. 1994).

In this regard, the research performed in the present Ph.D study contributed to a profound insight in the diversity of LAB population and the overall microbiota. We examined several samples of raw camel milk collected from different regions in Morocco, used state-of-the-art methodologies and provided an extensive collection of LAB isolates as a natural resource for further studies. In addition, this work allowed to assess the usefulness and limitations of new and existing approaches for the investigation of bacterial populations.

In the first phase of this study, we studied the LAB diversity present in twelve raw camel milk samples collected from eight regions in Morocco. The dominant LAB species recovered were *Leuc. mesenteroides* subsp. *mesenteroides* and *L. lactis* subsp. *lactis,* while the *Leuc. pseudomesenteroides*, *E. faecium, E. durans*, *Leuc. mesenteroides* subsp. *dextranicum, L. raffinolactis*, *L. lactis* subsp. *cremoris* and *Leuc. citreum* were isolated less frequently. Also *Leuc. mesenteroides* subsp. *dextranicum* and *Leuc. citreum* were present specifically in only one region of all eight analyzed sites. Repeat analyses of additional samples of these regions should reveal if this LAB microbiota is region specific. Four novel *Streptococcus* species, i.e., *S. moroccensis* sp. nov., *S. rifensis* sp. nov., *S. tangierensis* sp. nov., and *S. cameli* sp. nov (chapters 5.2 and 5.3) were recovered from one of the four samples collected from the region of Tangier. It remains

to be investigated whether these novel species are natural components of raw milk or environmental contaminants.

In the second phase of this study, we studied the overall microbiota present in four additional raw camel milk samples and detected a considerable bacterial diversity consisting of members of the phyla *Firmicutes*, *Proteobacteria*, *Actinobacteria* and *Bacteroidetes*. *L. lactis* subsp. *lactis* was again the dominant organism in each of the analyzed samples. Also *E. faecium* was dominantly present although it was not isolated from all analyzed samples. Other species were present less frequently such as *S. suis*, *E. italicus, Leuc. pseudomesenteroides, S. salivarius, E. faecalis* and *Lb. rhamnosus*. In addition, two novel LAB species were recovered, i.e., *E. bulliens* sp. nov., and *L. multifermentans* sp. nov. (chapters 6.2 and 6.3).

Both diversity studies therefore showed that the LAB community of raw camel milk produced in Morocco consists primarily of lactococci, leuconostocs, enterococci and streptococci (chapters 5.1 and 6.1) and is dominated by *L. lactis* subsp. *lactis*. Remarquably, lactobacilli were absent or virtually absent in both studies confirming results reported by Benkerroum et al. (2003), Merzouk et al. (2013) and Saidi et al. (2005). Simultaneously both studies revealed many differences in LAB species isolated. *Leuc. mesenteroides* subsp. *dextranicum, L. raffinolactis, L. lactis* subsp. *cremoris, Leuc. citreum* and *E. durans* were only recovered in the first study, while *E. italicus, S. suis, E. feacalis, S. salivarius* and *Lb. rhamnosus* were obtained solely during the second study. It is unclear how these differences can be explained. Repeat analyses of additional samples collected in the same regions will reveal if these differences are region specific or not. The variability observed may also reflect the traditional way of milking camels. During sampling, all farmers used hand milking. They further differed by their way of washing the milking equipment, by their premilking and postmilking udder preparation as well as the milk storage conditions. The combination of these practices may influence the microbial load and composition of the milk produced. In other studies these variations have been attributed to differences in feed, weather conditions and the health of the animal (Callon et al. 2007; Yagil, 1994).

Previous studies of the bacterial community composition in bulk tank raw cow milk (Weber et al. 2014) and raw goat milk (McInnis et al. 2015) demonstrated that the bacterial species identified belonged to the phyla of *Firmicutes, Proteobacteria, Actinobacteria* and *Bacteroidetes*, which corresponds to the results obtained in our study. Additionally, reports on LAB diversity of cow and yak milk revealed the dominance of

species belonging to the genus *Lactococcus*, *Leuconostoc*, *Enterococcus*, *Pediococcus*, *Lactobacillus* and *Weissella* (Ouadghiri et al. 2009; Watanabe et al. 2008; Yu et al. 2011), while LAB of raw goat milk were dominated by members of the genus *Enterococcus*, *Lactococcus*, *Lactobacillus*, *Leuconostoc* and *Streptococcus* (Alonso-Calleja et al. 2002; Callon et al. 2007; McInnis et al. 2015; Quigley et al. 2013). This demonstrates that these LAB communities differ from those of camel milk samples examined in the present study where members of the genera *Pediococcus*, *Lactobacillus* or *Weissella* were absent. Several other microorganisms have been reported in cow and goat milks including *Pseudomonas* spp., *Acinetobacter* spp., *Aeromonas* spp., *Kocuria* spp., *Staphylococcus* spp., *Pantoea* spp., *Micrococcus* spp., *Corynebacterium* spp., *Brevibacterium* spp., *Propionibacterium* spp., *Aerococcus spp.*, *Facklamia spp*., *Acetobacter* spp., *Bacillus* spp., *Clostridium* spp., *Listeria* spp., *Alcaligenes* spp., *Flavobacterium* spp., *Arthrobacter* spp., *Hafnia* spp., *Citrobacter* spp. or *Serratia marcescens* (Lafarge et al. 2004; McInnis et al. 2015; Quigley et al. 2013). Some of the latter species were detected in camel milk samples as well and include psychotrophic bacteria that flourish during cold storage, i.e., *Pseudomonas* spp., *Acinetobacter* spp., and *Aeromonas* spp., and other species such as *Kocuria* spp., *Staphylococcus* spp., *Citrobacter* spp., and *Serratia marcescens.*

Regarding the hygienic status of camel milk, the total bacterial counts (TBC) recorded in both studies were high which could arise from poor plant, tank or teat cleaning and can also be related to mastitis organisms, environmental contaminants, dirty milking equipment or failure of refrigeration (Blowey et al. 1995; Kelly et al. 2009). Hence, there is a potential hazard associated with its consumption. In addition, the presence of pathogenic bacteria, such as *Staph. aureus*, *Escherichia*/*Shigella* and *Serratia marcescens* subsp. *marcescens*, may reveal that camel herds rarely benefit from veterinary care and, hence, mastitis and other diseases are common among lactating females. This highlights the need for interventions to improve production practices through better hygiene and medical diagnosis of mastitic camels and their milk. The production of UHT camel milk and the establishment of collection centers and mobile dairy factories for processing pasteurized camel milk in the area of the production could be overcome part of these problems. In conclusion, results from the present study indicate that hygiene measures and sanitation of the ustensils used in the collection, storage and transportation should be improved to conform to standards for any other milk type destined for

human consumption. In addition, the awareness of health risks associated with consumption of raw camel milk should be promoted. Finally, additional studies should address the quality and microbial composition of raw camel milk, milking protocols and sanitizing programs.

7.1.2 Evaluation of $(GTG)_5$-PCR and MALDI TOF MS fingerprinting as dereplication tools

In the first phase of the present study, $(GTG)_5$-PCR fingerprinting analysis was successfully applied for preliminary grouping of LAB isolates from camel milk. Final identification of LAB isolates was obtained by sequence analysis of the 16S rRNA gene. The 16S rRNA gene is the most commonly used marker in bacterial genotypic identification. It is an ideal target due to its ubiquitous presence in prokaryotic organisms (Janda and Abbott, 2007; Olson et al. 2015; Tringe and Hugenholtz, 2008) but often lacks discriminatory power among closely related species (Doonan et al. 2015; Ghebremedhin et al. 2008; Janda and Abbott, 2007; Naum et al. 2008). The use of repetitive motifs in bacteria genomes, more specifically $(GTG)_5$, has proven to be a valuable and powerful tool in microbial ecology studies, molecular diagnostics, medical microbiology and epidemiological analyses (Ishii and Sadowsky, 2009). This technique has been used frequently for bacterial taxonomy purposes and has been applied successfully for reliable and fast identification of different bacterial groups such as lactic acid bacteria (Gevers et al. 2001; Rademaker et al. 2007; Švec et al. 2005). Disadvantages, however, are low rates of interlaboratory reproducibility (Johnson and Clabots, 2000) and as it is based on DNA fragment separation on agarose gels, analysis and interpretation are often time-consuming (Healy et al. 2005).

