Gembloux Agro-Bio Tech
Université de Liège

COMMUNAUTE FRANCAISE DE BELGIQUE
ACADEMIE UNIVERSITAIRE WALLONIE-EUROPE
UNIVERSITE DE LIEGE GEMBLOUX AGRO-BIO TECH

Fermentation du *kivuguto*, lait traditionnel du Rwanda: *mise au point d'un starter lactique*

Eugène KARENZI

Dissertation originale présentée en vue de l'obtention du grade de Docteur en Sciences agronomiques et Ingénierie biologique

Promoteur: Pr Philippe JACQUES
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A Dieu Tout-Puissant

Vous êtes et vous resterez toujours Grand.

Amen

A ma famille

Merci pour la résistance et la tenue des babies hors des soucis

A mon père

Que cette thèse soit un prolongement de votre travail sur les fermentations traditionnelles pour la pérennisation d'une tradition familiale depuis 1400 et un couronnement de vos leçons d'Arithmétique & français sur un tableau de morceau de bois conçu par vos propres mains.

Je ne peux être plus fier que de soutenir cette thèse de Doctorat en Bioindustries dans cette université de Liège Gembloux Agro-BioTech.

Sorry for delaying,
the things were so hard, the sky
was so cloudy, the weather so bad,
but people so nice
and job so exciting
Thanks for the good work you are doing now
We love you forever,

A ma mère,frères & soeurs

Pour l'absence prolongée et la peine endurée.

Karenzi E.

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SUMMARY/RESUME

Eugène Karenzi (2015). **Fermentation du *kivuguto,* lait traditionnel du Rwanda: *mise au point d'un starter lactique.*** Thèse de Doctorat. Université de Liège Gembloux Agro-Bio Tech. Belgique. 191 pages. 16 tables. 28 figures.

Résumé

Une recherche pour la production industrielle du *kivuguto,* lait caillé traditionnel du Rwanda a été entreprise par la sélection de micro-organismes responsables de la fermentation de ce lait normalement produit artisanalement. Pour ce faire, il fallait une mise au point d'un starter lactique à partir des isolats issus des échantillons de lait artisanal *kivuguto*.

Au terme de quatre échantillonnages, un dénombrement, un isolement et une purification ont abouti à conserver dans la Collection du CWBI 390 souches pures. Par des analyses phénotypiques (microscopiques, biochimiques), associées à des tests de résistance aux conditions extrêmes et à une analyse préliminaire des propriétés technologiques, 7 souches ont été pré-sélectionnées pour la poursuite du screening. Une caractérisation moléculaire par la méthode de 16S ADNr associé ou non à l'ITS 16S-23S ADNr a assimilé ces souches à deux *Lactococcus lactis*, deux *Leuconostoc mesenteroides* et trois *Leuconostoc pseudomesenteroides.* Des essais de formulation de laits fermentés par des mélanges de souches et leur conservation pendant 24 jours ont permis de formuler un lait fermenté semblable au lait artisanal *kivuguto* par l'association d'un *Lactococcus lactis*, d'un *Leuconostoc mesenteroides* et d'un *Leuconostoc pseudomesenteroides*. En effet, lors d'une analyse sensorielle discriminative, un jury de dégustation constitué de huit personnes est parvenu à identifier ce lait formulé parmi deux autres laits fermentés commercialisés aussi sur le marché à des différences significatives de p=0.05 dans une première série et p=0.01 dans une deuxième série. Des analyses technologiques proprement dites ont montré que ce lait formulé fermente après 14 heures avec une acidification de 73°D à pH4.6 et à 19°C, présente des caractéristiques d'un fluide visco-élastique. Son activité protéolytique est moyenne pour ne pas développer des peptides responsables de l'amertume en stockage. Son profil aromatique comporte cinq composés principaux 3-méthylbutan-1-ol, pentan-1-ol, acide acétique, furanméthan-2-ol et furan-2(5)H-one clairement identifiés par GC-MS. L'étude de la production et de la conservation de trois souches sélectionnées a montré une bonne stabilité sur trois mois à 4°C avec des viabilités cellulaires >90%, mais moins bonne à 20°C.

Eugène Karenzi (2015). **Fermentation of *kivuguto,* a rwandese traditional milk: making a starter culture** (Ph.D. Thesis). University of Liège - Gembloux Agro-Bio Tech, Belgium. 191 pages. 16 tables. 28 figures.

Summary

A research for the industrial production of *kivuguto* traditional curd of Rwanda was undertaken by conducting a selection of microorganisms involved in the fermentation of that milk normally produced artisanally by householders. For that purpose, the objective of this work was the development of a starter culture using strains isolated in the curd milk *kivuguto*. After four samplings, an enumeration, isolation and purification led to preserve in the CWBI Collection 390 pure strains. By phenotypic analyses (microscopic, biochemical) associated with resistance tests under extreme conditions, as well as a preliminary analysis of technological properties, 7 strains were pre-selected for a further screening. Molecular characterization by 16S rDNA method with or without the 16S-23S rDNA ITS assimilated these strains to two *Lactococcus lactis*, two *Leuconostoc mesenteroides* and three *Leuconostoc pseudomesenteroides*. Thereafter, these strains were used in mixed assays for milk formulation of fermented milks and their preservation over a 24 days period helped to make a fermented milk similar to the traditional *kivuguto*. An association of one *Lactococcus lactis*, one *Leuconostoc mesenteroides* and one *Leuconostoc pseudomesenteroides* developed a much closer *kivuguto* artisanal milk. Indeed, in a discriminative sensory analysis, a tasting panel of eight people reached its identification among two other fermented milks sold also on the local market with significant differences of p=0.05 in the first series and p=0.01 milk in a second series. Technological analyzes showed that this formulated milk ferments within 14 hours after acidification of 73 °D at pH4.6 and 19 °C, has the characteristics of a viscoelastic fluid. It presents a mean value of proteolytic activity, so that it can't develop bitter peptides in milk during storage. Its flavor profile includes five main compounds: 3-methylbutan-1-ol, pentan-1-ol, acetic acid, furanmethan-2-ol and furan-2(5)H-one clearly identified by GC-MS. The study of production and viability in storage of the three selected strains showed good stability over three months at 4 °C with cell viabilities >90% and less at 20°C.

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ABREVIATIONS

°C	Degré Celsius
°D	Degré Dornic
2D	Two Dimension
AAB	Acetic acid bacteria
ACE	Angiotensin-I Converting Enzyme
ADB	African Development Bank
Al-DH	Aldehyde dehydrogenase
API	Analytical profile index
ARDRA	Amplified ribosomal DNA restriction analysis
ATCC	American Type Culture Collection, Virginia, USA
ATP	Adenosine triphosphate
A_w	Water activity
BBL	Baltimore Biological Laboratory
BC	Before Christ
BLAST	Basic local alignment search tool
BMC	BioMed Central
BNR	Banque Nationale du Rwanda
BPR	Banques Populaires du Rwanda
BRD	Rwanda Development Bank
CA	California
CAADP	Comprehensive Africa Agriculture Development Program, Rwanda
CAS	Chemical Abstracts Service
CCUG	Culture Collection, University of Göteborg, Sweden.
CEPIL	Centre de formation permanente et de perfectionnement des cadres des industries du lait, Paris, France
cFDA	Carboxyfluorescein diacetate
CIP	Collection de l'Institut Pasteur
CNIEL	Centre national interprofessionnel de l'économie laitière
CNRS	Centre National pour la Recherche Scientifique
CPG	Chromatographie en phase gazeuse
CRC press	Chemical Rubber Company press

CSIRO	Commonwealth Scientific and Industrial Research Organisation
CUD	Communauté Universitaire pour le Développement, Belgium
CWBI	Centre Wallon de Biologie Industrielle, Belgium
D.C.	District of Columbia,
DMS	Development & Management Solutions
DNA-DNA	Deoxyribonucleic acid - Deoxyribonucleic acid
DNPH	2,4-dinitrophenylhydrazine
DSM=DSMZ	Deutsche Sammlung von Mikroorganismen und Zellkulturen, Germany
EAAP	European Association of Animal Production
EC	European Community
EI	Electron impact
EMBL	European Molecular Biology Laboratory
EPS	Exopolysaccharides
EU	European Union
EUFIC	European Food Information Council
EXCLI	Experimental and Clinical Sciences Journal
FAAs	Free amino acids
FAME	Fatty acid methyl esters
FAO	Food and Agriculture Organisation
FDA	Food and Drug Administration
FEMS	Federation of European Microbiological Societies
FID	Flame Ionization Detector
FIL-IDF	Fédération Internationale des Laiteries-International Dairy Federation
G'	Storage Modulus
G"	Loss Modulus
GC	Gas Chromatography
GC-MS	Gas Chromatography-Mass Spectrometry
GEA	Global Engineering Alliance
GNI	Gross National Income
GOS	α-glucooligosaccharides
HACCP	Hazard Analysis Critical Control Points
HCl	Hydrogen chloride
HP	Hewlett Packard

HPLC	High Performance Liquid chromatography
HS	Headspace
Hz	Hertz
ICSP	International Committee on Systematics of Procaryotes
ICTF	International Commission on the Taxonomy of Fungi
IFAD	International fund for agricultural development
IFO (now NBRC)	Institute for Fermentation, Culture Collection of Microorganizms (now NITE Biological Resource Center, Chiba, Japan)
IJSEM	International Journal of Systematic and Evolutionary Microbiology
ISAR	Institut des Sciences Agronomiques du Rwanda
ITIS	Integrated Taxonomic Information System
ITS	Internal Transcribed Spacer
IUPAC	International Union of Pure and Applied Chemistry
KF	Kenner-Faecal
KOH	Potassium Hydroxide
L/D	Large/Diameter
LAB	Lactic acid bacteria
LBS	Lactobacillus Selection
LMG (BCCM/LMG)	Collection of the Laboratorium voor Microbiologie en Microbiele Genetica, Gent, Belgium
MALDI-TOF	Matrix Assisted Laser Desorption/Ionization-Time Of Flight
MCCs	Milk collection centers
MD	Maryland
MINAGRI	Ministry of Agriculture and Animal Resources, Rwanda
MINICOM	Ministry of Trade and Industry, Rwanda
MRS	De Man, Rogosa and Sharp
MTT	Maaja Elintarviketalouden Tutkimuskeskus, Finnish Agrifood Research
NaCl	Sodium Chloride
NAD	Nicotinamide Adenine Dinucleotide
NADH	Nicotinamide Adenine Dinucleotide Hydrogene
NAEB	National Agriculture Export Development Board
NaOH	Sodium hydroxide
NC	North Carolina

NCDO	National Collection of Dairy Organisms, Karnal, India
NCFB	National Collection of Food Bacteria, UK
NCTC	National Collection of Type Cultures, London, UK.
NEPAD	New partnership for Africa's development
NGOs	Non Governmental Organisations
NISR	National Institute of Statistics of Rwanda
NIST	National Institute of Standards and Technology, MD, USA
NJ	New Jersey
$NO^{\cdot}$	Nitric oxide
NRIC	Nodai Research Institute Culture Collection, Tokyo, Japan.
NSOCPIBS	National Seminar on Current Perspectives in Biological Sciences
NY	New York
OCL	Oléagineux, corps gras, lipides
OD	Optical density
OECD	Organisation for Economic Cooperation and Development
ONG	Organisations Non Governementales
ONOO	Peroxynitrite
OPA	O-phthaldialdéhyde
PADEBL	Dairy Cattle Development Support Project
PAL	Palisade Complete Mass Spectral Library
PCR	Polymerase chain reaction
PDA	Potato dextrose agar
PDO	Protected Designation of Origin
PEP	Phosphoenolpyruvate
pH	Potential of hydrogen
PhD	Philosophical degree
PI	Propidium iodide
PPS	Strategic Planning & Program
PTS	Phosphotransferase system
RAB	Rwanda Agriculture Board
RAPD	Random amplification of polymorphic DNA
RARDA	Rwanda Animal Resources Development Authority
RBS	Bureau of Standards

RCA	Rwanda Cooperative Agencies
rDNA	Ribosomal deoxyribonucleic acid
RDP	Ribosomal DNA Project
RFLP	Restriction Fragment Length Polymorphism
RI	Retention index
RNA	Ribonucleic acid
RNS	Reactive nitrogen species
ROS	Reactive oxygen species
rRNA	Ribosomal ribonucleic acid
SFA	Saturated Fatty Acids
SM	Spectrométrie de masse
SR	Survival rate
Tm	Melting temperature
TPY	Trypticase Phytone Yeast
UFA	Unsaturated Fatty Acids
UHT	Ultra High Temperature
UK	United Kingdom
ULG	Université de Liège
US	United States
USA	United States of America
USD	United States Dollar
UV	Ultra-violet
VCs	Volatile compounds
Vm	Maximum velocity
YSI	Yellow Springs Instruments, OH, USA

LISTE DES FIGURES

Chapitre 7

LISTE DES TABLEAUX

Table des matières

Avant-propos

Cet essai est présenté sous forme d'articles scientifiques avec une étude théorique sur la fermentation du lait et les ferments utilisés en laiterie, le tout en huit chapitres.

Le **chapitre 1**, intitulé "**Milk fermentation**" précise la problématique de la recherche et le contexte global du secteur laitier à l'échelle mondial avec une projection sur la situation actualisé du secteur laitier du Rwanda de façon générale et, de façon particulière, sur les connaissances actuelles de la fermentation du *kivuguto,* lait fermenté du Rwanda.

Le **chapitre 2** "**Dairy starter cultures**" se charge de la sélection des micro-organismes utilisés dans les produits laitiers, en soulignant et en actualisant les principaux genres entrant en composition des ferments, leur taxonomie, leurs propriétés technologiques et leur production industrielle.

Le **chapitre 3** "**Selection of microbes in *kivuguto* milk**" marque le début des analyses expérimentales et se consacre à la sélection des micro-organismes véhiculant l'identité du *kivuguto* et le chapitre 4 "**Technological properties**" analyse les aspects technologiques de ces micro-organismes.

Les **chapitres 5 et 6** concernent la **caractérisation des souches sélectionnées** sous l'aspect production, procédés post-fermentaires et conservation pour formuler le starter lactique du *kivuguto*.

Le **chapitre 7** traite de la **production et de la stabilité du *kivuguto***, avec à la fin un **chapitre 8** pour **discuter les données essentielles issues** des chapitres précédents, suivi d'une **conclusion générale** pour mettre en évidence les éléments les plus importants de cette étude, leur impact et les **perspectives** pour des travaux futurs.

Chapter 1

Milk fermentation

*La fermentation du lait (**chapitre 1**) constitue un chapitre introductif. Il présente un panorama général de la production du lait, de sa répartition géographique mondiale et de l'état actuel des enjeux socio-économiques de ce produit **(General Aspects)**. Ensuite, ce chapitre se penche sur le contexte de ce travail. Il détaille les contours de cette recherche en mettant en exergue les objectifs et en structurant les stratégies pour les atteindre. En outre, il précise l'état actuel de la fermentation laitière au Rwanda **(Global context of milk fermentation in Rwanda)**. Pour cela, dans un article publié dans la revue BASE, il fait une étude descriptive de la fermentation traditionnelle du kivuguto, de la production laitière au Rwanda et de la situation actuelle de l'industrie laitière rwandaise.*

1. GENERAL ASPECTS

Milk is a white secretion of mammals produced by the mammary glands from many animals like cow, buffalo, goat, sheep, camel, horse, yak and zebu. It is very nutritious and serves primarily to nourish the young animals before being able to digest other food. In the present work, we will talk about milk from cow. Indeed, in regulatory terms, the word milk without indication of the animal species means cow's milk. Any other milk from other animals is coupled with the name of the animal it comes from, like buffalo milk, camel milk, goat milk (Decree of 25 March 1924 on milk and dairy products).

The Codex Alimentarius states that raw milk has not undergone heat treatment above 40°C or any other treatment having an equivalent effect. Nowadays, raw milk is defined by European regulations (Corrigendum to Regulation (EC) No 853/2004 of the European Parliament and of the Council of 29 April 2004) as milk produced by the secretion of the mammary gland of farmed animals and not heated above 40°C or subjected to treatment having equivalent effect (EUR-LEX, 2004). The Food and Drug Administration (FDA), under the US Public Health Services (USPHS), defines milk as the lacteal secretion, practically free from colostrum, obtained by the complete milking of one or more healthy cows, which may be clarified and may be adjusted by separating part of the fat therefrom; concentrated milk, reconstituted milk, and dry whole milk (Hickey, 2009).

Milk constituents are synthesized in specialized epithelial secretory cells known as mammocytes from molecules absorbed from blood (Fox and McSweeney, 1998) especially precursors of milk protein and carbohydrates. Other constituents like milk salts, some hormones and some proteins like immune-globulins come intact from blood. Therefore, the precursors of milk components enter the epithelial cells and follow the specific synthetic pathway.

Milk has a slightly acidic pH about 6.6 when left udder’s cow. The main constituent of milk is water at about 87%. It also contains proteins, fats, lactose, and various vitamins and minerals known also as milk salt or ash with calcium, sodium, potassium and magnesium as major components. There are two types of proteins in milk: casein proteins and whey proteins. About 90% of milk protein is composed of casein and is produced early in mammary glands as well as the whey proteins. This nutritious content makes it very perishable due to micro-organisms in like of this rich nutrients. When microorganisms are fermentative, milk changes

its structure in the process called coagulation, otherwise it spoils with pathogenic microbes contamination.

During coagulation, the fermentation process produces acidic dairy products by breaking down of milk complex organic substances into simple molecules. Lactose is the main source of energy in milk. For lactic acid fermentation, the electrons released by the huge molecule of lactose during glycolysis are passed to pyruvic acid to form two molecules of lactic acid. Lactic acid fermentation is carried out by many bacteria, mostly by the lactic acid bacteria used in the production of fermented milks and other dairy products. Fermentation is one of the oldest methods practiced by human beings for the transformation of milk into products with an extended shelf life.

The manufacturing of cultured dairy products represents the second most important fermentation industry (after the production of alcoholic drinks) (Khurana and Kanawjia, 2007; Anon, 2003). The study of Euromonitor international showed that global sales of dairy products in 2002 reached 211.5 billion USD (Adwan, 2003). In 2010, world milk production (**figure1)** is approaching 711 million tons, marking a slight increase of 2.6% compared to 2009.

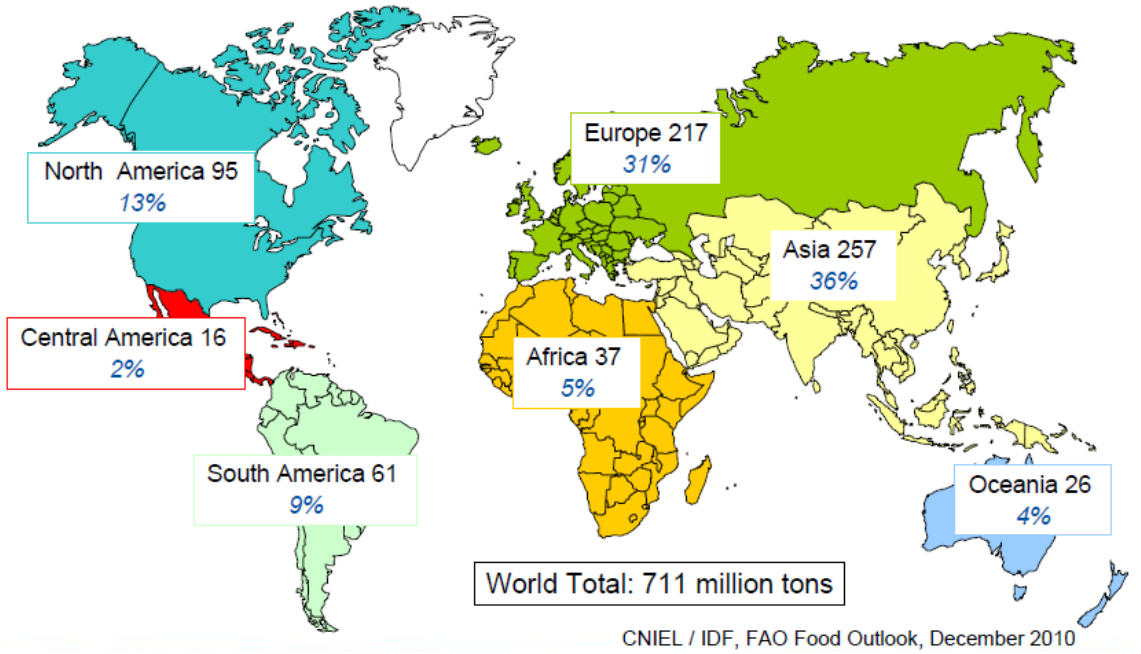

Figure1. Distribution of world milk production (millions tons) in 2010 (Rouyer, 2011).

Global production is dominated by cow's milk with 83% of the amount produced in 2010 (CNIEL, 2013). Behind, buffalo milk accounts for 13%. From the female buffalo, or 'wild ox',

the buffalo milk is not popular in Europe and is mainly collected in Asian countries (India, Pakistan, China); followed by goat milk (2%), sheep (1%) and other mammals, such as camels (0.2%) (CNIEL, 2013). However, popularity of dairy products, westernisation of diets and the increasing range of dairy products continue to be the key drivers underpinning dairy markets worldwide (OECD/FAO, 2011). The global dairy market is up to now dominated by western countries marked by a wide variation in liquid milk consumption per capita with about 50 to 100 kg per year. On that group of countries, we can add some countries like Brazil, Australia and New Zealand. Other countries are less than 50 kg per capita and per year. The same figures are similar both in production and trade (**figure 2**). But the new trend released by the 2012 World dairy situation shows an increase in import and consumption of Russia, Brazil, India and China (**figure 3**) as emerging countries driving now global economy, but the main suppliers remain the European Union, USA, New Zealand and Australia.

Fermented milks are manufactured throughout the world and approximately 400 generic names are applied to traditional and industrialized products (Khurana and Kanawjia, 2007; Kurmann et al., 1992) but unknown traditional milks lack on the list, especially in Africa and other developing countries.