An identification method to be routinely used should preferably meet following criteria: simplicity, reliability, uniformity in analysis of various groups of microorganisms, and cost-effectiveness. Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry has been recognized as a bacterial chemotaxonomic method fulfilling these requirements and has already been used in various microbiological identification studies (Bizzini and Greub, 2010; De Bruyne et al. 2011; Ghyselinck et al. 2011). Its widespread application in food microbiology will require purpose-built databases, for instance of microorganisms relevant in food fermentation processes. Its high-throughput capacity can however be exploited already for the dereplication of large numbers of isolates.

In the second phase of the present study, the total cultivable bacterial camel milk microbiota was characterized using a combination of variety of culture conditions and MALDI-TOF MS as a dereplication tool. Spectra were exported into the BioNumerics version 5.1 software package (Applied Maths), which lacked an efficient peak-picking algorithm. Therefore, curve-based analysis methods were used for dereplication to reduce the initial number of isolates to a subset of representative reference isolates. For this purpose two up to five representative isolates were selected from each MALDI-TOF MS cluster (depending on the size of the cluster), to do sequence analysis of protein encoding genes for further identification using the sequence data available from public databases. MALDI-TOF MS appeared to be an efficient tool that is powerful, fast, reliable and cost effective for the dereplication and identification of the overall microbiota from raw camel milk. Its throughput capacity was much higher than that of the $(GTG)_5$-PCR fingerprinting. It was possible to prepare 200 bacterial cell extracts and even to analyze them in an 8 h working day, a number that could even be significantly increased when coupled to process automation. In contrast, in the present study, $(GTG)_5$-PCR analysis allowed only 15 samples per gel, including molecular ladders for normalization, positive controls and blanks. In addition, the combination of a lengthy GES pitcher DNA extraction (Gevers et al. 2001) recommended for a good quality and more standardized DNA extract, PCR analysis and electrophoresis, made $(GTG)_5$-PCR much more time consuming since only 20 bacterial DNA extracts could be obtained in an 8 h working day.

7.1.3 Bacterial culturomics: a novel approach for maximal detection of camel milk microbiota

'Culturomics', a novel type of analysis first depicted by Lagier et al. (2012) represents a new approach to the study of complex microbial ecosystems, that i) has the potential to detect minority populations, ii) is not restricted to organisms of the domain *Bacteria*, and iii) provides strains that allow extensive characterization of new species and the study of interactions between different bacterial strains present in a given microbiota. Culturomics may be defined -by analogy with metagenomics- as an approach allowing an extensive assessment of the microbial composition by high-throughput and miniaturized application of numerous classical growth media for the isolation of microorganisms. Thus, Lagier et al. (2012) identified as many as 32,500 different colonies recovered from three human stool samples. Interestingly, a study performed by Pfleiderer

and colleagues using a culturomics approach has led to the identification of 11 new bacterial species from a single anorexia nervosa stool sample and another culturomics study of a human gut microbiome found not less than 31 new species (Pfleiderer et al. 2013). Furthermore, Dubourg et al. (2013) showed that culturomics could outperform bar-coded sequence analysis for the discovery of the rare microbiota in the gut microbiome of an immunocomprised patient. This high-throughput identification was only possible because of the availability of MALDI-TOF MS as for this purpose, sequence-based identification methods are too slow and expensive. It has been anticipated that MALDI-TOF MS may be coupled to smart incubators and automated colony-picking systems to constitute the next generation of culturomic approaches (Lagier et al. 2012. Pfleiderer et al. 2013).

In the present study we performed a modest culturomics study to characterize the total cultivable bacterial camel milk microbiota, using a combination of variety of culture conditions and MALDI-TOF MS as a dereplication tool. Final identification of the microbiota was obtained through sequence analysis of 16S rRNA and of protein encoding genes. With this combination of identification methods and the use of diverse cultivation condition we also aimed to isolate and recognize novel bacteria. As a result we discovered six novel LAB species, i.e., *S. moroccensis, S. rifensis, S. tangierensis, S. cameli, E. bulliens* and *L. multifermentans* (chapters 5.2, 5.3, 6.2 and 6.3, respectively). Only one species, i. e. *L. lactis,* was isolated from all four camel milk samples studied and it was recovered using 6 out of 10 sets of isolation conditions. The majority of the isolated species (52.9%) were cultured from only one camel milk sample, which highlighted the large inter-sample diversity of the raw camel milk microbiota. Thus, the use of 10 sets of isolation conditions in this limited culturomics study allowed the detection of a high number of different species (chapters 6.1; 6.2 and 6.3). The use of modifications of general purpose media such as ACA+2% milk powder, TSA+4.5% NaCl and PCA+2% milk powder allowed to isolate some unusual species such as *Chryseobacterium arthrosphaerae, Empedobacter falsenii, Kocuria salcisia, Rothia endophytica* and *Rothia marina,* which, as far as we know, have never been associated with the production of raw camel milk. Also the identification of single isolates as for instance *Moraxella osloensis, E. faecalis* and *S. salivarius* suggests that these species represent rare microbiota members which we detected since these organisms grow well on several of the isolation media used.

In conclusion, future culturomics studies of similar types of biological samples may benefit from using the set of conditions described in chapter 6.1 although the use of additional less common culture conditions may further expanded the repertoire of cultivated bacteria through the isolation of less abundant bacteria.

7.2 Perspectives

7.2.1 Combination of culture-dependent and culture-independent methods for the identification of raw camel milk microbiota

The successful integration of different techniques for the identification of LAB and the overall microbiota of camel milk in our work emphasizes the importance of a polyphasic approach as the optimal way for proper bacterial identification. The various data facilitate the generation of a consensus identification for each isolate where the limitations of each technique are overcome and the reliability of the identification improves in a cost-effective manner. Further research using cultivation-independent analyses of the same camel milk samples will reveal to which extent the overall microbiota described in the present study is complete, or if additional (novel) species await discovery. More generally speaking it is clear that a combination of multiple complementary techniques including culture-based and culture-independent methods and a cautious interpretation of the results remains the best approach in microbial diversity studies (Lagier et al. 2012). In the latter studies, culture-independent community fingerprint techniques were traditionally combined with standard cultivation methods (Dolci et al. 2010; Scheirlinck et al. 2008; Van Der Meulen et al. 2007; Wouters et al. 2013). In recent years, however, more advanced and high-throughput culture-independent methods, such as bar-coded amplicon sequencing and metagenomics, are gradually replacing traditional community fingerprint methods, such as the denaturating gradient gel electrophoresis (DGGE) approach. For instance, Spitaels et al. (2014) determined the microbiota involved in the fermentation of traditional lambic beers using culture-dependent and culture independent based on DGGE analysis. In contrast, Jost and colleagues determined the bacterial diversity in breast milk using standard cultivation methods and Sanger sequencing and 454-pyrosequencing as culture-independent methods (Jost et al. 2013).

The latter approaches have now been widely adopted because they provide greater ease of use and higher throughput than culture-based approaches. There has been a meteoric

rise in the use of metagenomics to investigate complex microbial communities over the past decade. Indeed, the term 'metagenomics' is applied to the direct sequencing of DNA extracted from a sample without culture or target-specific amplification or capture (Pallen et al. 2014). Unfortunately, the term metagenomics is often misleadingly and it is used loosely to cover any culture independent sequence-based profiling of microbial communities, particularly amplification and sequencing of molecular barcodes, such as sequences from rRNA genes (Pallen et al. 2014). The rapid development and increasing throughput of next-generation sequencing platforms have made it possible to generate large metagenomic data sets, incorporating many individual samples (Human Microbiome Project Consortium; 2012). The main advantage of this approach is that it allows simultaneous monitoring of a diversity of microorganisms, not only the species that grow readily in the laboratory. In addition, it is also possible to assemble complete or almost complete genomes from existent metagenomics datasets, particularly from the most abundant members of the microbiota (Albertsen et al. 2013). Indeed, these metagenomic techniques are also superior to classical fingerprint-based culture-independent techniques such as PCR-DGGE because of their ability to detect low abundant species in the communities (Bokulich & Bamforth, 2013). Given that for some of these metagenomic approaches there is no PCR step, these are than obviously also free of some of the biases introduced by DNA amplification. A further limitation is that it can be difficult to detect only metabolically inactive cells without detecting dead cells (Hong et al. 2009; Yuan et al. 2012). Therefore, although these modern culture independent analyses provide a more in-depth analysis of the microbial community composition, they are not without limitations and do not reveal which species are metabolically most active.

7.2.2 The use of LAB isolated from raw camel milk as starter cultures for fermented camel milk products

The research performed in this Ph.D. study provided an extensive set of LAB isolates for further studies. Several of their characteristics should now be studied more in detail. The dominant and subdominant microorganisms present in raw milk can have a variety of influences on the flavour, taste and texture of raw milk-derived products. A number of these microorganisms also have the potential to contribute to health through the production of antimicrobials or possessing other probiotic-associated traits. Previous re-

search has shown that LAB are the main group of bacteria that are considered technologically important in the fermentation and ripening of fermented dairy products worldwide (Akweya et al. 2012; Fernández et al. 2015; Garabal, 2007; Kongo, 2013; Wedajo, 2015; Widyastuti et al. 2014). The effectiveness of lactic acid bacteria in suppressing the multiplication of undesirable microorganisms is largely attributed to the production of organic acids (Nguyen, 2012). However, other factors include the production of bacteriocins and hydrogen peroxide and more general effects include competition for essential nutrients (Nguyen, 2012; Woodburn, 1992). In addition, LAB are able to produce amino acids from casein (Mc Sweeny and Fox, 1997). These amino acids are the key precursors for the essential cheese flavor by enzyme activities converting them to aldehydes, alcohols, ketones, esters, amino acids and sulfur containing compounds which all contribute to the cheese flavor (Hemme et al. 1982; Urbach, 1995). Finally, lipid degradation provides fatty acids that also contribute to flavour characteristics (Abeijón Mukdsi et al. 2009; Kenneally et al. 2002).

Raw camel milk could therefore be a source of LAB (Jans et al. 2012; Khedid et al. 2009), with specific functional properties, which might be used for the development of new starter cultures and innovative food products (Akhmetsadykova et al. 2014; Khedid et al. 2009). The diversity and high numbers of LAB in the analyzed samples indicate that they have a role either positive or detrimental in the fermentation of camel milk. Therefore, the abundance of some species, ie., *L. lactis* subsp. *lactis*, *E. faecium* or *Leuc. mesenteroides* subsp. *mesenteroides* supports their possible use as starter culture in the manufacture of camel fermented milk products. Strains of *L. lactis* subsp. *lactis* are important in food technology (Garvie, 1984; Salama et al. 1991) as bacteriocin producing lactococci have been used successfully in starter cultures for cheese to improve the safety and quality of the product (Delves-Broughton et al. 1996; Ryan et al. 1996). In addition, although enterococci are the most controversial group of food-associated LAB, they can be applied as starter cultures and probiotics (Fernández et al. 2015; Giraffa, 1995). They play an important role in cheese making and contribute to the development of the sensory characteristics of many varieties of cheese (Centeno et al. 1996; Giraffa, 2003; Manolopoulou et al. 2003). Enterococci from dairy products have also been reported to produce bacteriocins (enterocins) having antimicrobial activity against a broad spectrum of spoilage and pathogenic organisms such as *Listeria monocytogenes, Staphylococcus aureus, Clostridium* spp., and *Bacillus* spp. (Giraffa, 1995; Ennahar and Deschamps, 2000; Sarantinopoulos et al. 2002b; De Vuyst et al. 2003; De

Kwaadsteniet et al. 2005; Ghrairi et al. 2008). Leuconostocs are also frequently used as starter cultures for buttermilk, butter, cream, and cheese (Leroy and De Vuyst, 2004; Tamime and Marshall, 1997).

The use of starter cultures has become common in many countries, including developing countries such as Morocco where for instance, soft white cheese (Jben), skimmed milk (Lben) or fermented butter (Smen) are made with starter cultures (Benkerroum and Tamime, 2004; Hamama, 1992; Hamama and Bayi,1991; Ouadghiri et al. 2005; Ouadghiri et al. 2009; Tantaoui Elaraki et al. 1983a). In the starter culture preparations used for dairy products, *Lactococcus* strains are the most common (Fernández et al. 2015; Khedid et al. 2009;Widyastuti et al. 2014). These strains tolerate a high salt level and can be active in the temperature range used during fermentation (Huggins, 1984; Khedid et al. 2009; Sandine, 1988; Sharpe, 1979). In addition, they confer the expected palatability characteristics and prevent the production of harmful components (Woodburn, 1992). For camel milk, however, starter culture research is in its infancy (Brezovečki et al. 2015; Desouky et al. 2013; Khan et al. 2004; Nikkhah, 2011; Salih et al. 2011). It can be boosted by the thorough description of additional typical LAB community in this type of food and the availability of an extensive camel milk strain collection from the present study. Therefore, our study forms the basis for further research to search strains with outstanding features for the production of fermented camel milk products.

7.2.3 Yeasts in raw camel milk

Finally, it should be mentioned that yeasts too can represent important microbial populations within raw milk (Lagneau et al. 1996) and their presence can be influenced by the physiological state of the animal, as well as the weather, feeding and season (Callon et al. 2007; Vacheyrou et al. 2011). As with bacteria, the extent of the yeast population in raw milk and dairy products is often underestimated. In previous studies, Benkerroum et al. (2003) reported the presence of yeasts in Moroccan camel milk. In addition, Adugna et al. (2013), El-Ziney and Al-Turki (2006), Njage et al. (2011) and Omer and Eltinay, (2008) reported the presence of yeasts in camel milk from Eastern Ethiopia, Saudi Arabia and East Africa.

This presence of yeasts indicates that they are able to proliferate during milk fermentation (Pereira-Dias et al. 2000; Suzzi et al. 2003; Romano et al. 2001) and implies that they play either potentially beneficial or detrimental roles to both the quality and safety

of milk (Jespersen, 2003; Gadaga et al. 2001; Lopandic et al. 2006; Pereira-Dias et al. 2000; Suzzi et al. 2003). Defects such as gas production, yeasty flavour and other off-flavours, discolouration and changes of texture may result from their growth (Jakobsen and Narvhus, 1996). However, yeasts can play a major role in dairy fermentations due to a number of physiological and biochemical characteristics, including the ability to utilise lactose or galactose (Van den Tempel & Jakobsen, 2000), a high proteolytic or lipolytic activity (Sacristan et al. 2012) and the ability to grow at low temperatures and to tolerate high salt concentrations. Yeasts are important as part of the starter cultures for the development of sensory properties in milk products such as kefyr or koumiss (Fleet, 2006; Narvhus and Gadaga, 2003) and many types of cheeses (Lopandic et al. 2006). Yeasts secrete enzymes that play a key role in texture and produce various aromas during ripening. In addition, although fermented milk products are regarded as predominantly lactic acid fermentations, the frequent co-occurrence of yeasts and LAB has led to the suggestion that interactions may occur that can influence product characteristics and quality (Gadaga et al. 2001; Narvhus and Gadaga, 2003). Such interactions may be stimulation or inhibition of growth of one, or both, of the co-cultured strains (Marshall, 1987; Viljoen, 2001). The co-cultured organisms may compete for growth nutrients or they may produce metabolic products that inhibit each other's growth. Yeasts may produce vitamins that enhance the growth of LAB. Furthermore, mutual influence of the microorganisms on each other's metabolism may lead to different profiles of organoleptically important compounds in the fermented milk, which underscore the importance of the study of yeast diversity in camel milk samples.

In our study of the LAB diversity of raw camel milk samples (chapter 5.1), a total of 102 presumptive yeasts were isolated from the twelve analyzed samples (data not shown). These isolates were obtained on DYPA agar media incubated at 25°C under aerobic conditions, subcultured and stored at -80°C using 20% glycerol until further use. As a first step towards the study of the yeast diversity in raw camel milk samples from Morocco, we plan to identify these isolates using M13-PCR fingerprinting as dereplication tool followed by identification using rRNA and/or protein-coding gene sequencing (D1/D2 LSU rRNA, ITS rRNA, partial ACT1 and COX2 genes), as previously described by Ouadghiri et al. (2014).