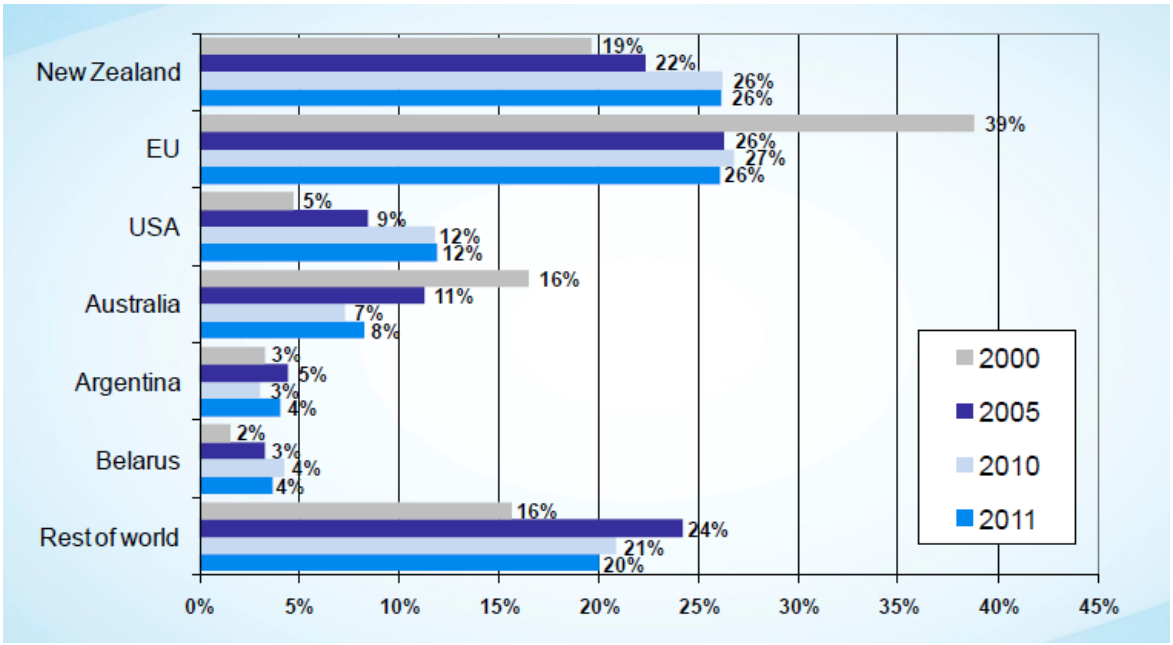

Figure 2. World milk trade: market shares (Krijger, 2012).

Nevertheless, many authors agree that many of these names actually refer to the same sort of preparation and that "only" something like 300 different preparations can really be identified

(Thormahlen et *al.*, 2005). However, among all of those milks, there are those well known and well characterized, but the first fermented milk well researched may be the yogurt and is produced worldwide using two strains of *Streptococcus thermophilus* and *Lactobacillus delbrueckii subsp. bulgaricus*.

The fermentation of milk is first an ancient practice of preservation and is believed to date around 9000 BC in Irak, and this technique has spread to other regions with the migrations of people to Europe and Asia. The products of this fermentation are fermented milks, but also cheese, butter, buttermilk. Until very recently, the nutritional values remained unknown, but already in Eastern Europe, around the 19th century, people see in fermented milks more than nutritional virtues, even better therapeutic attributes and well-being, able to give longevity to people whose diet is fermented products based. Yogurt, kefir and koumiss are fermented products of this region. Scientists found therefore an object of study and with new data that provides microbiology as an emerging science at that time, they began to make the first tests on yogurt with Professor Metchnikoff in collaboration with the Pasteur Institute.

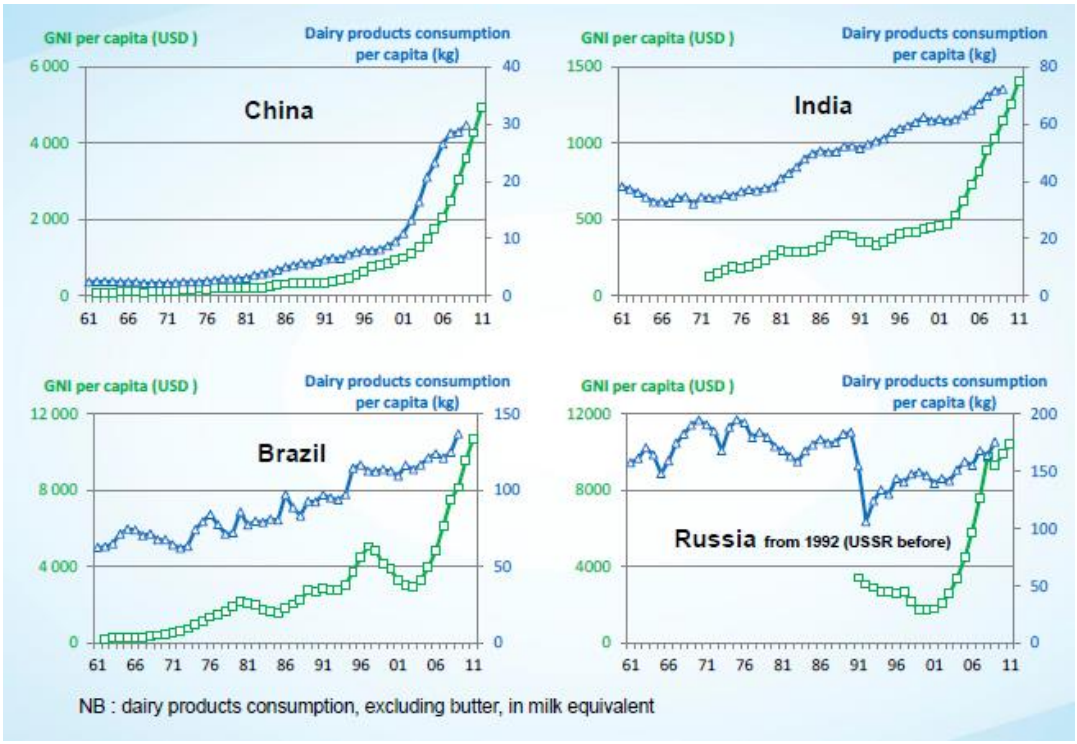

Figure 3. Strong relation between Gross national income (GNI) and dairy consumption per capita (Krijger, 2012).

Around 1920, two strains appear to be the bearers of the identity of yogurt. Much later, other fermented milks have been studied in Europe and Asia and their biochemical formulations were well established. At that time, with the tools of molecular biology, microbial selection

techniques have given birth to a wide variety of products with distinct and pleasant flavors.

In Africa, research on microbial selection allowed knowledge of fermented milks of Maghreb, but also from Senegal, Sudan, Zimbabwe, South Africa, Ethiopia, Ghana, etc. The fermented milk produced depends so far on the type of microbial cells involved, and subsequently on the environment conditions. The well known fermented milks are yogurt ("yog" means thick, "urt" milk) and kefir. The yogurt fermentation is a homolactic fermentation (Nauth, 2004) in which the lactose utilization involves lactose transport into the cells via proteins cytoplasmic lactose permeases. In this process, the bacilli yogurt cell coagulates milk rapidly, giving it a strongly acidic flavor (Metchnikoff, 1908), but the curdled milk presents a very pleasant flavour due to the association with a *Streptococcus* genus. Kefir is an acid-alcoholic dairy beverage originated in Eastern Europe. It is traditionally produced with kefir grains which are small, irregularly shaped, yellowish masses (Zajsek and Gorsek, 2010). These Kefir grains have a varying and complex microbial composition that includes species of yeasts, lactic acid bacteria (LAB), acetic acid bacteria (AAB), and mycelial fungi (Witthuhn et *al*., 2005). There are two types of fermented milks, the traditional and the nontraditional. Traditional fermented milks have a long history and are known and made all over the world whenever milk animal were kept (Gaden et *al.*, 1992). There are two main types: (1) products prepared with a defined culture and (2) products with a non-defined or empirical culture (Kurmann et *al.*, 1992). In contrast, nontraditional fermented milks are recently developed. They are based on known scientific principles, their microbial cultures are known, and their quality can be optimized (Gaden et *al*., 1992). In many countries, over the centuries there has been a gradual shift from uncontrolled fermentation procedures, in which the microflora is not defined, towards a new generation of fermented milks in which the microflora is defined (Danone, 2005).

In Rwanda, the *kivuguto* fermented milk, the main topic of this study is well known for several centuries. Nevertheless, it is only very recently that scientists began to think about the industrial production of that fermented milk well appreciated both by citizens and foreigners. Although its specificity and its appreciation against other fermented milks produced industrially are obvious, the *kivuguto* quality is lacking in stability as far as space and time are concerned. The primary objective of this work is therefore the development of a starter culture from microorganisms selected in the natural environment of *kivuguto* for industrial production of that foodstuff. To understand and to overcome this issue, four strategies were adopted:

Strategy 1. Selection of microbial starters (Chapter 3)

- Sample milk in two regions of Rwanda, away from areas with exotic strains
- Isolate and characterize phenotypically fermentative microorganisms
- Characterize genotypically by means of the 16S rDNA and/or 16S-23S rDNA coupled to PCR and sequencing

Strategy 2. Technological properties of microbial starters (Chapter 4)

Characterize technologically micro-organisms responsible for the formulation of *kivuguto* starter culture:

- Acidification & cell viability
- Rheology
- Production of flavors
- Proteolysis
- Sensory analysis

Strategy 3. Production and preservation of microbial starters (Chapters 5 & 6)

• Production in 20 liter bioreactor in batch and/or fed-batch, followed by down-stream process consisting in centrifugation, cryoprotection, freeze-drying and/or spray-drying.

• Preservation of micro-organisms by vacuum packaging freeze-dried cells and/or spray-dried cells on three months.

Strategy 4. Production and stability of *kivuguto* made with selected strains (Chapter 7)

• Production of *kivuguto* in 2 liters and 20 liters

• Studying the stability of *kivuguto* under refrigeration for 36 days to assess the evolution of acidity, microbial viability, rheology, proteolysis and volatile compounds (VCs).

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2. GLOBAL CONTEXT OF MILK FERMENTATION IN RWANDA

Fermented milk in Rwanda is called *kivuguto* and is consumed since ancient time, probably with the first introduction of cattle in the country. Since then, it is produced by traditional method used also to prepare butter or buttermilk.

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Kivuguto traditional fermented milk and the dairy industry in Rwanda

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ABSTRACT

***Kivuguto* traditional fermented milk and the dairy industry in Rwanda.** Traditional methods of fermenting milk involve the use of indigenous microorganisms, leading to the production of a variety of tastes in fermented milk products. *Kivuguto* is a fermented milk product, which is popular in Rwanda. *Kivuguto* is produced by traditional spontaneous acidification of raw milk by a microflora present both on utensils and containers used for milk preservation and in the near environment of cattle. Thus, this method does not allow the shelf stability of the product. Faced to such a situation, modern dairies now produce fermented milk and other dairy products using exotic strains. The main objectives of this paper are firstly, to provide documentation on the traditional production of *kivuguto*, as well as its by-products, and secondly, to describe the current situation of the dairy industry in Rwanda.

Keywords. Lactic fermentation, traditional technology, cultured milk, dairy industry, *Lactococcus lactis*, *Leuconostoc*, Rwanda.

RESUME

Lait fermenté traditionnel *kivuguto* et l'industrie laitière au Rwanda (synthèse bibliographique). La fermentation traditionnelle du lait, de loin la plus pratiquée au Rwanda, est effectuée par des micro-organismes indigènes. Cela conduit à la production d'une variété de saveurs dans les produits laitiers fermentés. Le *kivuguto*, un produit laitier obtenu par cette fermentation, est caractérisé par un manque de maitrise de qualité aussi bien dans l'espace que dans le temps. Elle est produite par l'acidification spontanée du lait cru par une microflore rencontrée dans les ustensiles utilisés pour la conservation du lait et dans l'environnement proche du bétail. Face à une telle situation, les laiteries modernes produisent du lait fermenté et autres produits laitiers en utilisant des souches exotiques. L'objectif principal de ce travail est de documenter la production traditionnelle du *kivuguto* et ses sous-produits, et d'extrapoler à la situation actuelle de l'industrie laitière au Rwanda.

Mots-clés. Fermentation lactique, technologie traditionnelle, lait fermenté, industrie laitière, *Lactococcus lactis*, *Leuconostoc*, Rwanda.

1. INTRODUCTION

Rwanda is located between latitudes 1°04' and 2°51' South and longitudes 28°45' and 31°15' East. It is a landlocked country situated between Burundi in the South, Tanzania in the East, the Democratic Republic of Congo in the West and North-West and Uganda in the North. Its altitude above sea level varies from 1,000 m to 4,507 m, with the highest point situated at the Karisimbi volcano, one of six in a chain of volcanoes extending from the far east of Congo to the North-West of Rwanda. Rwanda has a temperate tropical highland climate, with temperature variations across the country of between 14 °C and 25 °C. There are two rainy seasons in the year, from February to June and from September to December, and two dry seasons from June to September and from December to February. Rainfall varies geographically, with twice as much average annual precipitation in the West as in the East. These environmental conditions give Rwanda a mild and cool climate, which guarantees good pasture, providing sustainable welfare conditions for animals. Animal husbandry has been an integral part of the Rwandese culture for many centuries. Cattle, in particular, have served as a symbol of political power and have been the traditional mainstay of the Rwandese economy (Adekunle, 2007). Rwanda is faithful to its agro-pastoral tradition and, as such, over the centuries, the cow has taken on an importance in all areas of the country's culture. In all the social activities associated with cows (language, ceremonies, speeches, customs and taboos in their honor), the symbolic value of the animals transcends their economic value. Nevertheless, cows have been exploited for centuries by the Rwandese people for their meat, skin and milk. It is believed that milk has been consumed since the domestication of cattle. Milk represents an important food, and its nutritional value as well as its many health benefits have been well known to Rwandese society since ancient times. Milk consumption used to be the privilege of a few rich families and, as such, cows have played a somewhat divisive role in Rwandese society. Today, attempts are being made to build a new society where all people share the same values. As part of this approach, opportunities are being created and different policies are being implemented in order to develop the livestock sub-sector and the dairy industry in particular. Agriculture now contributes 40% to Rwanda's national GDP (Gross Domestic Product), of which 8.8% comes from livestock (animal resources). According to Rutamu (2008), annual milk production in Rwanda in 2005 was estimated at 178,598 tons of milk, and this quantity is not sufficient to satisfy the needs and requirements of the population. The

average milk consumption in Rwanda is 0.035 l per person per day (13 l of milk per person per year) and 75% of that milk is consumed in rural areas. Given this situation, the country is forced to import milk from Uganda and Kenya. The Ministry of Agriculture (MINAGRI) has set up strategies and programs to improve the dairy farmer's capacity and organizational skills, with an emphasis on the dairy chain and on the strengthening of the institutional framework at central and local levels (MINAGRI, 2009). In the present paper, an overview of traditional technology for producing *kivuguto* and its by-products is presented. New trends for the development of the dairy industry in Rwanda as well as information on milk consumption and the milk market are also discussed.

2. *KIVUGUTO* AND ITS BY-PRODUCTS

2.1. *Kivuguto* processing technology

In the traditional method of producing *kivuguto,* once the cow has been milked, the milk is placed in a jar called an *inkongoro*. After this, the milk is poured into a big, clean, wooden jar called an *icyansi* and is left at room temperature in a warm and clean place called an *uruhimbi*. In order to keep the product safe, the jar is usually covered either with a straw-woven lid known as an *umutemeri* or with a lid made from a calabash. A fermentation period of at least 2 to 3 days is required in order to obtain good *kivuguto*. **Figure 1** shows a flow chart for the traditional processing of *kivuguto* and its by-products.

The typical flora that creates the traditional sour milk, *kivuguto*, was not, to date, specifically selected and applied in producing the drink in dairy. This flora is very complex and varies from one location to another, as it is the case for all traditional curds. In addition, the lactic flora lives alongside a pathogenic flora, which also varies depending on the level of the personal hygiene of those preparing the *kivuguto*. Nevertheless, despite the disadvantages of the process, traditional fermentation remains the most convenient method in Rwanda. The microorganisms used are indigenous strains, which are left to develop naturally during the production process. However, in order to standardize this process, the need has arisen for the selection and application of specific strains.

These microorganisms are responsible for the acidification, texture, flavor and other organoleptic characteristics, which give the *kivuguto* curd its highly appreciated taste.

However, in order to standardize this process, the need has arisen for the selection and application of specific strains. These microorganisms are responsible for the acidification, texture, flavor and other organoleptic characteristics, which give the *kivuguto* curd its highly appreciated taste. This microflora is found in containers commonly used for storing milk. Its habitat is also easily located in the near environment of cattle (pasture, paddock, cattle dander, air, etc.). The preparation of the *kivuguto* curd varies between regions. In most cases, raw milk is stored directly in a vessel until it ferments spontaneously *via* microorganisms contained in the milk. These microorganisms come either from inside container or from the air in the surrounding environment. Note that in all methods of *kivuguto* production, the vessel is not hermetically sealed. Also, from time to time, the *kivuguto* of a previous batch is used in small quantity as a starter added into new fresh milk. In other cases, raw milk is first heated to boiling point and then cooled to room temperature. It is then stored for 24-36 h before coagulation.

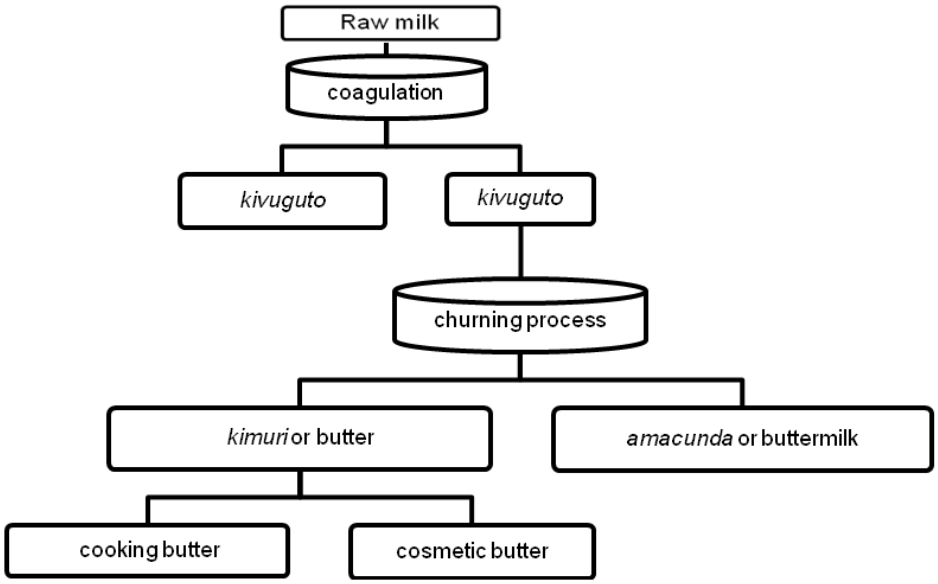


Figure 1. Diagram for processing of *kivuguto* and its by-products –
Schéma de préparation du *kivuguto* et ses dérivés.

In areas of eastern Rwanda, the containers in which the milk is stored undergo special preparation: washing with the hot smoke derived from burning two species of plant, one of which is an herb of the *Poaceae* (grass) family, and the other a shrub called *Combretum molle*. The grass used is first stripped of its roots and all its leaves, leaving a single short stem, which is then dried in the sun. These dried stems (rods) are then collected into small bundles

and are stored for more than six months in the house. During this time, smoke from the kitchen covers the bundles of rods with a reddish layer. At the end of this period, these rods are burned in a small pot fitted with one large and one small opening. First, the rods are inserted into the large opening of this small pot and are burnt, second the mouth of a vessel – a small wooden vessel (*inkongoro*), or a small gourd – is placed over the smaller opening of the pot in order to capture all the smoke that emerges. After a while, the vessel is removed and the inside is wiped with a kind of cloth made out of tree bark. The vessel is then placed back over the emerging smoke, then removed and wiped inside again, and so on. The exercise is repeated several times in order to make the inside of the vessel very smooth. At the end of the procedure, the vessel is kept closed. When milk is placed in a container prepared in this way, the fermentation process takes about 36 h, and a very good curd is produced, with a solid texture. For larger containers, the washing process is carried out using smoke obtained by burning a single large stem of the shrub, which has not been completely dried. The stem is burned and the vessel is held upside down directly over the smoking stem. The vessel is then wiped several times as before. In both procedures, the fermented milk stored in these containers has a smell and taste of smoke, which is highly appreciated both by Rwandese people and by others in the region. What is clear is that this smoking process ensures that these containers are clean, so that there are usually very few pathogens affecting the texture of the curd.

2.2. The traditional churning process

Milk stored in a small gourd will ferment to give *kivuguto* milk, which is then used in this liquid form. On the other hand, when milk is left to ferment in a large gourd (*igisabo*), or in a calabash (**figure 2**), additional processing is applied to the resulting *kivuguto* milk. Here the *kivuguto* is churned to produce *kimuri* (butter) or *amacunda* (buttermilk). In order to produce these products, the *kivuguto* is first of all stirred while it bubbles in a butter churn usually in the form of the calabash in which the milk was fermented. This churning process takes at least 2 h. During this time, the fat from the *kivuguto* coalesces and the separation between liquid and solids becomes more marked. The fat is removed and the liquid left behind in the churn is buttermilk, a product drunk as such. Buttermilk produced in this way has a characteristically sour taste caused by lactic acid bacteria involved in milk lactose hydrolysis during

fermentation. The extracted fat is then used in one of two ways. It may be heated with some natural perfuming additives to be used as a cosmetic body cream. On the other hand, the fat may be well preserved for a half-year or a full-year period, allowing the manufacture of a solid tasting yellow fat called butter or *amavuta akuze*, which is good for food preparation. Sometimes, this butter is seasoned with additional ingredients such as onions, garlic, etc., in order to add flavor to meals.

Picture: Karenzi E.