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SUMMARY

Camel milk is one of the most valuable food resources for pastoral people in arid and semi-arid areas, where it covers a substantial part of the quantitative and qualitative nutritional needs. The indigenous populations believe that raw camel milk is safe and even has therapeutic virtues as compared to that of other animal species. While many studies have investigated the microbiology of cow, sheep, and goat's milk, only a few studies have focused on the microbiology of camel milk. Consequently, information regarding camel milk is very limited. More in particular, in Morocco, few studies addressing the diversity and dynamics of lactic acid bacteria (LAB) in camel milk research have been carried out and these studies applied phenotypic characterization methods only. Previous research has shown that the beneficial microbiota of camel milk represented by LAB is a potential source of biological materials to be used in dairy technology. Therefore, in order to successfully extend the range of Moroccan raw camel milk applications to an industrial scale, this study aimed to investigate the overall microbiota of raw camel milk samples using state-of-the-art methodologies.

In the first phase of this work, a culture-based approach was used to investigate the diversity of LAB in raw camel milk collected from different locations in Morocco. A total of 129 LAB isolates were subjected to a stepwise identification approach combining $(GTG)_5$-PCR fingerprinting and 16S rRNA gene sequence analysis. Identification of the camel milk LAB isolates revealed the highest prevalence of *Leuconostoc* (*Leuc*). *mesenteroides* subsp. *mesenteroides* (41.1%) and *Lactococcus* (*L*). *lactis* subsp. *lactis* (31.8%). Lower numbers were observed for *Leuc. pseudomesenteroides* (6.9%), *L. lactis* subsp. *cremoris* (6.2%), *Leuc. citreum* (5.4%), *Leuc. mesenteroides* subsp. *dextranicum* (2.3%) and *Enterococcus* (*E*). *faecium* (1.6%). In addition, some isolates were identified as *E. durans* (0. 8%) and *L. raffinolactis* (0. 8%). The randomly investigated sites showed a strong LAB diversity but repeat analyses are needed to determine if the observed differences are region specific. Nevertheless, *Leuc. mesenteroides* subsp. *mesenteroides* and *L. lactis* subsp. *lactis* were the common LAB isolated from most of analyzed samples **(chapter 5.1)**.

During the taxonomic inventorisation of these camel milk LAB, isolates LMG 27682^T and LMG 27684^T could not be attributed to any of the known LAB species. Comparative 16S rRNA gene sequencing assigned these bacteria to the genus *Streptococcus*

(*S.*) with *S. rupicaprae* 2777-2-07^T as the closest phylogenetic neighbour (95.9% and 95.7% similarity, respectively). Both strains could be distinguished by multiple biochemical tests, sequence analysis of the phenylalanyl-tRNA synthase (*pheS*), RNA polymerase (*rpoA*) and ATP synthase (*atpA*) genes and by their matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) profiles demonstrating that they are two novel species from which the names *Streptococcus moroccensis* and *Streptococcus rifensis* were proposed **(chapter 5.2)**.

Similarly, two additional isolates CCMM B832^T and CCMM B834^T did not correspond to any recognized LAB. Phylogenetic analysis based on 16S rRNA gene sequences showed the unidentified organisms each formed a hitherto unknown sub-line within the *Streptococcus* genus, displaying a close affinity with *S. moroccensis*, *S. minor* and *S. ovis*. Further taxonomic analysis including DNA–DNA hybridization experiments, DNA G+C content determination, MALDI-TOF mass spectrometry and biochemical tests showed that these isolates represented two novel *Streptococcus* species for which the names *Streptococcus tangierensis* and *Streptococcus cameli* were proposed **(chapter 5.3)**.

A second objective of this study was to investigate the overall microbiota of raw camel milk collected from different regions in Morocco. A total of 808 isolates were retrieved from multiple culture conditions and subjected to a polyphasic identification approach combining MALDI-TOF MS fingerprinting as a fast and reliable method for dereplication and subsequent identification of representative isolates through comparative sequence analysis of 16S rRNA gene and/or protein encoding genes. The microbiota associated with camel milk was represented by thirty-four different bacterial species belonging to the phyla of *Firmicutes* (64.7%), *Proteobacteria* (30.0%), *Actinobacteria* (4.9%) and *Bacteroides* (0.4%). The *Firmicutes* comprised members of the *Streptococcaceae* (37.7%), *Enterococcaceae* (18.8%), *Staphylococcaceae* (7.1%), *Leuconostocaceae* (0.7%), and *Lactobacillaceae* (0.1%). The *Proteobacteria* included members of the *Enterobacteriaceae* (26.5%) and the *Pseudomonaceae* (3.7%). The phyla of *Actinobacteria* was represented by species belonging to the genus *Rothia* and *Kocuria.* Finally, the *Bacteroides* phylum (0.4%) was represented by species belonging to the *Flavobacteriaceae* **(chapter 6.1)**.

In the course of this study, four *Enterococcus* and two *Lactococcus* isolates remained unidentified after comparison with our in-house MALDI-TOF MS fingerprinting data-

base combined with sequence analysis of 16S rRNA and protein encoding genes including *pheS*, *rpoA* and *atpA*. These taxa were further studied using a polyphasic approach combining random amplified polymorphic DNA analysis, DNA-DNA hybridization experiments, determination of the DNA base composition (% G+C) and a detailed phenotypic characterisation which allowed the description of novel *Enterococcus* and *Lactococcus* species named *E. bulliens* sp. nov. and *L. multifermentans* sp. nov., respectively **(Chapters 6.2 and 6.3)**.

This research presented the first detailed investigation of the LAB microbiota of Moroccan raw camel milk, collected from different Moroccan areas, and identified using state-of-the-art molecular biology techniques. The extensive collection of LAB isolates can now be further studied to determine their food-technological characteristics and their potential as functional starters and probiotics. The present work also provided an extensive and detailed description of the overall microbiota associated with Moroccan camel milk for which, to the best of our knowledge, the bacterial community has never been studied deeply and using modern techniques. It demonstrated the applicability of a (limited) culturomics approach, in which multiple isolation conditions were used, coupled to dereplication with MALDI-TOF MS for a rapid assessment of the bacterial diversity of camel milk.

SAMENVATTING

Kamelenmelk is een van de meest waardevolle voedselbronnen voor herdersvolkeren in droge en semi-droge gebieden, waar het voorziet in een aanzienlijk deel van de kwantitatieve en kwalitatieve voedingsbehoeften. De inheemse bevolking is van mening dat rauwe kamelenmelk veilig en zelfs een therapeutische meerwaarde heeft in vergelijking met melk van andere diersoorten. Hoewel veel studies de microbiologie van melk van koeien, schapen en geiten hebben onderzocht, zijn er slechts een handvol studies die de microbiologie van kamelenmelk bestudeerden. Derhalve is de informatie over de microbiologie van kamelenmelk zeer beperkt. Meer in het bijzonder zijn er nauwelijks studies over de diversiteit en dynamiek van melkzuurbacteriën in kamelenmelk in Marokko. Deze weinige studies pasten daarenboven alleen fenotypische identificatiemethoden toe. Eerder onderzoek heeft aangetoond dat de melkmicrobiota, en in het bijzonder de melkzuurbacteriënfractie ervan, een potentiële bron is van biologisch materiaal voor toepassing in zuiveltechnologie. Om melkzuurbacteriën op industriële schaal te kunnen benutten had deze studie dan ook als doel om de microbiota van kamelenmelk in Marokko in kaart te brengen door middel van de best mogelijke methodologieën.

In de eerste fase van dit werk werd een cultuur-afhankelijke benadering gebruikt om de diversiteit van de melkzuurbacteriën in rauwe kamelenmelk, verzameld op verschillende plaatsen in Marokko te onderzoeken. Een totaal van 129 melkzuurbacterieisolaten werd onderworpen aan een stapsgewijze identificatie waarbij $(GTG)_5$-PCR fingerprinting en 16S rRNA gensequentieanalyse gebruikt werd. Identificatie van de kamelenmelk melkzuurbacteriën toonde aan dat *Leuconostoc* (*Leu*c). *mesenteroides* subsp. *mesenteroides* (41,1%) en *Lactococcus* (*L*). *lactis* subsp. *lactis* (31,8%) het meest geïsoleerd werden. Kleinere aantallen isolaten werden bekomen van *Leuc. pseudomesenteroides* (6,9%), *L. lactis* subsp. *cremoris* (6,2%), *Leuc. citreum* (5,4%), *Leuc. mesenteroides* subsp. *dextranicum* (2,3%) en *Enterococcus* (*E*). *faecium* (1,6%). Bovendien werden enkele isolaten geïdentificeerd als *E. durans* (0. 8%) en *L. raffinolactis* (0. 8%). De stalen van de verschillende onderzochte plaatsen vertoonden een sterke diversiteit in melkzuurbacteriën maar herhalingsstudies zijn nodig om te bepalen of de waargenomen verschillen regio-specifiek zijn. *Leuc. mesenteroides* subsp. *mesenteroides* en *L. lactis* subsp. *lactis* waren niettemin de gemeenschappelijke melkzuurbacteriën die van de meeste van de geanalyseerde monsters geïsoleerd werden (hoofdstuk 5.1).

Tijdens de taxonomische inventarisering deze kamelenmelk melkzuurbacteriën konden de isolaten LMG 27682^T en LMG 27684^T niet worden toegewezen aan één van de bekende soorten melkzuurbacteriën. Vergelijkende 16S rRNA gensequentie toonde aan dat deze bacteriën tot het geslacht *Streptococcus* (*S.*) behoorden met *S. rupicaprae* 2777-2-07^T als de dichtste fylogenetische buur (95,9% en 95,7% overeenkomst, respectievelijk). Beide stammen kunnen worden onderscheiden door verschillende biochemische testen, sequentieanalyse van de fenylalanyl-tRNA synthase, RNA polymerase en ATP synthase genen en hun MALDI-TOF MS profielen waaruit blijkt dat ze twee nieuwe soorten vertegenwoordigen waarvoor de namen *Streptococcus moroccensis* en *Streptococcus rifensis* werden voorgesteld (hoofdstuk 5.2).

Op een gelijkaardige wijze konden nog twee kamelenmelk melkzuurbacteriën (CCMM B832^T en CCMM B834^T) niet worden toegeschreven aan één van de bekende soorten. Fylogenetische analyse op basis van 16S rRNA gensequenties toonde aan dat de twee stammen een tot nu toe onbekende sublijn binnen het genus *Streptococcus* vormden en toonden een nauwe verwantschap met *S. moroccensis*, *S. minor* en *S. ovis*. Verdere taxonomische analyses met inbegrip van DNA-DNA hybridisatie-experimenten, DNA G + C-gehalte bepaling, MALDI-TOF massaspectrometrie en biochemische tests toonden aan dat beide isolaten nieuwe *Streptococcus* soorten vertegenwoordigden waarvoor de namen *Streptococcus tangierensis* en *Streptococcus cameli* werden voorgesteld (hoofdstuk 5.3).

Een tweede grote studie werd uitgevoerd om de totale microbiota van rauwe kamelenmelk verzameld uit verschillende regio's in Marokko te onderzoeken. Een totaal van 808 isolaten werden verkregen door middel van verschillende kweekomstandigheden en onderworpen aan een polyfasische identificatie met onder meer MALDI-TOF MS fingerprinting als een snelle en betrouwbare methode voor dereplicatie en daaropvolgende identificatie van representatieve isolaten door middel van vergelijkende sequentieanalyse van 16S rRNA en/of eiwitcoderende genen. De microbiota geassocieerd met kamelenmelk werd vertegenwoordigd door vierendertig verschillende bacteriesoorten die behoren tot de phyla van *Firmicutes* (64,7%), *Proteobacteria* (30,0%), *Actinobacteria* (4,9%) en *Bacteroides* (0,4%). De *Firmicutes* bestonden uit *Streptococcaceae* (37.7%), *Enterococcaceae* (18.8%), *Staphylococcaceae* (7.1%), *Leuconostocaceae* (0.7%), en *Lactobacillaceae* (0.1%). De *Proteobacteria* bestonden uit leden van de *Enterobacteriaceae* (26. 5%) en de *Pseudomonaceae* (3,7%). De phyla van *Actinobacteria*

werden vertegenwoordigd door soorten die behoren tot het geslachten *Rothia* en *Kocuria*. Tenslotte werd het *Bacteroides* phylum (0,4%) vertegenwoordigd door soorten die behoren tot de *Flavobacteriaceae* (hoofdstuk 6.1).

Tijdens dit onderzoek bleven vier *Enterococcus* en twee *Lactococcus* isolaten ongeïdentificeerd na vergelijking met onze eigen MALDI-TOF MS fingerprint databank gecombineerd met sequentieanalyse van 16S rRNA- en eiwitcoderende genen. Deze taxa werden verder onderzocht met behulp van een polyfasische identificatieaanpak waarbij RAPD analyse, DNA-DNA hybridisatie, het bepalen van de DNA base samenstelling (% G + C) en een gedetailleerde fenotypische karakterisering uitgevoerd werd. Dit liet toe om nieuwe *Enterococcus* en *Lactococcus* soorten te beschrijven waarvoor de namen *Enterococcus bulliens* en *Lactococcus multifermentans* voorgesteld werden (Hoofdstukken 6.2 en 6.3).

Dit onderzoek presenteerde derhalve de eerste gedetailleerde studie naar de melkzuurbacteriën microbiota van Marokkaanse rauwe kamelenmelk, verzameld in verschillende Marokkaanse gebieden, en geïdentificeerd met behulp van moderne moleculair-biologische technieken. De uitgebreide collectie van melkzuurbacteriën kan nu verder worden onderzocht om hun voedsel-technologische eigenschappen en hun potentieel als functionele starterculturen of probiotica te bepalen. Dit onderzoek leverde ook voor het eerst een uitgebreide en gedetailleerde beschrijving van de totale microbiota van Marokkaanse kamelenmelk en illustreerde de toepasbaarheid van een beperkte culturomics studie waarbij meerdere isolatieomstandigheden werden gebruikt, gekoppeld aan dereplicatie met MALDI-TOF MS voor een snelle bepaling van de bacteriële diversiteit van kamelenmelk.

APPENDIX

Tab. A. 1: List of isolates retrieved from raw camel milk samples analysed in the Laboratory of Microbiology at Ghent University