Figure 2. A traditional butter churn or *igisabo* - une baratte traditionnelle ou *igisabo.*

3. CHARACTERIZATION OF *KIVUGUTO*

3.1. Microflora

In a previous study (Karenzi et *al*., 2012), we investigated the characteristics of *kivuguto*. Using two samples of *kivuguto* from northern and southern provinces of Rwanda, we selected the microorganisms responsible for the fermentation of the product and formulated a starter culture composed of three strains: CWBI-B1466 *Lactococcus lactis* registered in the Genbank database under accession number JF313446, CWBI-B1465 *Leuconostoc mesenteroides* subsp. *mesenteroides*, accession number JF313445 and CWBI-B1470 *Leuconostoc pseudomesenteroides*, accession number JF313454.

The *kivuguto* has a lot of similarities with *kule naoto*, a traditional fermented milk of Kenya. The dominant lactic acid bacteria of *kule naoto* belong to the genus *Lactobacillus*, followed by *Enterococcus*, *Lactococcus* and *Leuconostoc* (Mathara et *al*., 2004). *Kivuguto* also presents some similarities with *urubu*, a traditional fermented milk of Burundi (Nzigamasabo et *al*., 2009). To date, the microorganisms of *urubu* have not been selected. Isono et *al*. (1994) found

that the major microorganisms of the Masai fermented milk in Northern Tanzania consist of *Lactococcus lactis* ssp. *lactis* and *Lactobacillus confus*. Since 1992, a similar study was carried out on traditional fermented milk of Zimbabwe, *amasi* (Feresu, 1992). Assays for formulating a starter culture for the production of *amasi* were conducted with isolates of *Lactococcus lactis* subsp. *lactis* biovar *lactis*, *Lactobacillus plantarum* and *Leuconostoc mesenteroides* subsp. *mesenteroides* (Mutukumira, 1996). However, a sensory panel was subsequently found to prefer *amasi* produced by a single strain of *Lactococcus* (Gadaga et *al*., 1999). *Lactococcus lactis* is the strain responsible for the fermentation of an israelian milk called *zivda*. The strain is found in many dairy products and is used mainly for its acidifying property.

In Sudan, a combination of lactic acid bacteria and yeasts is responsible for the fermentation of the traditional fermented milk, *rob*. The predominant lactic acid bacteria in rob fermentation have been found to be *Lactobacillus fermentum*, *Lactobacillus acidophilus*, *Lactococcus lactis* and *Streptococcus salivarius* (Abdelgadir et *al*., 2001). The predominant yeasts have been identified as *Saccharomyces cerevisiae* and *Candida kefyr* (Abdelgadir et *al.*, 2001). This association of lactic acid bacteria and yeasts is also found in the fermented milk of Ethiopia, and in the kefir and koumiss milks of Eastern European countries. The taste of these fermented milks displays an alcoholic flavor and is less sour, properties that are not well appreciated in Central and East Africa. The *kivuguto* also resembles to yogurt, the well-known example of a fermented milk product. However, the taste is different and the strains of fermenting bacteria used in yogurt are also different: *Lactobacillus bulgaricus* and *Streptococcus thermophilus*. The taste is the sole reason why people of the Great Lakes Region (Africa) continue to prefer the traditional *kivuguto*, although yogurt is also sold at the local market. Of all the fermented milks discovered to date, the one most similar to *kivuguto* is the *filmjölk* of Sweden, in terms of both consistency and flavor. When people from Central Africa stay in Europe, they usually consume this milk more than they do yogurt. *Filmjölk* is a mesophilic fermented milk, which, like *kivuguto*, is also composed of two strains of *Lactococcus lactis* and *Leuconostoc mesenteroides*. *Filmjölk* flavour is also very similar to *lben* of Morocco (Tantaoui-Elaraki et *al.*, 1987), a buttermilk fermented by *Lactococcus lactis* subsp. *lactis* biovar *diacetylactis*, *Leuconostoc lactis*, *Leuconostoc mesenteroides* subsp. *cremoris* and *Leuconostoc mesenteroides* subsp. *dextranicum*.

3.2. Acidification and physico-chemical properties of *kivuguto*

The acidification process of *kivuguto* is created by two strains: CWBI-B1466 *Lactococcus lactis* and CWBI-B1470 *Leuconostoc pseudomesenteroides*. The third strain responsible for the acidification of *kivuguto* – *Leuconostoc mesenteroides* subsp. *mesenteroides* – does not grow on its own in milk. In order to reach the isoelectric point at pH 4.6, CWBI-B1466 *Lactococcus lactis* takes 8 h whilst CWBI-B1470 *Leuconostoc pseudomesenteroides* takes 14 h. At that point, the titratable acidity is more than 80 °D. After 4 h of fermentation, a coagulum begins to form. For a period of up to 8 h, the *kivuguto* appears as a semi-solid pleasantly sour product. However, before it is suitable for drinking, it needs to be blended. *Kivuguto* keeps well for up to 36 days at 4 °C, maintaining its good flavor; its visco-elastic properties also increase with the duration of storage.

4. THE DAIRY INDUSTRY IN RWANDA

4.1. Dairy policy

The livestock (animal resources) sub-sector falls under the responsibility of the Ministry of Agriculture and Animal Resources (MINAGRI). Since 2006, the activities of this sub-sector have been implemented by the Rwanda Animal Resources Development Authority (RARDA), as part of the global program of the National Animal Resources Policy. In Rwanda, there is no dairy sub-sector as such, but cattle farming plays a very important role in the socio-economic activities of the country. Farming of goats, sheep, pigs, chickens, fish and rabbits is less well developed. To date, the livestock (animal resources) sub-sector has been represented mostly by cattle development projects. In 2003, the PADEBL was created as a separate project with the specific objective of satisfying domestic demand for milk and beef, as well as of increasing the income of farmers. This project contributed to the implementation of the "One cow per poor family" (Girinka) program, launched in 2006 to provide cows for poor families. PADEBL is also responsible for milk quality management: it created and now owns many milk collection centers (MCCs), each with its own cooling system. PADEBL is financed by the African Development Bank (ADB). From 2010, MINAGRI restructured its activities. MINAGRI comprises a Permanent Secretariat to coordinate the ministry's policies

and programs through two units at central level: the Strategic Planning & Program Coordination Secretariat (PPS) and the Internal Resource Management & Finance Unit; and through two boards at decentralized level: the Rwanda Agriculture Board (RAB), and the National Agriculture Export Development Board (NAEB). RAB comprises four departments: Agriculture Extension (formerly RADA – the Rwanda Agriculture Development Authority), Livestock (Animal Resources) Extension (formerly RARDA), Research (formerly ISAR) and Infrastructure & Mechanization. Within the new structure, the sub-sector coordinates the issuing of certificates authorizing imports of domestic animals, semen, fertilized eggs, seeds, plants and cuttings and other animal husbandry products. It also works in livestock research, training and information. In addition to inheriting the "One cow per poor family" (Girinka) program, the sub-sector is also responsible for the "One cup of milk per child" program, and the "Poultry and Hatchery, Insemination and Small Stock" program (RAB, 2012). The Girinka program aims at enabling every poor household to own and manage a productive dairy cow, enabling the family to improve their livelihood through increased milk and meat production and to improve the soil fertility of their land for their crops using the available manure (RAB, 2012). The "One cup of milk per child" program is a school milk program funded by the European Union (EU) to provide milk to children in nursery and primary schools in order to address the country's problem of severe child malnutrition (RAB, 2012).

4.2. Milk production and processing

Milk production in Rwanda was estimated to have reached 372,619 tons by 2009 (DMS, 2009), following tremendous efforts made by the government and other stakeholders. In order to promote milk production in the country, emphasis was placed on bovine genetic improvement (strengthening the use of artificial insemination, and the importing of exotic cows). Most of the milk in Rwanda (48%) is produced in traditional or extensive grazing system in the Eastern Province (Rutamu, 2008). The 2006 Agriculture Survey indicated that there were 1.2 million cattle in Rwanda, of which 13.6% were milking cows. The survey also indicated that there were a total of 157,479 milking cattle; improved breeds constituted 23% of that group, pure breeds 6% and crosses 17% (PADEBL, 2009). In 2010, the NISR (National Institute of Statistics of Rwanda) revealed the number of dairy cattle in the country to be 207,507, corresponding to an increase of 25%. The overall total number of cattle was confirmed to be 1,548,521, with 15.5% of these being represented by exotic breeds

(Modderman, 2010). The Rwandese dairy industry currently consists of five dairy companies: Inyange Industries, Nyanza Dairy, Eastern Savannah, Rubilizi Dairy, Masaka Farms plus several cheese processing units mainly located in the Western Province (20 cheese processing units), with a few others being found in other parts of the country (RBS, 2011). Two of these cheese processing units are located in Nyanza District and two more are found in Gasabo District. According to Rutamu (2008), the milk processing capacity available in Rwanda is estimated at 188,000 l per day, but only 3.3% of this capacity is used. Today, these data may be revised, if we take into consideration milk processed for cheese making and the existence of new dairy processing units. Milk technology is applied in the Rwandese dairy industry in the processing of the following dairy products: pasteurized milk, skimmed milk, cream, flavored milk, fermented milk and yogurt, UHT milk, and cheeses such as Gouda, butter, ice cream.

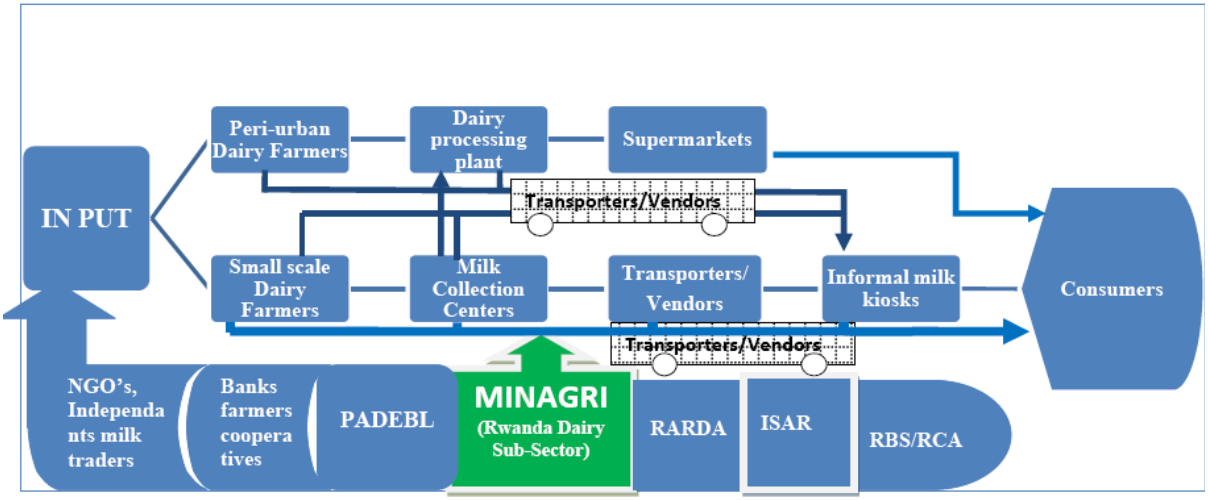
4.3. Milk consumption and importation

High population growth in Rwanda has led to an increase in demand for livestock products that is higher than can be satisfied by domestic production. This has forced the country to invest its few financial resources in the massive importation of milk (Rutamu, 2008). From 1999 to 2007, the trend in milk imports decreased from 1,280 t to 450 t (MINAGRI et al., 2007). It is very difficult to make an accurate estimation of milk consumption in Rwanda, because a large volume of milk is sold in the informal market. In 2007, approximately 62,000,000 l were consumed on farms and about 35% of raw milk was lost due to spoiling before reaching the market or being processed (TechnoServe, 2008). In 2008, a chain analysis estimated that 96% of all dairy products are sold in the informal market, *e.g.* in small informal milk shops. Kigali city has more than 1,500 milk shops (Modderman, 2010). Milk is consumed either fresh or after coagulation. As Rwandese people prefer fermented milk, it is very difficult to estimate the percentage of fresh milk consumed out of the 96% of all dairy products sold at the informal market.

4.4. The dairy value chain in Rwanda

Michael Porter was the first to use the concept of the “value chain”. Within a firm or organization, both the service provided and the product made add value according to the

activities and processes employed by the organization. Value chains traditionally refer to the chain of activities that products pass through as they gain value before reaching the consumer (TechnoServe, 2008). Along the chain, the organization is split into primary activities and support activities (Porter, 1998). The dairy value chain in Rwanda follows two main segments of actors, with the segments being composed of multiple nodes. The two segments are linked to input providers (**figure 3**) organized by the Ministry for Agriculture and Animal Resources (MINAGRI) through the Rwanda Livestock sub-sector and other stakeholders (other state organizations, NGOs and independent traders).



Source: Karenzi E.

Figure 3: –**Dairy Value Chain scheme of Rwanda** –The diagram is an adapted Porter's Generic Value Chain (Porter, 1998) where the primary value chain activities are divided in two segments according to the farmers feeding system. The primary value chain activities are supported by the Ministry of Agriculture through its specialized services, the banks, the farmer's cooperatives, NGO's and independent milk traders.

–**Chaîne de valeur de la filière laitière au Rwanda** –La figure est un adapté de la chaîne de valeur générique de Porter (Porter, 1998) où les activités primaires de la chaîne de valeur sont divisées en deux segments en fonction du système d'élevage. Cette chaîne de valeur génère des activités primaires grâce au soutien du Ministère de l'Agriculture à travers ses centres et instituts spécialisés, ainsi que des banques, des ONG, des coopératives des fermiers et des commerçants de lait.

Dairy value chain input providers. Since 2006, under the Ministry of Agriculture, RARDA (Rwanda Animal Resources Development Authority) and ISAR (Institute of Agronomic Sciences of Rwanda) have supported the dairy value chain at different levels. The mission of RARDA was to contribute towards the growth of animal production through the development of appropriate technologies, and by providing advisory, outreach and extension services to stakeholders in the animal resources sector (RARDA, 2010). Since 2003, PADEBL (Dairy Cattle Development Support Project) has contributed to a sustainable increase in milk and meat production, through the introduction of appropriate technological packages into

stockbreeding in villages and peri-urban areas (MINAGRI, 2009). ISAR was the body responsible for research and development at MINAGRI. ISAR's mandate was to carry out research on agronomy, and on animal and environmental sciences (ISAR, 2010). The Rwanda Bureau of Standards (RBS), under the Ministry of Trade and Industry (MINICOM), is the statutory body charged with the enforcement of standards and the certification of all products and services in the country (RBS, 2011).

The Rwanda Cooperative Agencies (RCA) is a public institution in charge of the promotion, registration and regulation of cooperatives in the country. The livestock sub-sector receives a lot of support from RCA for the dairy development, since many cooperatives are involved in the dairy chain. Other stakeholders in the livestock sub-sector are ADB (African Development Bank), BNR (Banque Nationale du Rwanda), BPR (Banques Populaires du Rwanda), BRD (Rwanda Development Bank) and other commercial banks; NISR (National Institute of Statistics of Rwanda); independent traders (veterinarians, transporters, etc.); farmers' cooperatives and many NGOs (non-governmental organizations). Today, the three support institutions of MINAGRI: RARDA, ISAR and PADEBL are part of the Livestock sub-sector within the new structure and they have the same mission as previously in terms of the dairy development program.

Dairy value chain actors. Informal actors are present throughout the two segments. From the farmer to the consumer, dairy activities are conducted by dairy processors, supermarkets, collection centers, transporters, and traders and retailers. Farmers are categorized according to the feeding system they use: free-grazing, semi-grazing or zero-grazing. Individual cattle farmers generally practice free-grazing and they produce milk for domestic consumption. Sometimes, surplus milk is sold to a retailer, a transporter or a cooling system owner. This cattle husbandry system represents the vestige of the old system; MINAGRI discourages this system and supports instead zero grazing. Zero grazing is practiced mostly by peri-urban farmers. These farms are well equipped around Kigali city, and the owners have invested both in large numbers of cattle and in equipment in order to supply dairy processing units. Semi-grazing systems have sufficient land and, with a low level of investment, they are able to produce milk to supply a retailer, a transporter, a cooling system owner or a processor. The processor then supplies dairy products to supermarkets. Semi-grazing and free-grazing husbandry farmers are categorized as "small scale farmers" along the dairy value chain. Cooling systems mainly belong to PADEBL and to some farmers' cooperatives. These

cooling systems are held in milk collection centers (MCCs). Transporters are mainly traders, who use either bicycles or vehicles to transport milk. However, some transporters are paid by farmers only for collecting milk from the farm and then taking it to the collection center or to a retailer. These transporters supply milk from small farmers to milk shops or directly to consumers. Those with vehicles sell milk to processors, direct from the collection centers. Milk retailers consist of bicycle milk traders selling milk from door to door, milk shops selling only milk or tea, and ordinary shops selling milk alongside other household products. TechnoServe (2008) estimated the profit margin within each node of the dairy value chain to be as follows: 15% for input providers, 62%-28%-44% for dairy farmers depending on how cows are fed respectively open- semi- zero- grazing, 15-25% for transporters, 6% for cooling system owners, 16% for processors, 10% for sellers of raw milk and 15-20% for supermarkets. To date, the dairy value chain in Rwanda has been scattered; nevertheless an organized structure is in the process of being established. PADEBL began a reorganization of the dairy industry in 2009 by setting up some milk collection centers equipped with cooling systems. However, thus far, the number of MCCs equipped in this way has remained below the level needed.

5. MILK DEVELOPMENT STRATEGIES

The Rwandese government development program, Vision 2020, aims to transform agriculture from a subsistence level to a productive, high-value and market-oriented farming industry, which is environmentally friendly and has an impact on other sectors of the economy (RARDA, 2011). In this regard, a new value chain will take into account the quality of milk along the value chain. Vision 2020 takes into account the growing pace of the Rwandese economy in general as well as the Livestock sub-sector, focusing on the target of exporting dairy products. Consequently, the Livestock sub-sector will set up a developmental program. It will also accelerate the restructuring of the dairy industry in the whole country with regard to the cold chain, as milk is a very temperature sensitive product. At the same time, distributing and trading milk in ordinary plastic material and on bicycles will be prohibited. The establishing of MCCs in all the dairy basins of the country is seen as the best solution. Mobile cold storage system respecting HACCP standards may replace the current set of standards for milk transport. Establishing cold-storage systems on farms will provide another

solution in the fight against microorganisms that pose a threat to human health. *Escherichia coli*, *Listeria monocytogenes* and *Salmonella* are destroyed by pasteurization or by simple boiling. However, microorganisms such as *Staphylococcus aureus*, despite being killed by pasteurization, are still harmful due to the toxins they produce. Thus, the protection system close to the farm must be based on a strategy of prevention. RBS must assist with the implementation of standards from the cow on the farm to the consumer. All these measures will be made possible by improving firstly, cattle breeding, genetics and nutrition and secondly, standards of milk preservation, storage, packaging and transportation. For that purpose, a business plan for the period 2008 to 2020 has been established as part of a dairy developmental program introduced by the Livestock sub-sector, based mainly on the identified dairy basins. The new structure of the Livestock sub-sector has served to highlight the targets fixed from 2008 to 2020 in this dairy development program, aimed at stimulating the development of a modern livestock industry in Rwanda through sustainable growth, increasing value added, and access to markets. Five pillars have been identified for the support of this program (Umar et al., 2011): – community livestock infrastructure support; – public livestock infrastructure support; – support for the "One cow per poor family" program; – support for productivity enhancing technologies; – capacity building. The program has planned certain objectives, with Rwanda being exporters of milk and milk by-products (**Table 1**) by 2020. The program will set up further milk collection centers, livestock watering systems and markets, veterinary clinics and quarantine stations in all dairy basins. Poor families will benefit from having cross-breed heifers to support food security and income generation. Training in genetic and feeding techniques will be provided for staff and farmers in the livestock industry, in the form of study tours, short courses, seminars, conferences and workshops. The ultimate goal is the integrated development of the dairy industry (DMS, 2009) in which all stakeholders must play a role in coordinating the production, processing and distribution of high quality milk and its by-products.

6. CONCLUSION

Little research has been conducted on *kivuguto*. In the present paper, the traditional technology used to make this fermented milk as well as its by-products has been described. The dairy industry in Rwanda has also been discussed. Proposed improvements in the

development of milk production and the dairy sub-sector in Rwanda at all levels and with all stakeholders have been outlined. Information provided on the value of the whole dairy chain raises some interesting issues in terms of trade in Rwanda and in the region. This study is part of a global research project on *kivuguto* milk, with the aim of producing this foodstuff in industry.

Table 1: Evolution projected supply of milk in relation to national needs until 2020- Evolution prévisionnelle de l'offre du lait par rapport aux besoins nationaux jusqu'à l'horizon 2020

Year	Rwanda population	National needs (tons)	National production (tons)	Imports (tons)			Total supply (tons)	Surplus / deficit compared to national needs (tons)
				UHT milk	Powdered milk	Milk-based products		
2008	9 674 975	386 999	364 084	500	100	5	387604	-23 520
2009	9 955 549	398 222	368 623	250	50	5	398527	-29 904
2010	10 244 260	409 770	379 642	125	50	5	409950	-30 308
2011	10 541 344	421 654	416 845	75	50	5	421784	-4 939
2012	10 847 043	433 882	490 052	50	50	5	433987	56 065
2013	11 161 607	446 464	518 224				446464	71 760
2014	11 485 293	459 412	549 312				459412	89 900
2015	11 818 367	472 735	583 521				472735	110 786
2016	12 161 099	486 444	621 078				486444	134 634
2017	12 513 771	500 551	738 743				500551	238 192
2018	12 876 671	515 067	790 003				515067	274 936
2019	13 250 094	530 004	845 922				530004	315 918
2020	13 634 347	545 374	906 857				545374	361 483

Source: DMS, 2009

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Chapter 2

Dairy Starter cultures

*Les starters laitiers (**chapitre deux**) abordent la thématique de la sélection microbienne des micro-organismes impliqués dans le procédé fermentaire des produits laitiers à l'aube du XXIè siècle. A cet effet, un article review a été élaboré pour publication (**Dairy Starter Cultures Selection**). L'article commence par un essai d'actualisation des micro-organismes que la littérature a mis à jour. L'article ne prétend pas être exhaustif, car tous les produits laitiers du monde ne sont pas encore répertoriés. Ensuite, l'article fait une brève description des techniques actuelles à travers leur caractérisation biochimique, moléculaire et technologique, et leur production industrielle.*

Dairy Starter Culture Selection, Review

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ABSTRACT

The present paper is an up-to-date review of the dairy starter cultures biodiversity. It relates recent data about the microbial selection. In a detailed description, the review underlines the characterization and taxonomy of the current and potential genera of lactic acid bacteria (LAB), non-LAB and fungi implicated in dairy products, their habitat, their technological properties and their industrial production for selection use. The genera implicated in milk and/or cheeses processes are elucidated and new species recently found in those dairy products are revealed.