R-number	Original Number	Genus ID	Species ID	Subspecies ID	Gene sequencing	Original sample	Isolation year
54787	CmT-m.ACA.28.AE.1	*Serratia*	*marcescens*	subsp. *marcescens*	*rpoB*	Tazarin, Morocco	2014
54802	CmT-m.M17.28.MA.1	*Serratia*	*marcescens*	subsp. *marcescens*	*rpoB*	Tazarin, Morocco	2014
54803	CmT-m.M17.28.MA.16	*Serratia*	*marcescens*	subsp. *marcescens*	*rpoB*	Tazarin, Morocco	2014
54880	CmT-m.VRBG.28.AE.2	*Serratia*	*marcescens*	subsp. *marcescens*		Tazarin, Morocco	2014
54788	CmT-m.ACA.28.AE.11	*Lactococcus*	*lactis*	subsp. *lactis*	*pheS*	Tazarin, Morocco	2014
54795	CmT-m.CBA.28.AE.12	*Lactococcus*	*lactis*	subsp. *lactis*	*pheS*	Tazarin, Morocco	2014
54800	CmT-m.M17.28.AE.2	*Lactococcus*	*lactis*	subsp. *lactis*	*pheS*	Tazarin, Morocco	2014
54801	CmT-M.M17.28.AE.4	*Lactococcus*	*lactis*	subsp. *lactis*	*pheS*	Tazarin, Morocco	2014
54805	CmT-m.M17.28.MA.5	*Lactococcus*	*lactis*	subsp. *lactis*	*pheS*	Tazarin, Morocco	2014
54807	CmT-m.M17.37.AN.5	*Lactococcus*	*lactis*	subsp. *lactis*	*pheS*	Tazarin, Morocco	2014
54808	CmT-m.MRS.28.AE.1	*Lactococcus*	*lactis*	subsp. *lactis*	*pheS*	Tazarin, Morocco	2014
54790	CmT-m.ACA.28.AE.16	*Lactococcus*	*lactis*	subsp. *lactis*		Tazarin, Morocco	2014
54789	CmT-m.ACA.28.AE.13	*Escherichia/Shigella*			*rpoB*	Tazarin, Morocco	2014
54806	CmT-m.M17.37.AN.4	*Escherichia/Shigella*			*rpoB*	Tazarin, Morocco	2014
54814	CmT-m.PCA+2%milk.28.AE.1	*Escherichia/Shigella*			*rpoB*	Tazarin, Morocco	2014
54819	CmT-m.PCA+2%milk.28.AE.3	*Escherichia/Shigella*			*rpoB*	Tazarin, Morocco	2014
54822	CmT-m.VRBG.28.AE.10	*Escherichia/Shigella*				Tazarin, Morocco	2014
54794	CmT-m.CBA.28.AE.1	*Escherichia/Shigella*				Tazarin, Morocco	2014
54824	CmT-m.VRBG.28.AE.17	*Escherichia/Shigella*				Tazarin, Morocco	2014
54798	CmT-m.M17.28.AE.1	*Klebsiella*	*oxytoca*		*rpoB*	Tazarin, Morocco	2014
54804	CmT-m.M17.28.MA.4	*Klebsiella*	*oxytoca*		*rpoB*	Tazarin, Morocco	2014
54818	CmT-m.PCA+2%milk.28.AE.1	*Serratia*	*marcescens*	subsp. *marcescens*	*rpoB*	Tazarin, Morocco	2014
54811	CmT-m.MSA.28.AE.20	*Staphylococcus*	*simulans*		*dnaJ*	Tazarin, Morocco	2014
54812	CmT-m.MSA.28.AE.5	*Staphylococcus*	*simulans*		*dnaJ*	Tazarin, Morocco	2014
54813	CmT-m.MSA.28.AE.6	*Staphylococcus*	*simulans*		*dnaJ*	Tazarin, Morocco	2014
54797	CmT-m.CBA.28.AE.2	*Staphylococcus*	*capitis*	subsp. *capitis*	*dnaJ*	Tazarin, Morocco	2014
54883	CmT-m.MSA.28.AE.1	*Staphylococcus*	*aureus*	subsp. *aureus*	*dnaJ*	Tazarin, Morocco	2014
54884	CmT-m.MSA.28.AE.25	*Staphylococcus*	*aureus*	subsp. *aureus*		Tazarin, Morocco	2014
54903	CmL-m.M17.28.AE.14	*Streptococcus*	*suis*		*pheS*	Laayoune, Morocco	2014
54906	CmL-m.M17.28.AE.6	*Streptococcus*	*suis*		*pheS*	Laayoune, Morocco	2014
54907	CmL-m.M17.28.AE.8	*Streptococcus*	*suis*		*pheS*	Laayoune, Morocco	2014
54908	CmL-m.M17.28.AN.5	*Streptococcus*	*suis*		*pheS*	Laayoune, Morocco	2014
54898	CmL-m.CBA.37.AE.11	*Streptococcus*	*suis*			Laayoune, Morocco	2014
54921	CmLm.PCA+2%milk.28.AE.19	*Streptococcus*	*suis*			Laayoune, Morocco	2014
54911	CmL-m.MRS.45.AN.17	*Enterococcus*	*faecium*		*pheS*	Laayoune, Morocco	2014
54912	CmL-m.MRS.45.AN.18	*Enterococcus*	*faecium*			Laayoune, Morocco	2014
54941	CmL-m.MRS.45.AN.10	*Enterococcus*	*faecium*			Laayoune, Morocco	2014
54913	CmL-m.MRS.45.AN.19	*Enterococcus*	*faecium*			Laayoune, Morocco	2014
54914	CmL-m.MRS.45.AN.5	*Enterococcus*	*faecium*		*pheS*	Laayoune, Morocco	2014
54890	CmL-m.ACA.28.AE.20	*Enterococcus*	*italicus*		*pheS*	Laayoune, Morocco	2014
54904	CmL-m.M17.28.AE.19	*Enterococcus*	*italicus*		*pheS*	Laayoune, Morocco	2014
54901	CmL-m.CBA.37.AE.7	*Enterococcus*	*italicus*			Laayoune, Morocco	2014
54928	CmLm.TSA+4,5%Nacl.28.AE.16	*Enterococcus*	*italicus*		*pheS*	Laayoune, Morocco	2014
54892	CmL-m.ACA.28.AE.8	*Lactococcus*	*lactis*	subsp. *lactis*	*pheS*	Laayoune, Morocco	2014
54905	CmL-m.M17.28.AE.4	*Lactococcus*	*lactis*	subsp. *lactis*	*pheS*	Laayoune, Morocco	2014
54920	CmL-m.PCA+2%milk.28.AE.17	*Moraxella*	*osloensis*		16S rRNA	Laayoune, Morocco	2014
54916	CmL-m.PCA+2%milk.28.AE.1	*Chryseobacterium*	*arthrosphaerae*		16S rRNA	Laâyoune, Morocco	2014
54922	CmL-m.PCA+2%milk.28.AE.2	*Chryseobacterium*	*arthrosphaerae*		16S rRNA	Laâyoune, Morocco	2014
54918	CmL-m.PCA+2%milk.28.AE.12	*kocuria*	*salsicia*		16S rRNA	Laâyoune, Morocco	2014
54919	CmL-m.PCA+2%milk.28.AE.14	*Empedobacter*	*falsenii*		16S rRNA	Laâyoune, Morocco	2014
54894	CmL-m.ACA+2%milk.28.AE.14	*Lactococcus*	*chungangensis*		*pheS*	Laayoune, Morocco	2014
54896	CmL-m.ACA+2%milk.28.AE.7	*Lactococcus*	*chungangensis*		*pheS*	Laayoune, Morocco	2014
54902	CmL-m.CBA.37.AE.8	*Lactococcus*	*chungangensis*		*pheS*	Laayoune, Morocco	2014
54934	CmL-m.VRBG.28.AE.14	*Raoultella*	*ornithinolytica*			Laayoune, Morocco	2014
54885	CmL-m.ACA.28.AE.1	*Raoultella*	*ornithinolytica*		*rpoB*	Laayoune, Morocco	2014
54932	CmL-m.VRBG.28.AE.11	*Raoultella*	*ornithinolytica*			Laayoune, Morocco	2014
54937	CmL-m.VRBG.28.AE.7	*Klebsiella*	*oxytoca*		*rpoB*	Laayoune, Morocco	2014
54924	CmL-m.PCA+2%milk.28.AE.3	*Enterobacter*	*xiangfangensis*		*rpoB*	Laayoune, Morocco	2014
54886	CmL-m.ACA.28.AE.10	*Enterobacter*	*xiangfangensis*		*rpoB*	Laâyoune, Morocco	2014
54927	CmLm.TSA+4.5%NaCl.28.AE.13	*Enterobacter*	*xiangfangensis*		*rpoB*	Laâyoune, Morocco	2014
54930	CmL-m.TSA+4.5%NaCl.28.AE.2	*Enterobacter*	*xiangfangensis*		*rpoB*	Laayoune, Morocco	2014
54915	CmL-m.PCA+2%milk.28.AE.13	*Enterobacter*	*xiangfangensis*		*rpoB*	Laayoune, Morocco	2014
54893	CmL-m.ACA+2% milk.28.AE.16	*Enterobacter*	*xiangfangensis*		*rpoB*	Laayoune, Morocco	2014
54935	CmL-m.VRBG.28.AE.20	*Enterobacter*	*xiangfangensis*		*rpoB*	Laayoune, Morocco	2014
54909	CmL-m.M17.37.AN.7	*Enterobacter*	*xiangfangensis*		*rpoB*	Laayoune, Morocco	2014
54897	CmL-m.CBA.37.AE.10	*Enterobacter*	*xiangfangensis*		*rpoB*	Laayoune, Morocco	2014
54900	CmL-m.CBA.37.AE.19	*Enterobacter*	*xiangfangensis*			Laayoune, Morocco	2014