Keywords: selection, LAB, fungi, taxonomy, dairy starter, technological properties, prokaryote, eukaryote, 16S DNA, phylogeny, industrial production.

1. INTRODUCTION

Dairy starter cultures are active microbial preparations added intentionally to dairy bases in order to achieve desired modifications (Chandan *et al.*, 2009). They have various functions (**Annexe 1**) in fermented dairy products which confer the identity and quality to the dairy product. These functions must satisfy both the requirements of products quality during their manufacturing and their utilization. According to Chamba *et al.* (1994), these strains have to:

- be propagated, stored and distributed in satisfactory technical and economic conditions.
- be easy to use and possess the requirements specific to each industrial use.

For selecting dairy starters, samples are collected from the dairy product or its close environment, and the strains are isolated, purified and identified correctly on the basis of microbial taxonomy of the most common microorganisms in dairy products. The pure cultures are then preserved at -80°C in a small collection for further analyses. Next, they are then subjected to preliminary tests to eliminate those not meeting the criteria of microbial selection in dairy products, and thereafter, they pass the tests of technological properties to check their acidifying, proteolytic, flavoring, texturizing behaviors and possibly their resistance to inhibitors and bacteriophages. Finally, they are tested to conditions of production and preservation.

2. CHARACTERIZATION OF DAIRY STARTER CULTURES

For over 8000 years, the fermentation has been used to make many products including fermented dairy products (cheese, yogurt, butter, cream, sauerkraut,...). The selection of dairy starter cultures depends on the desired product in terms of acidity, texture, proteolysis and flavor attributes in general through the fermentation process. In addition, this process must insure a good yield along the production and the storage processes. Remember that the task of the selection is a final product that respects the requirements of the consumer. This condition may be in agreement with the technological, economic and cultural aspects. For that purpose, selected starters may exhibit good growth in dairy product for which it will be used (milk, cheese, cream, etc.), without any negative interaction with other microorganisms of the formulated starter culture.

2.1. Pre-selection process

The sampling is performed in dairy product preferably or in its near environment. Samples are cultivated on selective media used usually for isolation and purification of dairy starter cultures with regard to culture conditions (pH, temperature, incubation time, aeration) specific to each microorganism. There are a lot of commercial media for isolation, cultivation or identification of lactic acid bacteria & fungi: media for lactobacilli (MRS, acidified MRS, Rogosa); media for Lactococcus (Turner, M17, Elliker, Tomato Juice); media for streptococci (MRS, Elliker, M17); media for Enterococci (MRS, Barnes Medium, KF Medium, Rothe & Lisky medium); media for Pediococci (MRS, MRS+NaCl4%); media for Leuconostoc (MRS, Mayeux medium); media for Bifidobacteria (TPY, Casitone, LBS); medium for Propionibacteria (Trypticase medium-BBL- at 1%) and medium for yeasts and molds (Potato dextrose agar, Sabouraud glucose). Upon the isolation and purification, a pre-selection of potential starters is performed by a screening process consisting of morphological observations, biochemical analyses and a preliminary technological evaluation.

2.2. Taxonomy & Identification

The taxonomy and/or Systematics is a scientific process giving names of appropriate taxonomic rank to the classified organisms with common properties or relationships and the determination of species they belong to, according to conventional rules. Classification, identification and nomenclature are subdisciplines of Systematics. The taxonomy is based on the direct comparison of characteristics (phenotypic, phylogenetic) to known taxonomic groups (taxa). Different techniques are used for the identification of a microorganism and for a complete characterization, a complementary analysis of different data, step-by-step, may be applied. Commercial media for isolation, differentiation and purification of lactic acid bacteria, non-lactic acid bacteria, yeasts and molds are used for specific culture conditions. Microscopic observations are the first step, followed by biochemical methods for phenotypic analyses. For lactic acid bacteria, the most commonly criterion studied are:

- morphology, mobility, production of capsule or pigment (microscopic examination after staining);
- Growth in aerobic or anaerobic conditions and at different pH, temperatures and NaCl

concentrations, in the presence of bile, methylene blue, ethanol or other inhibitors;

- CO_2, H_2, H_2S, H_2O_2 production in glucose medium or CO_2 and H_2 from lactate, NH_3 from arginine, acetoin from glucose or citrate, polysaccharides;
- Optical isomer of lactic acid produced by fermentation of glucose;
- Hydrolysis of arginine, hippurate, esculin, starch, urea;

Generally, micromethods are applied for profile fermentation of sugars by many identification galleries. The most known are API galleries kits of BioMérieux (France) for microbial identification. The most used kits are:

- API Gram positive Identification: for identification of clinical staphylococci and micrococci;
- API 20 Strep for the identification of streptococci and enterococci
- API Yeast Identification with API 20C AUX for yeasts identification
- API LAB Identification with the API 50 CH for lactic acid bacteria identification.

For more resolution, molecular techniques with DNA extraction, followed by PCR & sequencing genes (ARDRA, RFLP, RAPD, ITS, 16S rDNA,…) allow a good identification. If necessary, they are complemented by some analytical methods of GC content, the isomer of lactic acid, the composition of peptidoglycan cell membrane or the sequencing of protein-encoding genes, etc. If the differentiation is not complete, the DNA-DNA hybridization is the most resolutive and robust method. This process is called the polyphasic approach. Note that for the best identification, a direct analysis by DNA extraction through a fresh substrate is the best method. The taxonomic ranks are species, genus, family, order, class, phylum, domain and the most commonly used are species, genus and domain, with the species as the basic unit of the taxonomy. But a species is sometime divided into subspecies and in practice, the strain is the microbial individual. Dairy starter cultures are generally assembled in two groups: lactic acid bacteria (LAB) and fungi. LAB are prokaryotes whereas fungi are eukaryotes. Historically, there have been many tentative nomenclatures of bacteria, but scientists usually failed to reach an agreement on the same code of bacteria. In 1975, there has been the revision of the International Code of Nomenclature of Bacteria (Lapage *et al.*, 1975). The publication of the first Approved Lists of Bacterial Names (International Journal of Systematic Bacteriology, 30, 225-420), which contained all the bacterial names having standing in nomenclature on January 1st, 1980 was a starting point in renewal of bacterial nomenclature (Euzéby, 2013). Nowadays, the International Committee on Systematics of Procaryotes

(ICSP–http://www.the–icsp.org) regulates the bacterial taxonomy and the "Approved Lists of Bacterial Names" are regularly updated and published in the International Journal of Systematic and Evolutionary Microbiology (IJSEM) (the former International Journal of Systematic Bacteriology) with an online version. The classification of prokaryotes was based on their phenotype (morphology, staining reactions, biochemistry, substrates/products, antigens etc.) in the first nomenclatures of bacteria. Nowadays, there is an agreement that the DNA, RNA and some proteins could be the reference standard to determine taxonomic groups. Therefore, modern classification is based on the gene's information and the phylogeny. Indeed, the phylogeny and the study of specific genes showed that there are conserved and variable regions on genes. And it becomes easy to recognize genes with common ancestor. The most studied rRNA genes are the 16S in prokaryotes or 18S in eukaryotes. The homology of two DNA (**Annexe 2**) from two species with the same content of guanine + cytosine (GC%) is determined by a reassociation ≥70% and a difference in the melting temperature (ΔTm) equal to 5°C or lower (Wayne *et al*., 1987). However, two species having the same mole % G+C are not necessary of the same species. Note that this concept of species is not the same to eukaryotes (fungi). As prokaryotes, the taxonomy of the Fungi in the old classifications was based on morphological features; but since 2007, the classification of Kingdom Fungi was the result of molecular phylogenetics analyses. It recognizes seven phyla, three of which, the Ascomycota, the Basidiomycota and the Zygomycota comprising the most genera dairy starters fungi. For more search, websites Index Fungurum, I, the Mycobank Initiative and ICTF (International Commission on the Taxonomy of Fungi – http://www.fungaltaxonomy.org) are good records of current names of fungal species with references to old synonyms.

2.3. Major genera of dairy starter cultures

The microorganisms used in dairy technology are generally lactic acid bacteria (LAB). However, dairy starter cultures comprise also various micro-organisms. Yeasts and molds are used in cheeses ripening as well as in the manufacture of many dairy products (Simpson *et al.*, 2004; Simpson *et al.*, 2003; Guiraud and Galzy, 1980). Fermented milks like kefir (Beshkova *et al.*, 2003), kumis (Koro and Saastamoinen, 2010), ergo (Gonfa *et al.*, 2010), rob (Abdelgadir *et al.*, 1998) are both fermented by bacteria and yeasts. In addition,

Propionibacterium shermanii and *Bifidobacterium* spp. which are not lactic acid bacteria, although bifidobacteria species produce lactic acid, are also used (Mullan, 2001). During ripening of certain cheeses with Munster character, adding *Staphylococcus xylosus* as starter culture is widely used for the aromatic properties of this species as well as for its orange pigmentation (Frisoni, 2007). However, *Staphylococcus* is not a lactic acid bacteria, as the taxonomy of Lactic Acid Bacteria has been based on the Gram reaction, catalase reaction and the production of lactic acid from various fermentable carbohydrates (Khalid, 2011). The yeasts are generally introduced into milk during milking.

2.3.1. Lactic acid bacteria

Lactic acid bacteria (LAB) are Gram positive, non-spore forming, cocci or rods and they produce lactic acid as the sole or the major end product during the fermentation of carbohydrates. Using the homologous genes of 16S ribosomal RNA gene, the LAB are differentiated from other organisms on the basis of the GC content by phylogenetic method (**Annexe 3**). Stiles and Holzapfel (1997) suggested that the lactic acid bacteria comprise the *Leuconostoc, Pediococcus, Streptococcus, Tetragenococcus, Vagococcus* and *Weissella.* They all belong to the domain of Bacteria, phylum Firmicutes, class Bacilli, order Lactobacillales (Garrity *et al.*, 2004), and dispatched in 5 families: Enterococcaceae (Enterococcus, Tetragenococcus, Vagococcus), Lactobacillaceae (Lactobacillus, Pediococcus), Leuconostocaceae (Leuconostoc, Oenococcus, Weissella), Aerococcaceae (Aerococcus) and Streptococcaceae (Streptococcus, Lactococcus). The genus Bifidobacterium, traditionally listed among LAB, is only poorly phylogenetically related to LAB and its species use a metabolic pathway for the degradation of hexoses different from those described for "genuine" LAB (Felis and Dellaglio, 2013). They have their origins in vegetation for *Lactococcus* and *Leuconostoc* (Sandine *et al.*, 1972) and in the intestinal tract for *Bifidobacterium spp*., *Enterococci* and *Lactobacillus*. Nowadays, four types of habitat niches of LAB species are identified: human gastrointestinal tract, human oral flora, dairy fermentation and other fermentations of beer, wine, plants or meat (Zhang *et al.*, 2011). Two categories of LAB exist according to lactose catabolism: homofermentative and heterofermentative. The homofermentative LAB include some lactobacilli and most species of enterococci, lactococci, pediococci, streptococci, tetragenococci, vagococci and use the

Embden-Meyerhof pathway. The leuconostocs, some lactobacilli, oenococci and weissella species which utilize the phosphoketolase pathway form the heterofermentative LAB.

- **Enterococcus**

Enterococci are Gram-positive, catalase-negative, non-spore forming, facultative anaerobic bacteria, which usually inhabit the alimentary tract of humans in addition to being isolated from environmental and animal sources (Fisher and Phillips, 2009). Manero and Blanch (1999) developped a biochemical key for the identification of all recognized *Enterococcus* spp. Usually, the intestines of humans and other animals are recognized as the natural ecological niche for *Enterococcus* species. However, *Enterococci* are ubiquitous and can be found free-living in soil, on plants or in dairy products (Manero and Blanch, 1999; Devriese *et al*., 1991; Flahaud *et al*., 1997; Leclerc *et al*., 1996). Although *Enterococcus* strains are presumptive pathogenic species, current findings revealed interesting functional properties. Quiros *et al*. (2007) identified in milk fermented by *Enterococcus faecalis* novel peptides with demonstrated antihypertensive activity. Other biological activities are the angiotensin-I converting enzyme (ACE) inhibitory and immunomodulatory activities (Regazzo *et al*., 2010) analyzed in milks fermented by *Enterococcus faecalis* TH563 or *Lactobacillus delbrueckii subsp. bulgaricus*, two bacterial strains isolated from italian dairy products. A Colombian fermented milk, kumis was found also as a good source of non-virulent dominant Enterococci able to produce fermented milks with ACE-inhibitory activity (Chaves-Lopez *et al.*, 2011). In fact, whereas *Enterococci* are used as probiotics to improve the microbial balance of the intestine, on the other hand, Enterococci have become recognised as serious nosocomial pathogens causing bacteraemia, endocarditis, urinary tract and other infections (Franz *et al*., 1999).

- **Tetragenococcus**

Tetragenococci are Gram-positive, catalase negative and oxidase negative. The genus was first described as halophilic bacterium *Pediococcus halophilus* by Mees in 1934 and became the genus *Tetragenococcus* after reclassification by Collins *et al.* (1990) as *Tetragenococcus halophilus*. Tetragenococcus strains are currently used in several industrial applications in

eastern Asia countries, especially as a starter culture in fermentation processes (Röling & van Verseveld, 1996; Holzapfel *et al*., 2006) of soy sauce, miso, fish sauce, but not yet in dairy industry. Tetragenococcus species are typically cells with a high level of salt tolerance up to 26%. The genus counts for the moment 5 species: *Tetragenococcus halophilus* (Collins *et al*., 1993), with two subspecies *Tetragenococcus halophilus subsp. halophilus* (Justé *et al*., 2012) and *Tetragenococcus halophilus subsp. flandriensis* (Justé *et al*., 2012), *Tetragenococcus koreensis* (Lee *et al.*, 2005), *Tetragenococcus muriaticus* (Satomi *et al*., 1997), *Tetragenococcus osmosphilus* (Justé *et al*., 2012) and *Tetragenococcus solitaries* (Collins *et al*., 1989) (Ennahar and Cai, 2005).

- **Vagococcus**

The six isolates shared 100% 16S rRNA gene sequence similarity, and representative strain CD276^T formed a branch that was distinct from the type strains of the six recognized species of the genus *Vagococcus* (*Vagococcus fluvialis* CCUG 32704^T, *Vagococcus salmoninarum* NCFB 2777^T, *Vagococcus lutrae* CCUG 39187^T, *Vagococcus fessus* M2661/98/1^T, *Vagococcus carniphilus* ATCC BAA-340^T and *Vagococcus elongatus* PPC9^T). The genus habitat is found in human clinical samples *i.e.* blood, peritoneal fluid and wounds, a root-filled tooth with periradicular lesions (Wang, 2010); domestic animals e.g. pigs-swine clinical samples and swine manure storage pit (Lawson et *al.*, 2007), cattle-in ground beef (Shewmaker *et al*., 2004), horses and cats; seal and harbour porpoise carcasses, common otter, fish with peritonitis like atlantic salmon, rainbow trout and brown trout (Wallbanks *et al*., 1990). *Vagococcus lutrae* and *Vagococcus fessus* were isolated from other aquatic animals (Hoyles *et al*., 2000; Lawson *et al*., 1999) *and Vagococcu fluvialis* was originally isolated from chicken fæces and river water (Hashimoto *et al*., 1974). And very recently, the 7th species *Vagococcus teuberi* was isolated from *fénè* milk, a fermented milk of Mali (Wullschleger, 2009).

- **Lactobacillus**

The genus Lactobacillus is the largest among the LAB, with over 145 recognized species (Euzéby, 1997). The lactobacilli are Gram+, non-spore-forming microorganisms. Considering

cellular shape, they can occur as rods or coccobacilli (Felis and Dellaglio, 2013). They are fermentative, microaerophylic and chemo-organotrophic, requiring rich media to grow (Liong, 2011). They are catalase negative, even if pseudocatalase activity can sometimes be present in some strains. Considering DNA base composition of the genome, they usually show a GC content of lower than 54 mol% (Tannoc, 2005). Lactobacillus species play a major role in fermented dairy products and also contribute to the therapeutic aspects of human health (Jayalalitha *et al*., 2013) like *Lactobacillus acidophilus, Lactobacillus amylovorus, Lactobacillus crispatus, Lactobacillus johnsonni, Lactobacillus gasseri, Lactobacillus casei, Lactobacillus paracasei subsp. paracasei, Lactobacillus rhamnosus, Lactobacillus reuteri, Lactobacillus plantarum* (Holzapfel *et al*., 1998; Klein *et al*., 1998; Reid, 1999). *Streptococcus salivarius ssp. thermophilus* and *Lactobacillus delbrueckii* ssp. *bulgaricus*, strains of yogurt, are also classified as probiotics. Other Lactobacilli species involved in milk processes are *Lactobacillus acidipiscis* (Fontana *et al*., 2010), *Lactobacillus brevis* (Pedersen *et al*., 1962), *Lactobacillus coryniformis subsp. coryniformis* (Hegazi and Abo-Elnaga, 1980), *Lactobacillus delbrueckii subsp. delbrueckii* (Etchells, 1964), *Lactobacillus delbrueckii subsp. lactis* (Lazos, 1993), *Lactobacillus kefiri* (Kandler and Kunath, 1983), *Lactobacillus kefiranofaciens subsp. kefiranofaciens* (Fujisawa *et al*., 1988), *Lactobacillus kefiranofaciens subsp. kefirgranum* (Tazikawa *et al*., 1994), *Lactobacillus nodensis* (Masoud *et al*., 2010), *Lactobacillus parabrevis* (Pedersen *et al*., 1962), *Lactobacillus paraplantarum* (Manalopoulou, 2003), *Lactobacillus parakefiri* (Tazikawa et al., 1994), *Lactobacillus pentosus* (Poittevin de De Cores and Carrasco, 1966), *Lactobacillus perolens* (Ongol and Asano, 2009), *Lactobacillus plantarum subsp. plantarum* (Orillo and Pederson, 1968), *Lactobacillus salivarius subsp. salivarius* (Coulin, 2006), *Lactobacillus tucceti* (Massoud *et al*., 2010).

- **Pediococcus**

Pediococci are lactic acid bacteria Gram+, strictly homofermentative. The cells are spherical, organized in diplococci or tetrads, and they never appear in chains. Some species produce DL lactic acid and others L (+) from hexoses. They are found in various niches, like plants, dairy products, alcoholic beverages and many fermented foods. These bacteria are widely used in food biopreservation and pediocine is a bacteriocine from Pediococcus. *Pediococcus*

acidilactici and *Pediococcus pentosaceus* are used in the fermentation of dairy products (Tserovska *et al.*, 2002; Irmler *et al*., 2012; Bhowmik & Marth, 1990; Litopoulou-Tzanetakis & Tzanetakis, 1989), as well as *Pediococcus inopinatus* (El-Baradei *et al.*, 2007). They are phylogenetically homogenous to the Lactobacillus genus (**Annexe 4**). The genus Pediococcus currently consists of 14 species of Pediococcus: *Pediococcus stilesii* (Franz *et al*., 2006), *Pediococcus siamensis* (Tanasupawat *et al*., 2007), *Pediococcus pentosaceus* (Mees, 1934), *Pediococcus parvulus* (Gunther and White, 1961), *Pediococcus lolii* (Doi *et al*., 2009), *Pediococcus inopinatus* (Back, 1988), *Pediococcus halophilus* (Mees, 1934), *Pediococcus ethanolidurans* (Liu *et al*., 2006), *Pediococcus dextricus* (Coster and White, 1964; Back, 1978), *Pediococcus damnosus* (Claussen, 1903), *Pediococcus claussenii* (Dobson *et al*., 2002), *Pediococcus cellicola* (Zhang *et al*., 2005), *Pediococcus argentinicus* (De Bruyne *et al*., 2008) and *Pediococcus acidilactici* (Lindner, 1887). Pediococcus was found among dominant strains fermenting ergo, an Ethiopian soured milk (Gonfa *et al*., 2001).

- **Oenococcus**

The genus Oenococcus is represented by the type strains *Oenococcus oeni* (Garvie, 1967) (Dicks *et al*., 1995), which is phylogenetically homogeneous on the basis of 16S-23S rDNA intergenic sequence analysis (Björkroth and Holzapfel, 2006). Another strain of this genus is *Oenococcus kitahare* a non-acidophilic and non-malolactic-fermenting Oenococcus isolated from a composting distilled shochu residue (Endo and Okada, 2006). To date, no Oenococcus genus is reported in dairy products.

- **Leuconostoc**

Leuconostocs are heterofermentative lactic acid bacteria that occur naturally in milk, grass, herbage, grapes and many vegetables (Benkerroum *et al*., 1993; Teuber and Geis, 1981). They are more frequently associated with milk and may cause undesirable effects in fresh milk. However, selected Leuconostocs are of special value in dairy starter cultures (Dworkin *et al*., 2006). They play their principal role of metabolizing citrate to form flavor compounds, particularly for imparting a desirable "butter" aroma (diacetyl). Generally, they are not fermentative but some species are milk acidifiers. They are found in butter and cream,

cheeses, fermented milks (amasi, maziwa lala, leben, filmjölk, kefir, pindidam, smetanka, etc.) (Hemme and Foucaud-Scheunemann, 2003). According to Ogier *et al.* (2008), *Leuconostoc spp*. have an interesting property of exclusive production of D-lactate from glucose since most other lactic acid bacteria produce DL-lactate. The genus consists of the following species: *Leuconostoc argentinum* (Dicks *et al*., 1993), *Leuconostoc carnosum* (Shaw & Harding, 1989), *Leuconostoc citreum* (Farrow *et al*., 1989), *Leuconostoc fallax* (Martinez-Murcia and Collins, 1992), *Leuconostoc gasicomitatum* (Björkroth *et al*., 2001), *Leuconostoc gellidum* (Shaw and Harding, 1989), *Leuconostoc holzapfelii* (De Bruyne *et al*., 2007), *Leuconostoc inhae* (Kim *et al*., 2003), *Leuconostoc kimchii* (Kim *et al*., 2000), *Leuconostoc lactis* (Garvie, 1960), *Leuconostoc mesenteroides* species which has been divided into 4 subspecies: *Leuconostoc mesenteroides subsp. cremoris* (Garvie, 1983), *Leuconostoc mesenteroides subsp. dextranicum* (Garvie, 1983), *Leuconostoc mesenteroides subsp. mesenteroides* (Garvie, 1983) and *Leuconostoc mesenteroides subsp. suionicum* (Gu *et al*., 2012); *Leuconostoc miyukkimchii* (Lee *et al*., 2012), *Leuconostoc palmae* (Ehrmann *et al*., 2009), *Leuconostoc pseudoficulneum* (Chambel *et al*., 2006) and *Leuconostoc pseudomesenteroides* (Farrow *et al*., 1989) (Euzéby, 2013). Among those species, those frequently used in dairy technology are *Leuconostoc citreum, Leuconostoc lactis, Leuconostoc mesenteroides subsp. mesenteroides, Leuconostoc mesenteroides subsp. dextranicum, Leuconostoc mesenteroides subsp. cremoris, Leuconostoc pseudomesenteroides*. El Baradei *et al.* (2007) have also identified another Leuconostoc species in Domiati, a traditional Egyptian cheese *Leuconostoc carnosum.* Only four Leuconostoc species are included in the Bergey's Manual of Systematic Bacteriology (Garvie, 1986), the species *Leuconostoc mesenteroides* comprising the three subspecies mesenteroides, dextranicum and cremoris. The two main facts concerning Leuconostoc are the creation of the genus Weissella that comprises *Weissella paramesenteroides* (previously *Leuconostoc paramesenteroides*) and some heterofermentative species of the genus Lactobacillus (Collins *et al*., 1993; Samelis *et al*., 1993) and the assignment of *Leuconostoc oenos* as a new genus, *Oenococcus oeni* (Dicks, Dellaglio & Collins, 1995). Additionally, eleven new species have been described: *Leuconostoc gelidum* and *Leuconostoc carnosum* isolated from meat products, *Leuconostoc citreum* and *Leuconostoc pseudomesenteroides* from clinical isolates and milk (Karenzi *et al*., 2012; Sengoun *et al*., 2009; Cibik, 2000), *Leuconostoc fallax* from sauerkraut, *Leuconostoc*

argentinum from Argentine raw milk, *Leuconostoc gasicomitatum* associated with meat spoilage, *Leuconostoc kimchii* and *Leuconostoc inhae* from kimchi and *Leuconostoc ficulneum*. Strains found in the microflora of the wasp *Vespula germanica* present 90% homology with known Leuconostoc species and may constitute a new taxon (Reeson *et al*., 2003).

- **Weissella**

Currently, the genus Weissella consists of the 12 following species: *Weissella beninensis,* (Padonou *et al*., 2010); *Weissella ceti* (Vela *et al*., 2011); *Weissella cibaria* (Bjorkroth *et al*., 2002), *Weissella confusa* (Holzapfel and Kandler, 1969) (Collins *et al*., 1994), *Weissella fabalis* (Snauwaert *et al*., 2013), *Weissella fabaria* (De Bruyne *et al*., 2010), *Weissella halotolerans* (Kandler *et al*., 1983) (Collins *et al*., 1994), *Weissella hellenica* (Collins *et al*., 1993), *Weissella minor* (Kandler *et al*., 1983), *Weissella paramesenteroides* (Garvie, 1967), *Weissella thailandensis* (Tanasupawat *et al*., 2000) and *Weissella viridescens* (Niven and Evans, 1957; Kandler and Abo-Elnaga, 1966). Phylogenetically, Weissella spp. are those species previously called *Leuconostoc paramesenteroides*. Only *Weissella viridescens* was rarely found in dairy products. It was reported in zabady, an Egyptian fermented milk (Loones, 1994) and in maziwa lala, a fermented milk of Kenya (Miyamoto *et al.*, 2005).

- **Streptococcus**

The most known streptococci in dairy industry is *Streptococcus salivarius subsp. thermophilus* of yogurt. In past, this genus was classified based on serological differentiation and the formerly Lancefield group N streptococci diverged and formed the Lactococcus genus with species widely used in dairy fermentation. Others groups composed currently the genera Enterococcus (group D) and Streptococcus (groups A, B, C mainly pathogenic streptococci). Nowadays, only two streptococci are involved in dairy processes: *Streptococcus gallolyticus subsp. macedonicus* (Hansen, 2011) and *Streptococcus salivarius subsp. thermophilus* in cheeses (Hansen, 2011; Hutkins, 2006) and yogurt.

- **Lactococcus**

Lactococcus (Schleifer *et al*., 1986) is classified as a lactic acid bacterium naturally found in vegetable matters, dairy products and fishes. The type species is *Lactococcus lactis* transferred from the *Streptococcus lactis* species. The genus counts currently 7 species: *Lactococcus chungangensis* (Cho *et al*., 2008), *Lactococcus fujiensis* (Cai *et al*., 2011), *Lactococcus garvieae* (Schleifer *et al*., 1986), *Lactococcus piscium* (Williams *et al*., 1990), *Lactococcus plantarum* (Schleifer *et al*., 1986), *Lactococcus raffinolactis* (Schleifer *et al*., 1988), and the species *Lactococcus lactis* (Schleifer *et al*., 1986) with 4 subspecies: *Lactococcus lactis subsp. cremoris* (Schleifer *et al*., 1986) and *Lactococcus lactis subsp. lactis* (Schleifer *et al*., 1986) isolated from dairy products; *Lactococcus lactis subsp. hordniae* (Schleifer et al., 1986) isolated from the leafhopper *Hordnia circellata* and from dairy product by Pu *et al*. (2002); *Lactococcus lactis subsp. tructae* (Pérez *et al*., 2011) isolated from the intestinal mucus of brown trout (*Salmo trutta*). *Lactococcus lactis* is critical for manufacturing cheeses such as Cheddar, cottage, cream, Camembert, Roquefort and Brie, as well as other dairy products like cultured butter, buttermilk, sour cream and kefir (Todar, 2013). *Lactococcus lactis* is also responsible of the fermentation of many fermented milks like filmjolk, leben, zivda, amasi, *kivuguto*, etc.

- **Aerococcus**

Aerococcus viridans and *Aerococcus urinae* are the most known species of the genus Aerococcus. The cell is in form of immobile sphere of 1 to 2µm diameter, which tends to form clusters, but also pairs and tetrads. The Aerococci are little acidifying, Gram-positive and catalase-negative or pseudocatalase, microaerophilic. They are also involved in the production of lactic acid fermentation by lactic acid (De Roissart and Luquet, 1994). For the moment, the genus Aerococcus counts 7 species: *Aerococcus viridans* (Williams *et al*., 1953) found as one of the dominant LAB in Domiati cheese (El-Baradei *et al*., 2007), *Aerococcus christensenii* (Collins *et al*., 1999), *Aerococcus sanguinicola* (Lawson *et al*., 2001), *Aerococcus suis* (Vela *et al*., 2007), *Aerococcus urinae* (Aguirre and Collins, 1992), *Aerococcus urinaeequi* (Garvie, 1988) (Felis *et al*., 2005), *Aerococcus urinaehominis* (Lawson *et al*., 2001).

- **Bifidobacterium**

The genus Bifidobacterium counts 47 species and 9 subspecies. It belongs to the phylum Actinobacteria, class Actinobacteria, order Bifidobacteriales, family Bifidobacteriaceae. Most of cells of this genus are classified as probiotics and degrade hexoses through the fructose-6-phosphate pathway, in presence of the fructose-6-phosphoketolase enzyme. Generally, they are used in dairy fermentation in co-culture with some lactic acid bacteria. *Bifidobacterium bifidum* (Orla-Jensen, 1924) (Approved Lists 1980) (Skerman *et al.*, 1980) is the type species. The species found in dairy products are: *Bifidobacterium animalis subsp. lactis, Bifidobacterium bifidum, Bifidobacterium longum, Bifidobacterium adolescentis, Bifidobacterium animalis subsp. animalis, Bifidobacterium infantis, Bifidobacterium breve* (Holzapfel *et al*., 1998; Klein *et al*., 1998; Reid, 1999), *Bifidobacterium thermophilum* (Xiao, 2010) and *Bifidobacterium pseudolongum subsp. pseudolongum* (Rabiu *et al.*, 2001).

- **Non-LAB species**

Either they participate in the pigmentation of cheese or especially produce sulfur-containing compounds that enhance the characters "cheese" and "matured" to a cheese (Rattray and Fox, 1999) or produce aroma compounds giving good tastes to cheeses. These genera are Staphylococcus, Propionibacterium, Brevibacterium, Micrococcus, Microbacterium, Arthrobacter, Brachybacterium and Corynebacterium, etc.

- **Staphylococcus**

The genus comprises about 47 species and 24 subspecies, most of them are not pathogenic. Cells of Staphylococcus present a shape of clustered cocci. They are Gram positive. According to the presence of coagulase, the genus is separated in two groups. The group of positive coagulase can be implicated in human infections and the most known is *Staphylococcus aureus*. About 33 species are negative coagulase and generally non pathogenic. This group is frequently found in sausage, milk and cheeses. *Staphylococcus equorum* was originally isolated from the skin of healthy horses and its cell wall is similar to that of *Staphylococcus xylosus*. Strains of *Staphylococcus equorum* have been also isolated

from sausage and strains comprising subspecies of this species have been isolated from Swiss mountain cheeses (Place *et al.*, 2003). *Staphylococcus xylosus* is used for flavoring *Munster* cheeses (Hansen, 2011; Law and Tamime, 2011). *Staphylococcus sciuri subsp. carnaticus* (Kloos *et al.,* 1997) and *Staphylococcus caseolyticus* (Schleifer *et al*., 1982) are used also in cheese (Hansen, 2011). Various non-pathogenic species of Staphylococcus play important roles in the development of the rinds on naturally aged cheeses. Incredibly little is known about the ecology or evolution of these Staphylococcus species in cheese process: *Staphylococcus succinus (white, wrinkly), Staphylococcus xylosus (orange, wrinkly), Staphylococcus saprophyticus (cream, round)* and *Staphylococcus equorum (white round)* (Wolfe, 2013) or *Staphylococcus cohnii* (Drosinos, 2007), *Staphylococcus fleurettii* (Vernozi-Rozand *et al*., 2000), *Staphylococcus sciuri subsp. sciuri* (O'Halloran, 1998), *Staphylococcus vitulinus* (Bannerman *et al*., 1994).

- **Propionibacterium**

Species of Propionibacterium found in dairy products are: *Propionibacterium acidipropionici* in cheese (Hansen, 2011), *Propionibacterium freudenreichii subsp. freudenreichii* (Hansen, 2011; Place *et al*., 2003), *Propionibacterium freudenreichii subsp. shermanii* in Emmental cheese (Hansen, 2011; Riley, 2005; Hutkins, 2006), *Propionibacterium jensenii* and *Propionibacterium thoenii* in cheese (Van Niel, 1928).

- **Corynebacterium and Brevibacterium**

Coryneform bacteria were identified on the surface of cheese and the most species found were *Corynebacterium ammoniagenes* (Bockelmann and Hoppe-Seyler, 2001), *Corynebacterium flavescens* (Brennan *et al*., 2002), *Corynebacterium casei, Corynebacterium variabile* (Bockelmann *et al*., 2005). The species of Brevibacterium (**Annexe 5**) belongs to the class Actinobacteria, Order Actinomycetales. It is a combination of coryneform bacteria used to create vitamins, aminoacids for chemical production. These bacteria also present as rod-coccus shape along their exponential phase, but they turn into their normal rod-shaped structure at the end.

- **Micrococcus and Microbacterium**

The genus Micrococcus counts 16 species among which *Micrococcus luteus* was found in Limburger cheese (Hansen, 2011), *Microbacterium gubbeenense* in Limburger cheese and in Tilsit cheese (Law & Tamime, 2011), as well as in smear-ripened cheese (Hansen, 2011; Law & Tamime, 2011).

- **Brachybacterium and Carnobacterium**

The genus of Brachybacterium has two species of *Brachybacterium alimentarium* and *Brachybacterium tyrofermentans* (Schubert *et al*., 1996). And for the genus Carnobacterium, we found in dairy products *Carnobacterium divergens* (Hammes and Hertel, 2009) and *Carnobacterium maltaromaticum* (Afzal *et al*., 2010).

- **Arthrobacter and Kocuria**

Starter organisms of this group include *Arthrobacter arilaitensis* (Mounier *et al*., 2005), *Arthrobacter bergerei* (Irlinger *et al*., 2005), *Arthrobacter globiformis* (Fox, 2000), *Arthrobacter nicotianae* (Smacchi *et al*., 1999), *Kocuria rhizophira, Kocuria kristinae*, *Kocuria halotolerans* (El-Baradei, 2007) and *Kocuria varians* (O'Mahony, 2001).

2.3.2. Fungi

Fungi are present naturally in milk or can be added as starter cultures (Forquin, 2010). By their biochemical abilities, molds play a key role in the formation of sensory characteristics of cheeses. According to Seiler (2002), the most fungi species often found in dairy products (**table 1**) are *Candida intermedia, Candida parapsilosis, Cryptococcus curvatus, Debaryomyces hansenii, Galactomyces geotrichum, Issatchenkia orientalis, Kluyveromyces marxianus, Kluyveromyces lactis* (both sometimes referred to as *Kluyveromyces marxianus subsp. marxianus, Kluyveromyces marxianus subsp. lactis, Kluyveromyces marxianus subsp. bulgaricus, Candida kefyr), Pichia farinosa, Pichia fermentans, Pichia membranaefaciens, Pichia anomala, Trichosporon beigelii* and *Yarrowia lipolytica*. The use of *Penicillium*

camemberti and *Penicillium roqueforti* (Le Dréan *et al.*, 2010) is also very frequent during ripening of model cheese curd and surface mold-ripened cheeses. Studies on the diversity of the naturally fermented milk from the Tibetan Plateau of China (Bai *et al*., 2010) highlighted the dominance of yeast species as following: *Issatchenkia orientalis, Kazachstania unisporus, Rhodotorula mucilaginosa, Candida pararugosa, Geotrichum fragrans, Debaryomyces hansenii, Trichosporon gracile, Pichia membranifaciens, Torulaspora delbrueckii* and *Yarrowia lipolytica*.

Table 1. Non-exhaustive list of fungi encountered in dairy products.

Phylum	Family	Species	References
Ascomycota	Cordycipitaceae	*Lecanicillium lecanii*	Lund *et al*., 1995
	Dipodascaceae	*Galactomyces candidum* *Galactomyces geotrichum* *Geotrichum candidum* *Geotrichum fragrans* *Yarrowia lipolytica*	Mounier *et al*., 2008 Akabanda *et al*., 2013 Riley, 2005 Bai *et al*., 2010 Law & Tamime, 2011
	Microascaceae	*Scopulariopsis flava*	Moreau, 1979
	Netriaceae	*Fusarium domesticum* *Fusarium venenatum*	Hansen, 2011 Thrane, 2007
	Saccharomycetaceae	*Candida pararugosa*	Bai *et al*., 2010
		Candida kefyr *Candida intermedia*	Seiler, 2002
		Candida parapsilosis *Candida tropicalis*	Akabanda *et al*., 2013
		Candida etchellsii	Coton *et al*., 2006
		Candida rugosa *Candida versatilis* *Candida zeylanoides*	Seiler and Busse, 1990
		Pichia kudriavzevii	Bai *et al*., 2010
		Pichia occidentalis	Seiler and Busse, 1990
		Pichia farinosa	

		Pichia fermentans *Pichia membranaefaciens* *Pichia anomala*	Seiler, 2002
		Cyberlindnera jabnii	Thrane, 2007
		Debaromyces hansenii	Besançon *et al*., 1995
		Kazachstania exigua *Kazachstania unisporus*	Zhou *et al*., 2009
		Kluyveromyces marxianus *Kluyveromyces lactis* *Saccharomyces cerevisiae*	Roostita & Fleet, 1996
		Zygotorulaspora florentina	Boekhout&Robert, 2003
	Sarcosomataceae	*Torulaspora delbrueckii*	Law & Tamime, 2011
	Trichocomaceae	*Penicillium camemberti*	Moreau, 1979
		Penicillium caseifulvum	Lund *et al*., 1998
		Penicillium chrysogenum *Penicillium commune*	Lund *et al*., 1995
		Penicillium nalgiovense	Farber & Geisen,1994
		Penicillium roqueforti	Hansen, 2011
	Wallemiaceae	*Sporendonema casei*	Ropars *et al*., 2012
Basidiomycota	Cystofilobasidiaceae	*Cystofilobasidium infirmominiatum*	Early, 1998
	Tremellaceae	*Cryptococcus curvatus*	Seiler, 2002
	Trichosporonaceae	*Trichosporon gracile*	Bai *et al*., 2010
		Trichosporon beigelii	Seiler, 2002
	Sporidiobolaceae	*Rhodotorula mucilaginosa*	Bai *et al*., 2010
		Rhodotorula minuta	Law & Tamime, 2011
Zygomycota	Mucoraceae	*Mucor mucedo*	Oterholm, 2003
		Mucor plumbeus *Mucor racemosus*	Hansen, 2011

The dominance of yeast species was also observed beside LAB in *nunu* fermented milk of Ghana. The yeasts involved were *Candida parapsilosis*, *Candida rugosa*, *Candida tropicalis*, *Galactomyces geotrichum*, *Pichia kudriavzevii* and *Saccharomyces cerevisiae*, with *Pichia kudriavzevii* and *Saccharomyces cerevisiae* being the dominant yeast species (Akabanda *et al*., 2013).

3. TECHNOLOGICAL PROPERTIES

3.1. Acidification

The acidification activity of a starter culture is the first technological property needed in dairy industry. It is a characteristic of the species and/or the strain. The acidification of milk reduces the pH and consequently, the production of lactate with/without other metabolites depending of the microorganisms used. Briefly, three main phenomena occurred by milk acidification:

- The production of lactic acid from lactose allows the coagulation of casein mastrix by reducing its pH, which impacts on the milk rheology.
- The degradation of lactose by the glycolytic or phosphoketolase pathways leads to production of lactate which brings an acidic taste or influences the activity of flavoring micro-organisms. By glycolysis, some starters produce also exopolysaccharidic molecules which give a characteristic texture.
- The lowering pH by starter cultures inhibits some spoilage microorganisms. This action on the milk preservation is very important along the post-acidification and is strain dependent.

3.2. Aroma production

The flavor is paramount for food acceptability by consumers and for most dairy products, it is the result of the combination of a large number of aroma compounds produced by the metabolism of milk nutrients (caseins, lipids, lactose and citrate, etc.) by dairy micro-organisms. The main pathways of flavor compounds development in dairy products (glycolysis, lipolysis, proteolysis, citrate) begin by the lactate formation step, from which many volatile compounds (VCs) (hydrocarbons, aldehydes, ketones, alcohols, esters, fatty

acids and sulphur compounds) are produced as illustrated in **figure 1**. The genesis of those compounds depends on the microorganisms used.

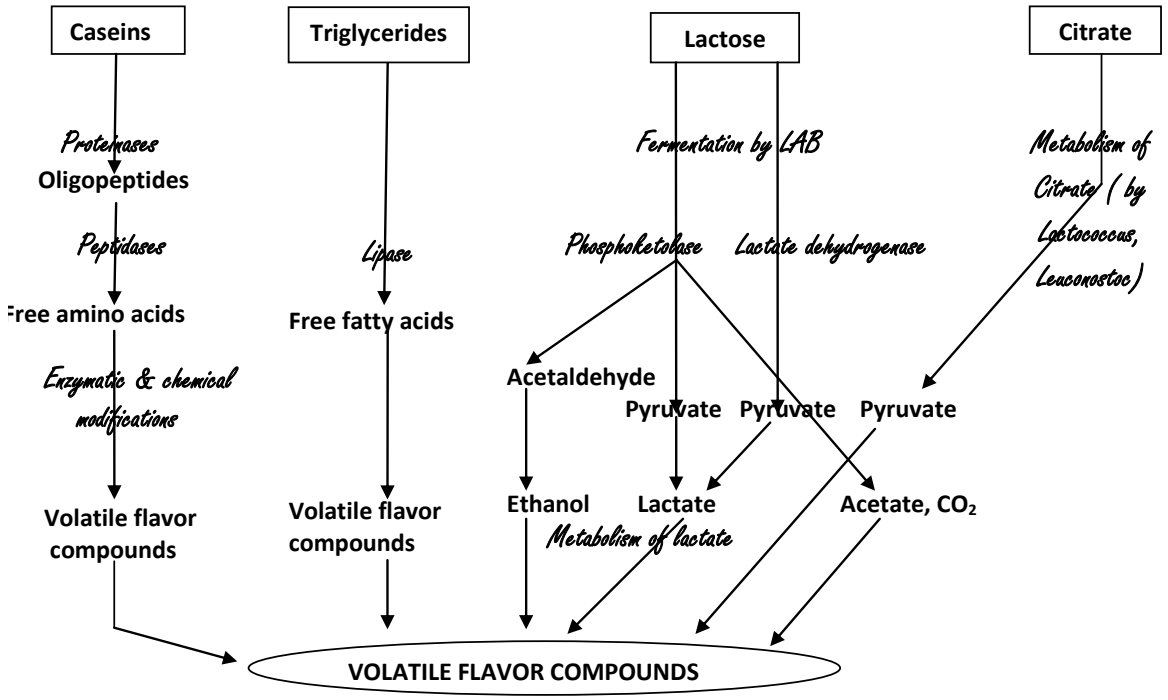

Figure 1. Aroma generation in fermented milk

3.3. Textural activity

Some starter cultures produce exopolysaccharides in dairy products, which determine the rheological characteristics. But the rheology of fermented milk is also an action of the structure and mass molecular of those exopolysaccharides, as well as their interaction with milk casein during fermentation process (Marshall and Rawson, 1999). In order to characterize the structure of the dairy product, the rheological and textural measurements are performed. Three parameters may be analyzed: viscosity, thixotropy and elasticity according to the texture of the dairy product.