54917	CmL-m.PCA+2%milk.28.AE.11	*Enterobacter*	*xiangfangensis*		*rpoB*	Laayoune, Morocco	2014

Tab. A. 1: Continue

R-number	Original Number	Genus ID	Species ID	Subspecies ID	Gene sequencing	Original sample	Isolation year
54933	CmL-m.VRBG.28.AE.12	*Enterobacter*	*xiangfangensis*		*rpoB*	Laayoune, Morocco	2014
54923	CmLm.PCA+2%milk.28.AE.20	*Enterobacter*	*asburiae*		*rpoB*	Laayoune, Morocco	2014
54942	CmL-m.MSA.28.AE.3	*Enterobacter*	*hormaechei*		*rpoB*	Laâyoune, Morocco	2014
54938	CmTm.TSA+4,5%Nacl.28.AE.1	*Escherichia/Shigella*				Laayoune, Morocco	2014
54939	CmTm.TSA+4,5%Nacl.28.AE.5	*Escherichia/Shigella*			*rpoB*	Laayoune, Morocco	2014
54940	CmTm.TSA+4,5%Nacl.28.AE.6	*Escherichia/Shigella*				Laâyoune, Morocco	2014
54887	CmL-m.ACA.28.AE.13	*Aeromonas*	*caviae*		*gyrB*	Laayoune, Morocco	2014
54899	CmL-m.CBA.37.AE.13	*Aeromonas*	*caviae*		*gyrB*	Laayoune, Morocco	2014
54936	CmL-m.VRBG.28.AE.6	*Aeromonas*	*caviae*		*gyrB*	Laayoune, Morocco	2014
54888	CmL-m.ACA.28.AE.15	*Aeromonas*	*caviae*		*gyrB*	Laayoune, Morocco	2014
54895	CmL-m.ACA+2%milk.28.AE.4	*Aeromonas*	*caviae*		*gyrB*	Laayoune, Morocco	2014
54889	CmL-m.ACA.28.AE.17	*Citrobacter*	*freundii*		*rpoB*	Laayoune, Morocco	2014
54931	CmL-m.VRBG.28.AE.10	*Enterobacter*	*cloacae*	subsp. *cloacae*	*rpoB*	Laayoune, Morocco	2014
54926	CmL-m.TSA+4,5%Nacl.28.AE.11	*Enterobacter*	*cloacae*	subsp. *cloacae*	*rpoB*	Laayoune, Morocco	2014
54925	CmL-m.TSA+4,5%Nacl.28.AE.1	*Enterobacter*	*hormaechei*		*rpoB*	Laayoune, Morocco	2014
54985	CmD-m.ACA.28.AE.14	*Leuconostoc*	*pseudomesenteroides*		*pheS*	Dakhla, Morocco	2015
54986	CmD-m.PCA+2% milk.28.AE.11	*Leuconostoc*	*pseudomesenteroides*			Dakhla, Morocco	2015
54987	CmD-m.MRS.45.AN.13	Dead				Dakhla, Morocco	2015
54988	CmD-m.VRBG.28.AE.18	*Enterobacter*	*xiangfangensis*		*rpoB*	Dakhla, Morocco	2015
54989	CmD-m.CBA.37.AE.4	*Enterobacter*	*hormaechei*		*rpoB*	Dakhla, Morocco	2015
54990	CmD-m.VRBG.28.AE.2	*Enterobacter*	*hormaechei*		*rpoB*	Dakhla, Morocco	2014
54991	CmD-m.TSA+4,5%Nacl.28.AE.4	*Enterobacter*	*hormaechei*		*rpoB*	Dakhla, Morocco	2015
54992	CmD-m.TSA+4,5%Nacl.28.AE.19	*Enterobacter*	*hormaechei*		*rpoB*	Dakhla, Morocco	2015
54993	CmD-m.M17.37.AN.4	*Enterobacter*	*hormaechei*		*rpoB*	Dakhla, Morocco	2015
54994	CmD-m.M17.37.AN.1	*Enterobacter*	*hormaechei*		*rpoB*	Dakhla, Morocco	2015
54995	CmD-m.CBA.37.AE.1	*Enterobacter*	*hormaechei*		*rpoB*	Dakhla, Morocco	2015
54996	CmD-m.CBA.37.AE.2	*Enterobacter*	*hormaechei*		*rpoB*	Dakhla, Morocco	2015
54997	CmD-m.MRS.45.AN.7	*Enterococcus*	*faecium*		*pheS*	Dakhla, Morocco	2015
54998	CmD-m.MRS.45.AN.1	*Enterococcus*	*faecium*		*pheS*	Dakhla, Morocco	2015
54999	CmD-m.MRS.45.AN.5	*Enterococcus*	*faecium*		*pheS*	Dakhla, Morocco	2015
55000	CmD-m.M17.37.AN.20	*Enterococcus*	*italicus*		*pheS*	Dakhla, Morocco	2015
55001	CmD-m.M17.37.AN.19	*Enterococcus*	*bulliens*		*pheS*	Dakhla, Morocco	2015
55002	CmD-m.PCA+2% milk.28.AE.1	*Enterococcus*	*bulliens*		*pheS*	Dakhla, Morocco	2014
55003	CmD-m.ACA.28.AE.18	*Enterococcus*	*bulliens*		*pheS*	Dakhla, Morocco	2015
55004	CmD-m.M17.37.AN.5	*Enterococcus*	*bulliens*		*pheS*	Dakhla, Morocco	2015
55005	CmD-m.M17.37.AN.12	*Enterococcus*	*bulliens*		*pheS*	Dakhla, Morocco	2015
55006	CmD-m.TSA+4,5%Nacl.28.AE.18	*Enterococcus*	*bulliens*		*pheS*	Dakhla, Morocco	2015
55007	CmD-m.CBA.37.AE.18	*Enterococcus*	*bulliens*		*pheS*	Dakhla, Morocco	2015
55008	CmD-m.M17.37.AN.3	*Enterococcus*	*bulliens*		*pheS*	Dakhla, Morocco	2015
55009	CmD-m.M17.37.AN.7	*Enterococcus*	*bulliens*		*pheS*	Dakhla, Morocco	2015
55010	CmD-m.M17.37.AN.14	*Lactococcus*	*lactis*	subsp. *lactis*	*pheS*	Dakhla, Morocco	2015
55011	CmD-m.CBA.37.AE.16	*Streptococcus*	*suis*		*pheS*	Dakhla, Morocco	2015
55012	CmD-m.M17.28.AE.19	*Lactococcus*	*lactis*	subsp. *lactis*		Dakhla, Morocco	2015
55013	CmD-m.PCA+2% milk.28.AE.18	*Lactococcus*	*lactis*	subsp. *lactis*		Dakhla, Morocco	2015
55014	CmD-m.M17.37.AN.6	*Lactococcus*	*lactis*	subsp. *lactis*		Dakhla, Morocco	2015
55015	CmD-m.CBA.37.AE.19	*Lactococcus*	*lactis*	subsp. *lactis*		Dakhla, Morocco	2015
55016	CmDm.TSA+4,5%Nacl.28.AE.1	Dead				Dakhla, Morocco	2015
55017	CmD-m.M17.37.AN.11	*Lactococcus*	*lactis*	subsp. *lactis*	*pheS*	Dakhla, Morocco	2015
55018	CmD-m.ACA.28.AE.8	*Lactococcus*	*lactis*	subsp. *lactis*	*pheS*	Dakhla, Morocco	2015
55019	CmD-m.M17.28.AE.15	*Lactococcus*	*lactis*	subsp. *lactis*	*pheS*	Dakhla, Morocco	2015
55020	CmD-m.M17.28.AE.3	*Lactococcus*	*lactis*	subsp. *lactis*	*pheS*	Dakhla, Morocco	2015
55021	CmD-m.M17.37.AN.8	*Lactococcus*	*lactis*	subsp. *lactis*	*pheS*	Dakhla, Morocco	2015
55022	CmD-m.M17.28.AE.8	*Lactococcus*	*lactis*	subsp. *lactis*	*pheS*	Dakhla, Morocco	2015
55023	CmD-m.M17.28.AE.20	*Lactococcus*	*lactis*	subsp. *lactis*	*pheS*	Dakhla, Morocco	2015
55024	CmD-m.ACA+2% milk.28.AE.6	*Lactococcus*	*lactis*	subsp. *lactis*	*pheS*	Dakhla, Morocco	2015
55025	CmD-m.ACA.28.AE.17	*Lactococcus*	*lactis*	subsp. *lactis*	*pheS*	Dakhla, Morocco	2015
55026	CmD-m.ACA+2% milk.28.AE.12	*Lactococcus*	*lactis*	subsp. *lactis*	*pheS*	Dakhla, Morocco	2015
55027	CmD-m.PCA+2% milk.28.AE.10	*Lactococcus*	*lactis*	subsp. *lactis*	*pheS*	Dakhla, Morocco	2015
55028	CmD-m.M17.28.AE.5	*Lactococcus*	*lactis*	subsp. *lactis*	*pheS*	Dakhla, Morocco	2015
55029	CmD-m.CBA.37.AE.13	*Lactococcus*	*lactis*	subsp. *lactis*	*pheS*	Dakhla, Morocco	2015
55030	CmD-m.PCA+2% milk.28.AE.17	*Lactococcus*	*lactis*	subsp. *lactis*		Dakhla, Morocco	2015
55031	CmD-m.M17.37.AN.17	*Lactococcus*	*lactis*	subsp. *lactis*	*pheS*	Dakhla, Morocco	2015
55032	CmD-m.MRS.45.AN.19	*Lactobacillus*	*rhamnosus*		*pheS*	Dakhla, Morocco	2015
55033	CmD-m.VRBG.28.AE.19	*Enterobacter*	*xiangfangensis*		*rpoB*	Dakhla, Morocco	2015
55034	CmD-m.M17.37.AN.9	*Streptococcus*	*salivarius*	subsp. *salivarius*	*pheS*	Dakhla, Morocco	2015
55044	CmZ-m.CBA.37.AE.9	*Rothia*	*endophytica*		16S rRNA	Zagora, Morocco	2015
55045	CmZ-m.CBA.37.AE.20	*Rothia*	*endophytica*		16S rRNA	Zagora, Morocco	2015