3.4. Proteolysis

Dairy cultures are a dynamic biochemical system in which micro-organisms grow using nutrients found in the product. However, milk does not have enough of essential amino acids

needed for LAB growth in free form (Donkor *et al*., 2007). They have therefore proteolytic enzymes allowing hydrolysis of milk caseins into oligopeptides and thereafter in free amino acids, necessary for the growth of starter cultures. Proteolysis is a cascade process involving a number of steps including (i) an extracellular proteinase initiating degradation of casein into oligopeptides, (ii) transport systems that translocate peptides and amino acids across the cell wall, (iii) various intracellular peptidases for further degradation of peptides into amino acids, and (iv) different enzymes that convert liberated amino acids into various components (Kunji *et al*., 1996). And proteinases and peptidases are species/strains dependent, leading to different specificities. The dairy industry prefers strains with low proteolytic activity. In fact, strains with high proteolytic activity can cause the formation of hydrophobe peptides, source of organoleptic defects, creating bitterness in the final product. Such micro-organisms may be discarded during microbial selection, especially in single culture. In mixed culture, the aroma generation is a result of well balanced volatile compounds of strains involved in fermentation. Nevertheless, the selection task may pay attention of strains with high level of proteolytic activity.

3.5. Other technological properties.

The phage production in milk is a big challenge during fermentation. Bacteriophages destroy starter cultures and this causes imbalance in the dairy starter formulation. The selection of strains resisting to bacteriophage is paramount, because they can stop the fermentation (Emond *et al.*, 1997). Many LAB create antagonistic activity against other starters or non-starters bacteria during fermentation. It is better to evaluate such interaction for the product stability.

4. INDUSTRIAL PRODUCTION OF DAIRY STARTER CULTURES

Since the 1950s, there have been many developments in the field of starter culture technology (i.e. preservation, maintenance and production). Before, milk fermentation was performed by use of a previous culture (back slopping) or by liquid cultures. In order to improve shelf-life of cultures and to facilitate transport by post without any appreciable loss in their activity, the use of dried/frozen starters replaced the old method. Thereafter, since 1970s, the dairy

industry used freeze dried or frozen cultures according to the facility of each one. The traditional production of lactic acid bacteria has now given way to more efficient processes. The current techniques take into account the selection of well defined strains with high technological potential (acidification, flavoring, proteolysis, lipolysis, texture, ...). These techniques allow the manufacture of reproducibly, economic and qualitative biotechnological products. The back slopping technique is now supplemented by the method of pre-culture or mother culture. This method generally uses concentrated liquid starter culture. It also uses frozen and most frequently freeze-dried/spray-dried starter cultures. Depending on the needs and size of production, freeze-dried starter cultures are increasingly used nowadays with fermentors of various sizes, but the technology is limited by its high cost.

4.1. Preparation of media

The medium for the industrial production of lactic acid bacteria depends on the physiology of micro-organisms to be produced. The growth parameters (temperature, pH, aeration,...) are well known and nutrient requirements necessary for good multiplication contribute to correctly identify the ingredients of the medium. More specific to each organism characteristics, balance of carbon source (lactose, glucose, sucrose...) and nitrogen source (peptones, yeast extracts, meat, …) must be respected. They are complemented by minerals (Ca, Mg, Mn, Fe, Cu, Zn, Mo, Se), growth factors (vitamin B group), as well as compounds ensuring resistance to all steps of the starter culture development, even in the storage (e.g. ascorbic acid). Thereafter, the medium culture formed is prepared in the proportions of each nutrient, and then sterilized at 120°C under 2 bars. In addition, the entire system must undergo this treatment after a thorough cleaning between fermentations to limit any contamination (Lejard *et al*., 1994). In general, the industrial production follows the diagram as on **figure 2**.

4.2. Production in fermentor

The fermentor or bioreactor is a vessel capable of heat-treating the medium, aseptic seeding of medium, incubating the culture under suitable atmosphere and at controlled pH and stabilizing the culture by cooling (Branger et al., 1994). In addition, it allows a real-time control of the fermentation using probes (pH, temperature, etc.) and an addition of other

elements needed for growth. There are three methods of fermentation:

- The batch fermentation is a non continuous culture until the end of the fermentation and the culture is recovered in full with the whole biomass formed. Then there is an accumulation of toxic products of bacterial metabolism, which is a limiting factor for bacterial growth. The pH, the dissolved O2, the temperature are controlled throughout the process. It remains the most commonly used technology in industrial production and the bacterial growth is stopped at the limit of the exponential phase for most species.
- The fed-batch fermentation is a non continuous culture as the batch, but a renewal of the culture is carried out by adding a limiting substrate to sterile medium in relation to the cell, the rate of growth, the medium used and the inhibition of growth.
- The continuous fermentation is an open culture to allow control of the concentration of the substrates continuously. Indeed, the input flow rate of fresh medium is the same as the output rate of the fermented medium.

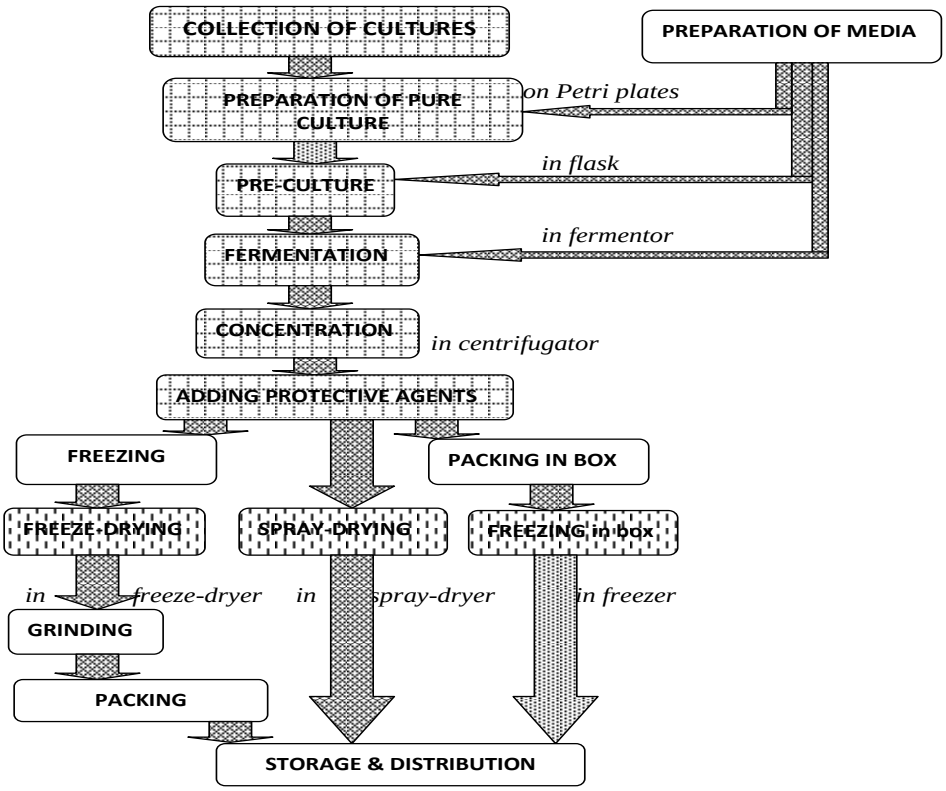

Figure 2. Diagram of starter cultures production

4.3. Down-stream processes

4.3.1. Cooling and biomass concentration

Industrial production of starter cultures is most often a batch process. At the end of culture, it is necessary to separate the biomass and the liquid medium. Centrifugation (separation by density difference) is the concentration method most widely used on an industrial scale (Mäyra-Mäkinen and Bigret, 1998), but the ultrafiltration or microfiltration (separation by size difference) are also used (Lejard *et al*., 1994).

4.3.2. Adding protective agents

The addition of cryoprotectants allows a strong reduction of cell death along with the down-stream processes, especially during the various stages of the freeze-drying and storage conditions. Some polysaccharides (sucrose, maltodextrin, ...), supplemented by peptides and polyols such as glycerol provide good protection against cellular oxidation of membrane lipids.

4.3.3. Freezing or Drying

Microbial concentrate with added protective agents is then stabilized for its long shelf life. It is therefore frozen and/or dried for an easy storage and transport. Freezing is carried out into blocks or particles of 2 to 5 mm in diameter. In blocks, sealed boxes are used, making difficult the mixing of strains after thawing. In particles, the starter distribution is carried out dropwise into a liquid nitrogen bath. The logistics of this technique is huge (cold room at -60°C, -80°C, liquid nitrogen at -96°C; strict cold chain) and complicated seeding conditions of this technique make it less used to the benefit of the drying techniques. Freeze-drying is the most widely practiced technology nowadays for the microbial preservation. The concentrate is frozen and dehydrated by sublimation in a freezer. The dry product is then ground in a fine powder. The cells mixtures are made very easily, depending on the intended formulation. The spray-drying is also used, but it requires strains highly resistant to high temperatures. For a complete drying, it can be complemented with a drying in fluidized bed.

4.3.4. Packaging and storage

The powders obtained can then be mixed according to the desired concentration of starter. They are then often aseptically vacuum-packed in metallo-plastic bags consisting of an oxygen-free atmosphere, which allows a long shelf life.

5. CONCLUSION

The natural biodiversity of dairy starter cultures is still wide and rich of bacterial and fungal species with potentials needed in dairy industry. And only a small proportion is already selected and used in many dairy cultured products. Knowledge of taxonomy and microbial ecology in various niches and fermentations; new molecular techniques, phylogeny, genomics and proteomics; biochemical processes and fermentation technology are important tools for microbial selection. The diversity of dairy starter cultures involves lactic acid bacteria (LAB) with five families and bifidobacteriaceae; non-lactic acid bacteria both Gram+ and Gram– with eight families: staphylococcaceae, brevibacteriaceae, corynebacteriaceae, microbacteriaceae, propionibacteriaceae, enterobacteriaceae, dermabacteriaceae, micrococcaceae; about 10 families of yeasts & molds: cordycipitaceae, dipodascaceae, microascaceae, nectriaceae, saccharomycetaceae, sarcosomataceae, trichocomaceae, wallemiaceae, cystofilobasidiaceae, mucoraceae. Considering their overall potentials to produce an enormous range of natural products with nutritional, healthy, antimicrobial or other biological activities, many species are selected and used in starter formulation for fermented milk and/or cheese manufacturing. A dairy product is a complex ecosystem in which different types of microbial interactions coexist, with many biochemical activities: glycolysis, proteolysis, lipolysis, etc. LAB produce lactic acid from lactose by glycolysis during fermentation to form many fermented milks depending on species used or/with associated LABs and/or other species. LAB are also used for their ability to produce flavor compounds and exopolysaccharides, which is very important in taste and texture formation of dairy products. Genera involved are mainly Lactococcus, Leuconostoc, Streptococcus and Lactobacillus. LAB are also very active in the first stages of cheese manufacturing. Their essential roles are to acidify milk and curd, to participate in the formation of taste (proteolysis, flavor production) and texture. Non-lactic acid bacteria are also involved actively

in the whole ripening process. They participate in the pigmentation of cheese or especially produce sulfur-containing compounds that enhance the characters "cheese" and "matured" to a cheese (Rattray and Fox, 1999) or produce aroma compounds giving good tastes to cheeses. These genera are Staphylococcus, Propionibacterium, Brevibacterium, Micrococcus, Arthrobacter, Brachybacterium, Microbacterium or Corynebacterium, etc. Yeasts develop to a greater extent to the surfaces of cheeses and inside. They are involved in the deacidification of the curd at the beginning of ripening by consumption of lactate and/or production of ammonia (Forquin, 2010). Deacidification is dependent on both the numbers and strains of yeasts present and yeasts produce compounds that are stimulatory to the growth of the bacterial smear flora. These compounds include products of proteolysis and vitamins synthesised by the yeasts (e.g. pantothenic acid, niacin, riboflavin) (Sheehan, 2000). Molds used in cheese production are non-toxic and are thus safe for human consumption; however, mycotoxins (e.g., aflatoxins, roquefortine C, patulin, or others) may accumulate because of growth of other fungi during cheese ripening or storage (Erdogan et *al*., 2004). The most known in cheese manufacture are Penicillium and Mucor. *Penicillium camemberti* is present on the surface of Camembert and *Penicillium roqueforti* is the internal mold of the Blue d'Auvergne (cow's milk) or Roquefort (sheep milk), whilest Mucor is found on the surface of the Tome de Savoie as the dominant mold.

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Chapter 3

Selection of microbes in *kivuguto* traditional milk

Ce chapitre présente les premiers résultats expérimentaux de cette recherche. Il décrit les méthodes utilisées pour la détection et la caractérisation taxonomique des micro-organismes impliqués dans le processus de fermentation du kivuguto, l'évaluation partielle des paramètres technologiques en vue du screening de souches performantes et les premiers essais de production pilote du kivuguto en laboratoire.

L'expérimentation a été conduite sur quatre échantillons de kivuguto issus de deux sites différents au nord et au sud du Rwanda. Un isolement, une purification et un dénombrement ont permis de constituer un souchothèque de 390 souches. Les analyses phénotypiques (microscopiques et biochimiques), puis le screening par des essais de croissance en milieux synthétiques et dans le lait, ainsi que la croissance dans des conditions environnementales extrêmes ont permis la pré-sélection de sept souches potentiellement impliquées dans la fermentation du kivuguto. Par la suite, ces souches ont été identifiées par la génétique moléculaire et les sept séquences envoyées à la base des données GenBank.

Enfin, des essais de production du kivuguto par le mélange de souches pré-sélectionnées, ainsi que la conservation des laits formulés pendant le temps légal de stockage ont mis en évidence la compatibilité, la stabilité et tous les aspects technologiques du kivuguto au moyen de trois souches: un lactocoque et deux Leuconostocs.

Au terme de cette étude, les résultats ont fait l'objet d'une publication, détaillée in extenso dans ce chapitre.

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Fermentation of *kivuguto*, a rwandese traditional milk: Selection of microbes for a starter culture

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ABSTRACT

A total of 390 bacterial strains were isolated from four *kivuguto* samples. Isolates were screened by phenotypic methods and molecular techniques using the PCR and sequencing of 16S rDNA and/or 16S-23S rDNA intergenic transcribed spacer (ITS) region. The rate of milk acidification and ability to grow under extreme environmental conditions were also examined for pre-selected isolates. Fermentation assays with mixed strains were performed to identify microbial formulations that produced milk similar to *kivuguto.* Such formulated milk was stored at 4°C on 24 days for shelf-life analysis to detect any organoleptic defect. Bacteria identified in *kivuguto* included *Lactococcus lactis* (strains CWB-B1466 and CWBI-B1469) and *Leuconostoc mesenteroides* subsp. *mesenteroides* (strains CWBI-B1467 and CWBI-B1465) and *Leuconostoc pseudomesenteroides* (strains CWBI-B1468, CWBI-B1470 and CWBI-B1471). One *Leuconostoc* sp. grew at low pH 2.5, in high sodium chloride concentration (up to 6.5%) and was resistant to heat (55°C). Fermentation experiments showed that *kivuguto* coagulates in 8 hours at 30°C. Three milk formulations produced using mixed strains gave good results. The best-tasting and longest shelf-life (35 days) *kivuguto* was produced by a mixture of two *Leuconostoc* spp. and *Lactococcus lactis.* These strains give *kivuguto* its characteristic properties.

Key words: *kivuguto,* phenotypic methods, PCR, sequence, 16S rDNA, 16S-23S rDNA ITS, fermentation, *Lactococcus*, *Leuconostoc*

RESUME

390 souches bactériennes ont été isolées à partir de quatre échantillons du lait *kivuguto*. Les isolats ont été sélectionnés par des méthodes phénotypiques et des techniques moléculaires par la PCR et le séquençage de l'ADNr 16S et/ou de la région intergénique 16S-23S ADNr (ITS). L'acidification du lait et la croissance dans des conditions environnementales extrêmes ont aussi été examinées pour les souches pré-sélectionnées. Des essais de fermentation avec des souches mixtes ont été effectués pour identifier les formulations microbiennes qui produisent du lait similaire au *kivuguto*. Afin de détecter des défauts organoleptiques et plus particulièrement l'apparition d'amertume, chaque lait formulé a été conservé sous

réfrigération pendant 24 jours pour évaluer la durée de vie. Au cours de ces analyses, les bactéries identifiées sont les suivantes: *Lactococcus lactis* (souches CWBI-B1466 et CWBI-B1469) et *Leuconostoc mesenteroides subsp. mesenteroides* (souches CWBI-B1467 et CWBI-B1465) et *Leuconostoc pseudomesenteroides* (souches CWBI-B1468, des CWBI-B1470 et CWBI-B1471). Un *Leuconostoc* sp. a montré sa tolérance à un faible pH 2.5, à une concentration élevée en chlorure de sodium (jusqu'à 6,5%) et était résistante à une température élevée (55°C). Ces expériences ont montré que le *kivuguto* coagule en 8 heures à 30°C. Trois formulations de lait produit en utilisant des souches mixtes ont donné de bons résultats. La meilleure formulation suivant le critère goût et la stabilité sous réfrigération (35 jours) a été réalisée par un mélange de deux *Leuconostoc* spp. et *Lactococcus lactis*. Ces souches donnent au *kivuguto* ses propriétés caractéristiques.

Mots clés: *kivuguto*, les méthodes phénotypiques, PCR, séquençage, l'ADNr 16S, 16S-23S ADNr ITS, la fermentation, *Lactococcus*, *Leuconostoc*

ملخص

تم عزل 390 سلالة بكتيرية من أربع عينات من حليب kivuguto . تم إنتقاء هذه العزلات بواسطة طرق الدراسة المظهرية و التقنيات الجزيئية عن طريق الPCR وتسلسل rDNA-S16 أو بين الجينات منطقة rDNA S23-S16 (ITS). وبحث أيضا تحمض الحليب والنمو في الظروف البيئية القاسية للسلالات المختارة. أجريت تجارب تخمر مع سلالات مختلطة لتحديد الصيغ الميكروبية التي تنتج حليب مماثل لkivuguto. للكشف عن العيوب الحسية وخصوصا ظهور المرارة، تم تخزين كل صيغ الحليب تحت التبريد لمدة 24 يوما لتقييم مدة الحياة. في هذه التحليلات، البكتيريا التي تم تحديدها هي: *Lactococcus lactis* (سلالات B1466-CWBI و B1469-CWBI), *Leuconostoc mesenteroides* subsp. *mesenteroides*(سلالة B1467-CWBI و B1465-CWBI) و *Leuconostoc pseudomesenteroides* (سلالة B1468- CWBI، B1470-CWBI، وB1471- CWBI). واحد من *Leuconostoc* sp. أظهر سماحية لانخفاض الرقم الهيدروجيني (2.5) و نسبة عالية من كلوريد الصوديوم (بزيادة تصل إلى 6.5٪) وكانت مقاومة لدرجات الحرارة المرتفعة (55 درجة مئوية). أظهرت هذه التجارب أن تخثر kivuguto تم في 8 ساعات عند 30 درجة مئوية. ثلاث صيغ من الحليب المنتج باستخدام سلالات مختلطة كانت ناجحة واستنتج أن أفضل صيغة من حيث معيار الذوق والاستقرار تحت التبريد (35 يوما) بواسطة مزيج بين إثنين من *Leuconostoc* spp. و*Lactococcus lactis*. هذه السلالات تعطي لحليب kivuguto خصائصه المميزة.

كلمات فاتحة: kivuguto، الطرق المظهرية، PCR، تحديد التسلسل، rDNA-S16، S23-S16، rDNA (ITS)، التخمر، *Lactococcus*، *Leuconostoc*

1. INTRODUCTION

Fermentation has been used for many years to preserve milk in a number of countries, including Rwanda. *Kivuguto* is the term for traditional rwandese fermented cow's milk. It is a very consistent and rich dairy product with high nutritional value. Fermentation is carried out spontaneously by natural milk microflora and environmental contaminants without the addition of any starter culture [1].

During fermentation, milk microorganisms produce a large number of metabolites such as amino acids, vitamins and minerals. The reported health benefits of such microorganisms include, among others, boosting the immune system and inhibiting the growth of pathogens and preventing diarrhoea from various causes [2]. Rwandese local markets are dominated by traditional milk. However, Rwanda has five milk-processing plants (Inyange Industries, Nyanza Dairy, Eastern Savannah, Rubirizi Dairy and Masaka Farms). Although modern dairies put other types of fermented milk on the Rwandese market, artisanal *kivuguto* is popular, because consumers prefer locally-made natural fermented milk to fermented milk made using an imported mesophilic starter culture [3]. This preference for naturally fermented milk led researchers to isolate and characterize wild strains able to produce milk with similar sensorial properties to those of traditional products [4]. Therefore, an investigation was begun to identify those characteristics in traditional *kivuguto* in order to meet the needs of the Rwandese and regional marketplaces. This will allow standardization of quality without changing the fundamental properties of *kivuguto.*

Artisanal *kivuguto* is made at the household level using raw milk. This makes the final product very variable and, as reported Tornadijo *et al.* [5], the lack of uniformity limits its acceptance and distribution on the market.

The purpose of this work definitely was to develop a starter culture from microbial strains selected from traditional *kivuguto*, with the ultimate objective of reproducing its major properties in dairy. In the present paper, we report the results of screening tests of isolates involved in *kivuguto* fermentation and the results of fermentation assays designed to identify a formulation with characteristics similar to *kivuguto*. To date, there are no reports of dairy starter formulations developed using this approach and no other report of microbial and technological study of *kivuguto* milk.

2. MATERIALS AND METHODS

2.1. Sampling and origin of strains

Prior to choosing the samples for this work, a short survey was conducted at two sites in order to determine which households produce high quality *kivuguto*. Sites selection was based on geography, ecology and the absence of exotic fermented milk in the area. Samples of *kivuguto* were collected in february and september at two sites in Rwanda: the Huye District in the southern province (Sample S) and the Musanze District in the northern province (Sample N). Sample preparation followed IDF Standard 122C [6].

2.2. Isolation and enumeration

Media were prepared according to IDF Standard 149A [7]. Decimal dilution of samples followed IDF Standard 122C [6]. Eight selective and differential media were inoculated with 100 µl of diluted sample, prepared with 0.1% peptone water. The culture conditions are listed in **Table 1**. Microorganisms were enumerated using the spread-plate method. Each experiment was carried out in triplicate. Single colonies (10 or 20) were picked randomly from each plate and subjected to Gram staining and assay for catalase activity. Gram staining followed the manufacturer's instructions (*BioMérieux, Marcy-l'Etoile, France*). Catalase activity was assayed by placing a drop of hydrogen peroxide solution on the colony. The presence of catalase was indicated by the formation of bubbles on the colony. Thereafter, Gram-positive and catalase-negative colonies were purified three times on MRS agar containing $CaCO_3$ for presumptive bacteria. Pure colonies were then stored at -80°C in Nalgene® cryogenic vials (Thermo Fisher Scientific Inc., Rochester, NY, USA) in 1 ml of 40% (v/v) glycerol.

2.3. Phenotypic and genotypic characterization

The colonies and cells of dominant isolates (Gram-positive and catalase-negative) were initially analyzed through observations. Five colonies from each dominant isolate group were analyzed using an API 50®CH gallery kit (*BioMérieux Marcy-l'Etoile, France*), according to

the manufacturer's instructions. Each dominant group was defined by the medium used for isolation. The API 50®CH galleries used API 50®CHL medium to identify isolates by characterizing their ability to ferment 49 carbohydrates. The database associated with APILAB PLUS V3.2.2 (*BioMérieux*) software was used to aid in the identifications. Strains were therefore grouped based upon comparison of their API 50®CHL profile to that of organisms listed in the database.

Table 1. Culture conditions, isolation and differentiation of microorganisms of *kivuguto.*

Enumeration media	Presumptive targeted microorganisms	Temperature of incubation (°C)	Duration of incubation (hours)	Incubation condition	pH
[1]**M17 agar**	total *lactococci*	30	48-72	Aerobiosis	7,15
Turner agar (arginine tetrazolium agar)	differentiation of *Lactococcus lactis ssp. lactis and L. lactis ssp. lactis bv. diacetylactis*	30	48-72	Anaerobiosis	6,0
Tomato juice	differentiation of *L. lactis ssp. lactis bv. diacetylactis*	25	72	Anaerobiosis	6,1
[2]**Mayeux agar**	total *Leuconostoc*	25	120	Aerobiosis	-
[3]**Rogosa agar**	total *Lactobacilli*	30	72	Anaerobiosis	5,4
Rogosa raffinose agar	differentiation of *Lb. plantarum*	30	72	Anaerobiosis	5,4
[4]**MRS agar**	differentiation of *Lb. acidophilus*	37	72	Aerobiosis	6,1-6,
[5]**PDA**	Yeasts	30	48	Aerobiosis	5,6

[1]The modified M17 agar [8] was prepared for the isolation of cocci (*Lactoccus* and *Streptococcus*); [2]Mayeux agar [9] for *Leuconostoc* spp., is an elective medium for the detection and enumeration of *Leuconostoc* in milk, dairy products and sweet foods. It was supplemented with sodium azide and sucrose. Sodium azide was used for inhibition of *Lactococci* and Gram negative bacteria. *Leuconostoc mesenteroides subsp. mesenteroides* and *Leuconostoc mesenteroides subsp. dextranicum* utilize the sucrose in the medium to synthesize polysaccharides (dextrans) which impart a gelatinous appearance to the colonies; [3]Rogosa agar [10] targeted all *Lactobacilli*. However, glucose was replaced by raffinose for presumptive *Lactobacillus plantarum* in Rogosa raffinose agar; [4]MRS [11] was modified and incubation was made in the conditions of *Lactobacillus acidophilus* [7]. [5]Potato dextrose agar (PDA) was used for yeasts.

The 16S rDNA and/or 16S-23S rDNA intragenic spacer (ITS) regions of pre-selected strains were sequenced after a polymerase chain reaction (PCR) according to the method of Drancourt *et al.* [12]. The following primers were used for PCR of 16S rDNA and 16S-23S rDNA: 16SPO/16SP6 and ITS R/ITS F, respectively. Amplifications were performed following the Fermentas Taq DNA Polymerase Protocol (Fermentas Life Sciences, MD, USA) in a My Cycler™ thermal cycler (Bio-Rad Laboratories, Inc., California, USA). The BigDye Terminator v1.1 Cycle Sequencing Kit Protocol [13] was used for the sequencing

reactions with 8 primers for the 16S rDNA and/or ITS R and ITS F for the 16S-23S rDNA ITS **(Table 2).** The data were analyzed using ABI Genetic Analyzer Sequencing Analysis v5.2 software. The 16S rRNA and/or the 16S-23S rRNA ITS sequences obtained were submitted for BLAST searching (http://www.ncbi.nlm.nih.gov/blast/blast.cgi) for comparison with sequences available in the GenBank databases.

Table 2. Oligonucleotides primers, length and function used for PCR and sequencing

Primers	Sequence	Technique	Sense	Source
16SPO	5'-AAGAGTTTGATCCTGGCTCAG-3'	PCR	Forward	[14]
6SP6	5'-CTACGGCTACCTTGTTACGA-3'	PCR	Reverse	[14]
ITS R	*5'-GTCCTTCATCGGCTBYTA-3'	sequencing	Reverse	-
ITS F	5'-TACACACCGCCCGTCAC-3'	sequencing	Forward	-
F1	*5'-CTGGCTCAGGAYGAACG-3'	sequencing	Forward	Sigma-Proligo
F2	*5'-GAGGCAGCAGTRGGGAAT-3'	sequencing	Forward	Sigma-Proligo
F3	*5'-ACACCARTGGCGAAGGC-3'	sequencing	Forward	Sigma-Proligo
F4	*5'-GCACAAGCGGYGGAGCAT-3'	sequencing	Forward	Sigma-Proligo
R1	5'-CTGCTGGCACGTAGTTAG-3'	sequencing	Reverse	Sigma-Proligo
R2	*5'-AATCCTGTTYGCTMCCCA-3'	sequencing	Reverse	Sigma-Proligo
R3	5'-CCAACATCTCACGACACG-3'	sequencing	Reverse	Sigma-Proligo
R4	*5'-TGTGTAGCCCWGGTCRTAAG-3'	sequencing	Reverse	Sigma-Proligo**

Note: ***** Degenerated sequences.
** Synthetic primers used for sequencing were deduced from alignment of 16S rDNA genes coding for bacterial RNA collected from EMBL (European Molecular Biology Laboratory) databases and have been supplied by Sigma-Proligo.

2.4. Effect of temperature, pH and sodium chloride on growth

The ability to grow at 10, 15, 37, 44, and 55°C in MRS for *Leuconostoc* strains and in M17 broth for *Lactococcus* strains was evaluated. Growth was also analyzed at pH 3, 4, 7, and 9. Media were adjusted to the desired pH using NaOH (3 N) or HCl (3 N). Salt tolerance was determined by culturing each isolate in MRS broth containing 2%, 4% or 6.5% NaCl. At the end of the incubation time, changes in the color and turbidity of cultures were visually recorded as a simple indication of growth. Each treatment was repeated in 3 separate experiments.

2.5. Acidification of milk

To prepare the pre-culture, 150 ml of sterile MRS broth was inoculated with each strain thawed from the stock culture, followed by streaking onto solid MRS. Fermented milk was prepared by inoculating 150 ml of Joyvalle UHT milk with the overnight (about 16 h) MRS broth pre-culture to a final concentration of 2% (v/v) in a 250 ml flask. The pH of the milk was determined every 4 h and the acidity was evaluated by titrating a 10 ml sample with NaOH (1/9N) using phenolphthalein as an indicator. This experiment was done in triplicate.

2.6. Fermentation with mixed strains and evaluation of storage stability

Flasks containing 150 ml of sterile Joyvalle milk were inoculated to 2% (v/v) with a mixture of overnight MRS broth cultures of strains that had been cultured separately and then mixed prior to inoculation. Milk was inoculated with two strains from the dominant Sample N groups and a single strain of flavoring bacteria. After coagulation, the fermented products were tasted to determine which microbial mixture produced a flavor similar to *kivuguto*. A total of four strains were used for the Sample S in the same manner. We also examined the effect of mixing two strains from Sample N milk with a single strain from Sample S milk. Milk formulations that tasted similar to *kivuguto* prepared using mixed strains were stored at 4°C for 24 days to examine their stability. The development of organoleptic defects, especially bitterness, was evaluated by tasting casually the milk on days 1, 8, 16 and 24. No trained taste panel or special taste analyses were employed for this experiment at this stage. Taste was evaluated by two individuals who were very familiar with the characteristics of *kivuguto*. A sensorial evaluation will follow the technological analyses in the next phase of this work. Two reference samples were used in the stability experiment: plain yogurt and Nyanza fermented milk made by the Nyanza Dairy in Rwanda.

2.7. Species interactions

To assess interactions between species during fermentation, milk samples were inoculated with three strains isolated from Sample N either individually or in co-culture. Each strain was pre-cultured overnight in MRS broth from colonies taken in cultures grown on MRS agar. In

order to standardize the inocula, 50 ml of each pre-culture was centrifuged for 20 min at 7333 x *g*. The pellet was prepared to adjust cultures to the same concentration and resuspended in 50 mL of milk containing 10% glycerol and stored at -20°C for later use in initiating fermentation. Milk samples (150 ml of Joyvalle milk) were inoculated with the pre-cultures to a final concentration of 2% (v/v). The pH was recorded at the start (T_0) and the end (T_1) of fermentation. Lactic acid bacteria (LAB) populations were differentiated and enumerated at T_0 and T_1 by plating an aliquot of each milk sample onto tomato juice agar, Rogosa agar, and Mayeux agar with appropriate dilutions. Plates were incubated as described in **Table 1**.

3. RESULTS

3.1. Isolation and enumeration of microorganisms

The pH and coagulation time of various milk samples were evaluated during preliminary analyses. The average pH of raw milk samples tested was 6.5-6.6, while the average pH of fermented milk was about 4.5. A total of 390 isolates were obtained from the milk samples. The isolates were purified on MRS agar with $CaCO_3$ and then stored in glycerol at -80°C. The enumeration showed that dominant strains grew on selective media such as tomato juice agar (3.57×10^7 cfu ml^{-1}), Rogosa (4.27×10^7 cfu ml^{-1}), Rogosa-raffinose (0.40×10^7 cfu ml^{-1}), and MRS (0.40×10^7 cfu ml^{-1}). Strains isolated on Mayeux agar (0.11×10^7 cfu ml^{-1}) were pre-selected for the flavor characteristics of *kivuguto* even if they were in low concentration.

3.2. Phenotypic and molecular characterization

In the first and the second samplings, all isolates were found to be *cocci*, Gram-positive, catalase-negative, and were therefore presumed to be LAB. Yeasts were present only at very low levels in all samples. About five similar dominant strain colonies were picked from each medium and subjected to API 50® CH testing. Colonies producing identical profiles were considered to be isolates of the same strain. However, a given strain could produce several profiles, differing only with respect to fermentation of 1 or 2 carbohydrates. API 50® CH testing of the microorganisms isolated during the first sampling (february) produced 7 profiles for Sample N and 5 profiles for Sample S. The profiles produced by the Sample N isolates

resulted in the identification of *Lactococcus lactis* ssp. *lactis* 1 (95.4%), *Lactobacillus helveticus* (68.1%) and *Leuconostoc mesenteroides* ssp. *mesenteroides/dextranicum* 2 (65.6%). The profiles produced by the Sample S isolates resulted in the identification of *Leuconostoc mesenteroides* ssp. *mesenteroides/dextranicum* 2 (99.7%), *Leuconostoc mesenteroides* subsp. *mesenteroides/dextranicum* 1 (95.7%) and *Leuconostoc lactis* (52.9%). A total of 7 profiles for Sample N and 5 profiles for Sample S were also obtained upon API 50® CH testing of microorganisms isolated during the second sampling (september). Analysis of Sample N resulted in identification of *Leuconostoc mesenteroides* subsp. *mesenteroides/ dextranicum* 2 (55.4%), *Leuconostoc mesenteroides* subsp. *mesenteroides/dextranicum* 1 (99.9%; 69.4%; 52.5%) and *Lactobacillus paracasei* subsp. *paracasei* 1 (92.4%). In Sample S, *Leuconostoc mesenteroides* subsp. *mesenteroides/dextranicum* 2 (99.9%; 90.3%; 85.8%) and *Lactococcus lactis* subsp. *lactis* 1 (87.4%) were identified. To confirm our phenotypic identifications, the 16S rDNA gene and/or 16S-23S rDNA ITS of pre-selected strains were sequenced using PCR **(Table 3)**. Sequencing indicated that the 16S rDNA of strain CWBI-B1466 had a high degree of identity (99%) with that of *Lactococcus lactis* subsp. *lactis* AB100805.1 (ATCC 13675). The strain was therefore identified as *Lactococcus lactis* and registered in GenBank under accession number JF313446. Strain CWBI-B1469 was assigned as *Lactococcus lactis* subsp. *lactis* AB008215.1 (97% identity) and the rDNA sequence was registered in GenBank under accession number JF313446. Strain CWBI-1467 was assigned as *Leuconostoc mesenteroides* subsp. *mesenteroides* CP000414.1 (97% identity) and was registered in GenBank under accession number JF313452. Strain CWBI-B1468 was 98% similar to strain *Leuconostoc pseudomesenteroides* AB290443.1. The accession number is JF313453. Strain CWBI-B1470 closely resembled to *Leuconostoc pseudomesenteroides* AB290443.1 (98% identity) and was registered in GenBank under accession number JF313454, while strain CWBI-B1471 was found to be comparable to *Leuconostoc pseudomesenteroides* AB290443.1 (99% identity) and was registered in GenBank under accession number JF313455. Finally, strain CWBI-B1465 may be *Leuconostoc mesenteroides* subsp. *mesenteroides* (GenBank accession number JF313445).

3.3. Effect of temperature, pH and sodium chloride on growth

The strains isolated from *kivuguto* were generally resistant to extreme environmental

conditions (**Table 4**). All isolates grew in broth containing 4% NaCl. *Lactococcus* strains could not grow at 45°C. The viability of *Leuconostoc mesenteroides* strain CWBI-B1465 at 55°C was determined after analysis of its growth at 44°C and the residual viable count was determined at pH 2.5 and pH 3.0. The viable count was determined by dilution and plate counting on MRS agar before and after 4 h incubation in MRS broth under one of the specified conditions (55°C, pH 2.5, pH 3.0). The survival rate at 55°C and pH 2.5, 3.0, and 4.0 were 38.63% 25%, 61.76% and 97.36%, respectively.

3.4. Acidification, milk formulation and storage stability

Strains used for acidification of milk were taken from dominant groups belonging to different profiles. In the first pre-selection, we identified those strains that produced the most rapid acidification. Two *Lactococcus* strains isolated from Sample N were rapid acidifiers (8 hours), as was a *Lactococcus* strain isolated from Sample S (9 hours). Two *Leuconostoc* spp. isolated from sample N were not fermentative when cultured alone in milk. Two *Leuconostoc* spp. isolated from Sample S coagulated milk in 14 hours, while 2 others acidified milk in 16 and 20 hours. Milk was fermented with mixed strains and then tasted casually to determine the mixture of organisms that best reproduced the taste of authentic *kivuguto*. The fermented formulations were then stored at 4°C for up to 24 days to test stability. In that preliminary study, we obtained milk formulations that were fermented in only 8 hours by mixed strains from Sample N and one of the formulations presented a bitter taste after 15 days of storage. Based on these data, the strain of *Lactococcus* that was used was eliminated for further investigations. One association of organisms isolated from Sample S acidified milk in 9 hours. Another formulation failed the test of shelf-life, because one of the *Leuconostoc* sp. involved was not viable in acidic milk. When used alone, this species coagulated the milk in 14 hours. But such milk can't coagulate another raw milk. Another strain was also discarded because the milk formulation exhibited changes in flavor after only 1 week. Two strains from Sample N in association with the best acidifier identified from among the *Leuconostoc* spp. isolated from Sample S made a very tasty *kivuguto*, with no organoleptic defects detected up to 35 days. These results were reproducible in 3 replicated experiments.

Table 3. API50 CHL Test vs molecular characterization of bacterial species selected in *kivuguto* milk

Strains	API 50CHL identification			16S rDNA Sequencing			16S-23S rDNA ITS Sequencing		
	Similar Species	% of identity	Sequence length (bp)	Accession Number	Similar species*	% of identity	Similar species	Accession Number	% of identity
CWBI-B1465	*Ln. mesenteroides subsp. mesenteroides/dextranicum2*	65.6%	1436	AB362705.1	*Ln. mesenteroides subsp. mesenteroides*	98%	-	-	-
CWBI-B1466	*Lactococcus lactis subsp. lactis1*	95.4%	1512	AB100805.1	*Lactococcus lactis*	99%	-	-	-
CWBI-B1467	*Lactobacillus helveticus*	68.1%	578	CP000414.1	*Ln. mesenteroides subsp. mesenteroides*	98%	*Ln . mesenteroides subsp. mesenteroides*	CP000414.1	97%
CWBI-B1468	*Ln. mesenteroides subsp. mesenteroides/dextranicum2*	91.9%	1422	AB326299.1	*Leuconostoc spp.*	99%	*Leuconostoc pseudomesenteroides*	AB290443.1	98%
CWBI-B1469	*Lactococcus lactis subsp. lactis1*	87.4 %	1550	AB008215.1	*Lactcoccus lactis*	97%	-	-	-
CWBI-B1470	*Ln.mesenteroides subsp. mesenteroides/dextranicum2*	99.9%	1489	AB326299.1	*Leuconostoc spp.*	99%	*Leuconostoc pseudomesenteroides*	AB290443.1	98%
CWBI-B1471	*Ln. mesenteroides subsp. mesenteroides/dextranicum2*	91.9%	1483	AB326299.1	*Leuconostoc spp.*	99%	*Leuconostoc pseudomesenteroides*	AB290443.1	99%

Ln.: Leuconostoc;* **AB100805.1 *Lactococcus lactis subsp.lactis*, Strain: ATCC 13675 ; **CP000414.1** *Leuconostoc mesenteroides subsp.mesenteroides*, Strain: ATCC 8293 complete genome; **AB290443.1** *Leuconostoc pseudomesenteroides*, 16S-23S rRNA intergenic spacer region, Strain: NRIC 1777.

Table 4. Morphological, physiological and biochemical characteristics of *kivuguto* strains

Characteristics	CWBI-B1466	CWBI-B1469	CWBI-B1465	CWBI-B1467	CWBI-B1468	CWBI-B1470	CWBI-B1471
Cells morphology	cocci	cocci	cocci	cocci	cocci	cocci	cocci
Gram staining	+	+	+	+	+	+	+
Catalase reaction	-	-	-	-	-	-	-
Growth in broth at:							
pH 3.0	+	+	+	-	+	-	-
pH 4.0	+	+	+	+	+	+	+
pH 7.0	+	+	+	+	+	+	+
pH 9.0	+	+	-	+	-	-	-
Survival rate at pH 2.5 for 4 h	0	0	25	ND	0	ND	ND
Survival rate at pH 3.0 for 4 h	[1]ND	ND	61.76	ND	0	ND	ND
Survival rate at pH 4.0 for 4 h	ND	ND	97.36	ND	77.61	ND	ND
Growth in broth at:							
10°C	ND	ND	+	+	+	+	+
15°C	+	+	+	+	+	+	+
37°C	+	+	+	+	+	+	+
44°C	-	-	+	-	-	-	-
Survival rate at 55°C	ND	ND	38.63	ND	ND	ND	ND
Growth in broth with:							
2.0% NaCl	+	+	+	+	+	+	+
4.0% NaCl	+	+	+	+	+	+	+
6.5% NaCl	ND	ND	+	-	-	-	-
Dextran production	ND	ND	+	-	+	+	+

[1]Not determined

3.5. Species interactions

Due to the presence of non-fermentative dominant species in Sample N, an examination of their interactions with the CWBI-B1466 *Lactococcus* strain was conducted to highlight their high biomass in the fermented milk. This examination revealed that the 2 non-fermentative *Leuconostoc mesenteroides* do not grow in milk when cultured singly. Instead, a high growth rate was observed for *Leuconostoc mesenteroides* strain CWBI-B1465 in co-culture. A similar result was obtained for *Leuconostoc mesenteroides* strain CWBI-B1467. A positive interaction thus occurs between *Lactococcus* and *Leuconostoc* species (detailed results not shown).