55046	CmZ-m.CBA.37.AE.4	*Rothia*	*endophytica*		16S rRNA	Zagora, Morocco	2015
55047	CmZ-m.CBA.37.AE.1	*Macrococcus*	*caseolyticus*		16S rRNA	Zagora, Morocco	2015
55048	CmZ-m.CBA.37.AE.5	*Macrococcus*	*caseolyticus*		16S rRNA	Zagora, Morocco	2015

Tab. A. 1: Continue

R-number	Original Number	Genus ID	Species ID	Subspecies ID	Gene sequencing	Original sample	Isolation year
55049	CmZ-m.M17.37.AN.17	*Macrococcus*	*caseolyticus*		16S rRNA	Zagora, Morocco	2015
55050	CmZ-m.M17.37.AN.8	Dead				Zagora, Morocco	2015
55051	CmZ-m.M17.37.AN.11	*Macrococcus*	*caseolyticus*		16S rRNA	Zagora, Morocco	2015
55052	CmZ-m.M17.37.AN.10	*Enterococcus*	*faecium*		*pheS*	Zagora, Morocco	2015
55053	CmZ-m.M17.37.AN.12	*Enterococcus*	*faecium*		*pheS*	Zagora, Morocco	2015
55054	CmZ-m.CBA.37.AE.6	Dead				Zagora, Morocco	2015
55055	CmZ-m.MRS.45.AN.4	*Enterococcus*	*faecium*		*pheS*	Zagora, Morocco	2015
55056	CmZ-m.CBA.37.AE.16	*Lactococcus*	*lactis*	subsp. *lactis*	*pheS*	Zagora, Morocco	2015
55095	CmD-m.MSA.28.AE.1	Dead				Zagora, Morocco	2015
55096	CmZ-m.ACA.28.AE.10	*Leuconostoc*	*pseudomesenteroides*		*pheS*	Zagora, Morocco	2015
55097	CmZ-m.ACA.28.AE.12	*Enterococcus*	*faecium*		*pheS*	Zagora, Morocco	2015
55098	CmZ-m.ACA.28.AE.13	*Rothia*	*endophytica*		16S rRNA	Zagora, Morocco	2015
55099	CmZ-m.ACA.28.AE.20	*Enterococcus*	*faecium*			Zagora, Morocco	2015
55100	CmZ-m.ACA.28.AE.8	*Rothia*	*endophytica*		16S rRNA	Zagora, Morocco	2015
55101	CmZ-m.ACA.28.AE.9	*Pantoea*	*vagans*		*rpoB*	Zagora, Morocco	2015
55102	CmZ-m.ACA+2%milk powder.28.AE.1	*Acinetobacter*	*bouvetti*		16S rRNA	Zagora, Morocco	2015
55103	CmZ-m.ACA+2%milk powder.28.AE.2	*Macrococcus*	*caseolyticus*		16S rRNA	Zagora, Morocco	2015
55104	CmZ-m.ACA+2%milk powder.28.AE.3	*Rothia*	*endophytica*		16S rRNA	Zagora, Morocco	2015
55105	CmZ-m.M17.28.AE.10	*Enterococcus*	*faecium*		*pheS*	Zagora, Morocco	2015
55106	CmZ-m.M17.28.AE.11	*Rothia*	*endophytica*		16S rRNA	Zagora, Morocco	2015
55107	CmZ-m.M17.28.AE.3	*Lactococcus*	*lactis*	subsp. *lactis*	*pheS*	Zagora, Morocco	2015
55108	CmZ-m.M17.28.AE.5	*Enterococcus*	*faecium*		*pheS*	Zagora, Morocco	2015
55109	CmZ-m.M17.28.AE.6	*Rothia*	*endophytica*		16S rRNA	Zagora, Morocco	2015
55110	CmZ-m.M17.28.AE.7	Dead				Zagora, Morocco	2015
55111	CmZ-m.M17.28.AE.9	*Pseudomonas*	*koreensis*		*rpoB*	Zagora, Morocco	2015
55112	CmZ-m.PCA+2%milk powder.28.AE.1	*Pseudomonas*	*koreensis*			Zagora, Morocco	2015
55113	CmZ-m.PCA+2%milk powder.28.AE.18	*Leuconostoc*	*pseudomesenteroides*		*pheS*	Zagora, Morocco	2015
55114	CmZ-m.PCA+2%milk powder.28.AE.2	*Citrobacter*	*werkmanii*		16S rRNA	Zagora, Morocco	2015
55115	CmZ-m.PCA+2%milk powder.28.AE.4	*Enterococcus*	*faecium*			Zagora, Morocco	2015
55116	CmZ-m.PCA+2%milk powder.28.AE.6	*Enterococcus*	*faecalis*		16S rRNA	Zagora, Morocco	2015
55117	CmZ-m.TSA+4.5%NaCl.28.AE.19	*rothia*	*marina*		16S rRNA	Zagora, Morocco	2015
55118	CmZ-m.VRBG.28.AE.1	*Acinetobacter*	*bouvetti*		16S rRNA	Zagora, Morocco	2015
55119	CmZ-m.VRBG.28.AE.10	*Pseudomonas*	*moraviensis*		*rpoB*	Zagora, Morocco	2015
55120	CmZ-m.VRBG.28.AE.14	*Pseudomonas*	*moraviensis*		*rpoB*	Zagora, Morocco	2015
55121	CmZ-m.VRBG.28.AE.16	*Pantoea*	*agglomerans*		*rpoB*	Zagora, Morocco	2015
55122	CmZ-m.VRBG.28.AE.2	*Citrobacter*	*freundii*		*rpoB*	Zagora, Morocco	2015
55123	CmZ-m.VRBG.28.AE.7	*Pantoea*	*agglomerans*		*rpoB*	Zagora, Morocco	2015

Dead: not grow