4. DISCUSSION

Raw milk typically has a pH of 6.6. Mathara *et al.* [15] found that the average pH of the Kenyan fermented milk product *kule naoto* is 4.4 (range 4.17-5.19). Only four *kule naoto*samples examined had a pH exceeding 4.5. Abdelgadir *et al.* [16] tested the pH of two

samples of *rob*, a Sudanese fermented milk, and found values of 3.87 and 4.02 when the pH was tested immediately upon sampling. The pH of *kivuguto* falls within the normal acidic pH range for fermented milk. People living in this region therefore typically consume acidic milk, and those who consume *rob* are consuming an even lower pH milk. For the purpose of this work, we put more emphasis on the dominant strains because the quality of a fermented milk product is judged by its flavor, appearance and consistency [17]. Nevertheless, the main technological property of strains fermenting milk is the acidifying activity, but also the growth in milk and survival in acidic milk [18]. And strains sustaining these properties are major strains at the end of the fermentation. Thus, the strains developed on tomato juice were acidifiers and represented by *Lactococcus lactis.* Mayeux agar and other media were used to isolate strains imparting both flavor and consistency, such as *Leuconostoc* spp., which produce aromatic compounds and dextran. The enumeration and the phenotypic characterization experiments demonstrated that the *kivuguto* microflora is composed primarily of *Lactococcus* and *Leuconostoc* species. The group of bacteria isolated from Rogosa agar was presumed to be composed of lactobacilli. Indeed, 3 strains were assigned as *Lactobacillus helveticus* by the API software, but at a low percentage (61.1%). It is known that leuconostocs grow on Rogosa agar, unlike *lactococci* [19]. Phase contrast microscopy indicated that these bacteria were not rods, and therefore could be *Leuconostoc* spp. It is typically quite difficult to differentiate heterofermentative lactobacilli and leuconostocs due to difficulty to distinguish bacilli to cocci [20]. Many authors have confirmed the difficulty of identifying *Leuconostoc* by phenotypic means due to heterogeneity in the biochemical and physiological characteristics of these organisms [21, 22]. Furthemore, some *kivuguto* strains exhibited unusual tolerance to high temperature and very low acidic media. McDonald *et al*. [23] showed that growth of *Leuconostoc mesenteroides* stops when the internal pH reaches a value of 5.4 to 5.7. This is a very useful characteristic in dairy technology, because the ability to grow at high temperature is a desirable trait as it could translate into an increase in the rates of growth and lactic acid production [24]. Such a strain could be preserved by freeze- or spray-drying. After isolation, enumeration and identification by phenotypic (morphology and API tests) techniques, molecular methods confirmed the identification for six pre-selected strains. The study was followed by tests of acidification because the primary function of dairy starter cultures is rapid acidification [25]. Following the acidification tests, the shelf-life of the fermented milk formulations was examined in order to prevent proteolysis and lipolysis, since some LAB produce peptidases that produce bitter-tasting peptides that detrimentally impact the organoleptic quality of milk [26]. Due to issues regarding strain compatibility, the first

starter formulations were composed of strains from the same sample. The choice of strains used was based on resistance to extreme environmental conditions and the functional role of the strain in the fermentation process. Using such an approach, complex and costly analyses were avoided. This is in agreement with a number of findings, suggesting that these species could be used as starters or starter adjuncts [27, 28]. Ultimately, selected strains showed that no yogurt strain enters in the *kivuguto* selected strains. The CWBI-B1466 *Lactococcus lactis* is found in *leben* of Morocco, in *zivda* of Israel, in Indian *dahi*, in Zimbabwean *amasi*, in sudan *rob* and in other types of fermented milk worldwide. With *Leuconostoc* sp., it composes the starter of *filmjölk* of nordic countries, which is the most similar both in consistence and taste. But the selected strains confer to *kivuguto* its sole biochemical identity.

5. CONCLUSION

In the present study, we isolated and identified the microorganisms that produce the fermented milk *kivuguto*. Phenotypic and molecular characterisation studies enabled a screening of seven strains used to formulate three *kivuguto* starters, and we found that based on fermentation time, organoleptic aspects, and shelf-life at 4°C, the best *kivuguto* was produced by a mixture of *Lactococcus lactis* CWBI-B1466, *Leuconostoc mesenteroides* CWBI-B1465, and *Leuconostoc pseudomesenteroides* CWBI-B1470. Using this formulation, milk fermented in 8 hours at 30°C, and no organoleptic defects were detected after 35 days of storage. Of particular interest to the dairy industry, most of the *Leuconostoc spp*. isolated ferment milk and produce dextran, and one of these isolates were particularly resistant to extreme environmental conditions, a characteristic that would be beneficial in terms of the cost of its production. Since the three strains in the present study are technologically interesting bacteria, development of a standardised processing method coupled to a sensorial analysis will ensure the production of a highest microbial and nutritional quality *kivuguto*.

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Chapter 4

Technological properties of *kivuguto* milk

Le chapitre 4 *se consacre à l'analyse expérimentale des principales propriétés technologiques sous-tendant tous les aspects de qualité d'un lait fermenté, en appui aux propriétés partielles traitées dans le chapitre précédent. Quatre propriétés ont été analysées: l'acidification, les propriétés rhéologiques, les arômes et le degré de protéolyse. Et en complément de ces analyses, une analyse sensorielle de validation a été réalisée.*

L'évaluation de l'acidification du kivuguto formulé a été faite par l'analyse du pH, de l'acidité titrable et de la vitesse d'acidification; l'analyse rhéologique a mis en évidence les propriétés visco-élastiques du lait formulé; les produits volatils du lait kivuguto ont été estimés au moyen de l'analyse par espace de tête statique (HS) utilisé en chromatographie en phase gazeuse (CPG) couplée à la spectrométrie de masse (SM) et la protéolyse a été mesurée par la méthode de Church et al. (1983) dont le principe est la réaction de l'OPA (O-phthaldialdéhyde) et du β-mercaptoéthanol. Au cours de cette réaction, les groupements α-aminés sont relâchés lors de l'hydrolyse des protéines du lait formant ainsi un complexe qui absorbe fortement à 340 nm. Pour compléter ces analyses, il fallait évaluer la ressemblance du lait formulé au lait traditionnel kivuguto. Un panel non entraîné de 8 personnes a été constitué par un groupe de consommateurs habituels du kivuguto et des laits fermentés se trouvant sur le marché local pour estimer par une analyse sensorielle le degré de similitude. Pour ce faire, le test triangulaire modifié a été adopté comme test de discrimination. Le niveau de similitude a été évalué à 87.5% pour une première série et de 75.0 % pour une deuxième série du test.

*Les résultats de cette étude ont été rassemblés dans un article "**Technological features of kivuguto selected strains during milk fermentation**" soumis pour publication et constituent le chapitre 4 de ce travail.*

Technological features of *kivuguto* selected strains during milk fermentation

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ABSTRACT

Selected strains in *kivuguto* artisanal milk were used for an investigation of technological properties for a further starter culture development. Acidification, proteolysis, flavor profile, rheology and sensory analyses of fermented milks were followed as the most indicators of the starter culture formulation. The acidification showed that the *kivuguto* milk ferments in 14 hours at 19°C with a titratable acidity of 73°D. The samples of CWBI-B1466 *Lactococcus lactis* and CWBI-B1470 *Leuconostoc pseudomesenteroides* reaches the fermentation time at 14 h and 20 h respectively. All samples were viscoelastic fluids and the most important flavor compounds found were two alcohols, one ester and two furans derivatives compounds. The proteolysis revealed low values ranging to 3.04-5.45 mg/L leucine equivalent which is very interesting in terms of taste acceptability. The three strains showed positive technological properties for *kivuguto* starter culture development and the data obtained are fully in agreement with the first results in partial technological analyses. Ultimately, the findings are regarded with reference to the traditional *kivuguto* as recognized by the panel in discrimination test for the formulation of *kivuguto* milk.

Key words: Technological features, *kivuguto,* milk fermentation, acidification, rheology, proteolysis, aroma compounds, sensory analysis.

1. INTRODUCTION

Fermented milks are manufactured throughout the world and approximately 400 generic names are applied to traditional and industrialized products (Kurmann et al., 1992). To reach the industrial scale, it needs the selection of starters cultures involved in fermentation process. Thus, a long study where biochemical and technological properties are well understood may reach to the starter culture development. In milk fermentation, the major biochemical reactions are the metabolism of residual lactose, lactate and citrate; liberation of free fatty acids (lipolysis), associated catabolic reactions and the proteolysis of the milk proteins (mainly caseins) in peptides and free amino acids and the catabolism of free amino acids (McSweeney and Sousa, 2000). Acidification is an important technological property in milk fermentation. It is performed by microbial starters used for that purpose and must stay in acceptable concentration during storage for quality stability. Fermentative starters reduce lactose to lactate and the pH decreases leading to several physicochemical changes. From pH 6.6 to 4.6, the caseins micelles through proteolysis broke down in smaller peptides, changing the texture of milk by coagulation. Some starter cultures also produce exopolysaccharides which increase the viscosity and texture and decrease susceptibility to syneresis (Zourari et al., 1992). This phenomenon leads to the gelation reached at pH4.6. In dairy technology, the knowledge of rheological properties is very necessary in order to characterize the fermented milk quality in terms of texture. In practice, the change in rheological properties affects the acceptance of the product by the consumer (Debon et al., 2010; Mohameed et al., 2004) positively or negatively. Moreover, the rheological properties influence the design and the sizing of the process for shear stress control (Debon et al., 2010; Chibane, 2008; Maskan and Gogus, 2000). More specifically, they influence the engineering calculations of the process, such as the flow, selection of pumps, determination of load loss in the tubes (Debon et al., 2010; Chibane, 2008; Steffe, 1996; Holdsworth, 1993), as well as during heat exchanges. The acidification also generates flavor compounds and this process is strain dependent. Aroma perception is one of the foremost criteria for the evaluation of fermented milk because of its influence on consumer acceptance (Alonso and Fraga, 2001). According to Thomas (1981), consumer acceptance and preference for milk as a beverage is influenced by its flavor more than any other attribute. The typical flavor of fermented milks derives from lactic acid and any carbonyl compounds are produced by starter cultures. Lactic acid is responsible for the sharp, refreshing taste of these products (Nursten, 1997). Other source of flavor compounds is

the thermal degradation of lipids, lactose and proteins during the heat treatment of milk before the fermented milk manufacture e.g. aldehydes, ketones, alcohols, lactones, sulfur compounds (Tamime and Deeth, 1980). The *kivuguto* milk is manufactured by spontaneous fermentation or by backslopping in Rwanda. To date, no investigations were conducted on the manufacturing techniques, neither on the physicochemical characteristics, nor on the organoleptic features, nor on any other technological parameter. In a previous study (Karenzi, 2012), three strains were selected for *kivuguto* starter formulation: CWBI-B1466 *Lactococcus lactis* registered under Genbank accession number JF313446, CWBI-B1465 *Leuconostoc mesenteroides subsp. mesenteroides*, accession number JF313445 and CWBI-B1470 *Leuconostoc pseudomesenteroides*, accession number JF313454. The present paper deals with the main technological properties to characterize the *kivuguto* milk using these strains. Additionally, the sensory analysis is performed by untrained panel as a validation test of similarities between the *kivuguto* produced by selected starter bacteria and the traditional one.

2. MATERIAL & METHODS

2.1. Bacterial strains and milk fermentations

The selected microorganisms fermenting *kivuguto* are preserved at -80°C in CWBI Collection and registered in Genbank databases. The three strains are: CWBI-B1466 *Lactococcus lactis* registered under Genbank accession number JF313446, CWBI-B1465 *Leuconostoc mesenteroides subsp. mesenteroides*, accession number JF313445 and CWBI-B1470 *Leuconostoc pseudomesenteroides*, accession number JF313454. Prior to this experiment, assays were made with many fractions of each strain to find fermented milk similar to *kivuguto*, and one formulation was retained for further analyses. Attention was paid to the enumeration data obtained during the selection, as a reference proportion. The incubation temperature was fixed to 19°C. The flasks were then incubated until the pH of ≈4.5 was reached and thereafter stored at 4°C before analyses. The preparation of samples was made on four milks:

1° M1: whole milk fermented with the mixed *kivuguto* starters composed of three selected strains CWBI-B1466 *Lactococcus lactis*, CWBI-B1465 *Leuconostoc mesenteroides* and CWBI-B1470 *Leuconostoc pseudomesenteroides* at a ratio of 40%, 35% and 25% respectively. The pre-culture was inoculated at 10^6 cells.g^{-1} as initial concentration. This inoculum was used to culture at 4% (vol/vol) two liters of UHT milk in a 5 liters flask.

2° M2: whole milk fermented with the strain CWBI-B1466 *Lactococcus lactis* at 10^6 cells.g^{-1} as initial concentration for pre-culture and used as inoculum at 4% (vol/vol) two liters of UHT milk in a 5 liters flask.

3° M3: whole milk fermented with the strain CWBI-B1470 *Leuconostoc pseudomesenteroides* at 10^6 cells.g^{-1} as initial concentration for pre-culture and used as inoculum at 4% (vol/vol) two liters of UHT milk in a 5 liters flask.

4° M4: UHT milk used for fermentation. Tests were carried out without inoculation.

2.2. Acidification and enumeration

Milk cultures of each sample were inoculated at ambient temperature and the pH (pHmeter WTW pH351i, Weilheim, Germany) and titratable acidity (°D) were measured after incubation time by titrating a 10 mL sample with NaOH (1/9N) using phenolphthalein as an indicator. The titratable acidity was measured in Dornic degrees (°D); in such conditions, 1°D is equivalent to 0.1 mL of NaOH, i.e 0.1 g lactic acid per kg of milk. For biomass count, suitable dilutions were made and plated on MRS agar medium. Results are the average of three independent measurements.

2.3. Rheological properties

The rheology characterization of the *kivuguto* milk by description of its viscoelastic behavior was performed using an oscillatory model rheometer. The rheological parameters storage modulus (G') and loss modulus (G'') were followed as a function of time at 10°C using a high resolution Bohlin CVO 120 rotational rheometer (Malvern Instruments, Worcestershire, UK). The measuring geometry employed was a rotating upper cone and a fixed lower plate (α=4°, ϕ=40 mm). The oscillation frequency was 1.0 Hz and the shear stress was 1Pa which was found to be within the linear viscoelastic region of fermented milk samples according to Stern et al. (2008). Three replicates were measured.

2.4. Proteolysis

The peptides/free amino acids (FAAs) accumulated in milk after the incubation time as a consequence of the proteolytic activity of the tested strains were determined using the o-phthaldialdehyde (OPA) method (Church et al., 1983). This method is based on the reaction

of OPA and 2-mercaptoethanol with the α-amino groups released during hydrolysis of milk proteins. They form then a complex which absorbs strongly at 340 nm. The OPA solution is obtained by combining the following reagents and completing a volume of 50 mL with water: 25 ml of 100 mM sodium tetraborate (Sigma Aldrich, Diegem, Belgium), 2.5 mL of 20% SDS (Merck, Darmstadt, Germany), 40 mg OPA (Sigma Aldrich, Diegem, Belgium) dissolved in 1 mL of methanol and 100 µL of 2-mercaptoethanol (Sigma Aldrich, Diegem, Belgium). This reagent should be prepared the same day. To measure the proteolytic activity, with milk protein as a substrate, a 150 µL aliquot (of a 1:25 dilution of milk in 1% SDS) was added to 3.0 ml of OPA reagent. The solution is stirred by inversion and incubated for 2 minutes at room temperature and the absorbance is measured at 340 nm using a Genesys 10S UV-VIS spectrophotometer (Thermo Fisher Scientific, Madison, WI, USA). The results in triplicate were calculated from a standard curve obtained from dilution of leucine in distilled water and expressed in leucine equivalent ($mg.L^{-1}$) (Sigma Aldrich, Diegem, Belgium) of milk.

2.5. Aroma compounds

Headspace sample preparation. Headspace (HS) samples were prepared manually. 10 g of milk sample were introduced in a 20-mL HS vial (Filter Service, Eupen, Belgium) sealed hermetically with a polytetrafluoroethylene-coated rubber septum and an aluminum cap (Filter Service, Eupen, Belgium). The samples were kept at 4°C after fermentation time before analysis for a short time. Unless, samples were kept at -20°C and put at 4°C the day before analysis. Samples were equilibrated for 65 min at 70°C prior to analysis and the volatile compounds trapped in the headspace region of the vial (2000 µL) were taken with a micro syringe (Filter Service, Eupen, Belgium) and analyzed by GC using direct gas injection.

Gas chromatography. Milk samples volatiles (2000 µL) were injected into an Agilent Technologies 7890A GC System (Agilent Technologies, Santa Clara, CA, USA) equipped with a flame ionization detector (FID) and a 30 m x 250 µm x 0.25 µm VF-WAX polar column (Agilent Technologies, Santa Clara, CA, USA) was used for the study. Helium was used as carrier gas, at a flow rate of 1.5 ml. min^{-1} and the splitless mode was used. The following temperature program was used: 50°C for 6 min, increased to 180°C for 5min at a rate of $8°C.min^{-1}$ and held 10 min at $15°C.min^{-1}$ from 180 to 250°C. The injector and detector temperatures were 220 and 250°C respectively.

Mass spectrometry analysis. The volatile compounds were identified by mass spectrometry using an Agilent Technologies 5875C with Triple-Axis Detector coupled to 6890 GC System (Agilent Technologies, Santa Clara, CA, USA). The MS was carried out in EI mode, the ionization potential 70 eV; ionization current 2 A; the ion source temperature 200°C, resolution 1000 and mass range 30 to 450 m/z.

Chemical identification. Compounds were identified by comparing recorded mass spectra with the Willey 275L mass spectra Library (Scientific Instrument Service, Ringoes, NJ, USA), the NIST MS Library (NIST, Gaithersburg, MD, USA), the PAL 600K mass spectra Library (Palisade Corporation, Ithaca, NY, USA) and those in literature, as well as comparison of their retention times with authentic standards of saturated *n*-alcanes standard solution (C_7-C_{30} alkanes) (Sigma Aldrich, Diegem, Belgium) as external references under the same chromatographic conditions, allowing calculation of Kovats index (Kovats, 1958) of the separated volatile compounds (Harris, 1987).

Standard solutions and quantification. Aqueous solutions of acetic acid, pentan-1-ol, methyl benzoate as an external standards were prepared from high purity chemicals (>99%) purchased from Sigma-Aldrich (Diegem, Belgium). 40 µL of each standard was accurately weighed and diluted in 100 mL in double distilled water and thereafter mixed at a ratio of 1:1. The prepared solution was hermetically sealed in 20 mL headspace vials at -20°C until they were used. Quantification of compounds was calculated by external standard technique. Mean results of three assays obtained were used to calculate the response factors corresponding to mean peak area for each standard compound and the amount of each compound in sample was calculated according to known amount standard and its peak area. Moreover, response factors of pentan-1-ol and methyl benzoate were used to calculate the concentration of 3-methylbutan-1-ol, furanmethan-2-ol and 1,7,7-trimethylbicyclo[2.2.1] hept-2yl acetate, furan-2(5H)-one respectively.

2.6. Sensory analysis

A modified triangle test allowed 8 untrained panelists but knowing very well the *kivuguto* milk to differentiate between samples the most similar to *kivuguto* according to De Lacharlerie et al. (2008) in a discriminative test. The test was sought to determine between two products A and B which was similar to *kivuguto*, one of the two being *kivuguto*. Samples

were presented in three-digit coded cups with two samples per set to each subject. The principle is that subjects receive three samples. Two are the same product (A or B), and the third is different. Therefore, there are two options of three samples: AAB or ABB. A randomised presentation order uses the six possible combinations of the triplet on each plate: AAB, ABA, BAA, BAB, BBA, ABB. Two sets were consecutively evaluated by the panelists and the first set was *kivuguto* (M1) *versus* a reference milk sold on the rwandese market, whereas the second set was *kivuguto* (M1) *versus* yogurt, also sold on the rwandese market. The UHT milk used to ferment the milk come also from Rwanda. Results were calculated using the table of critical values for triangle test for differences, table built on the binomial distribution with EXCEL IV for the estimation of statistic significance in sensory evaluation.

3. RESULTS & DISCUSSION

3.1. Cultures and fermentations

Fermentations assays were experienced with single or mixed *kivuguto* starters to ferment raw sterile milk. Both the mixed or single cultures start with initial inoculation of 10^6 freeze-dried cells in a pre-culture made in 250 mL flask. The best mixture was composed of 40% of *Lactococcus lactis*, 35% of *Leuconostoc mesenteroides subsp. mesenteroides* and 25% of *Leuconostoc pseudomesenteroides*. Single and/or mixed fermentations were carried out in two liters (4% vol/vol) of UHT milk. Acidification data obtained are presented in **table 1**.

Table 1. Acidification data of milk fermented by *kivuguto* selected strains at 19°C.

Culture	$\mathbf{T}_L$ (h)	$\mathbf{T}_f$ (h)	$\mathbf{V}_m$ ($g.L^{-1}.h^{-1}$)	$\mathbf{pH}_f$	$\mathbf{A}_f$ (°D)
kivuguto	4	14	0.26	4.5-4.6	73±4
Lactococcus lactis	4	14	0.25	4.5-4.6	72±5
Leuconostoc pseudomesenteroides	7	20	0.17	4.5-4.6	70±4

h=hour; T_L: lag phase; T_f: fermentation time at pH_f=4.5, time required for the pH to reach 4.5; V_m= maximum acidification velocity at pH_f in g of lactic acid per liter per hour; $\mathbf{A}_f$ =titratable acidity in °Dornic (°D) at pH_f

The characterization of the acidification activity used three parameters: the pH, the maximum acidification velocity and the titratable acidity (°D). The acidification activity is determined by the cell's capacity to reduce the pH of milk after inoculation. It is an indication of the physiology and the fermentative potential of a strain. The shorter the time of acidification, the

greater the acidification activity, the best is the cell. The fermentation time in hours ended at the time when the acidification reached pH≈4.55.

The data obtained showed that single cultures ended the fermentation at 14 h and 20 h for Lactococcus and Leuconostoc respectively. For the mixed fermentation *(kivuguto),* the end fermentation time was 14 h too. The maximum velocity V_m, i.e. the lactic acid produced per liter per hour after milk inoculation is equal for *kivuguto* and the best acidifier (Lactococcus), respectively 0.26 and 0.25 g/L/h. This data is an indication of a positive interaction of Leuconostoc cells in milk with Lactococcus, as the percentage of the inoculum of Leuconostoc cells is 60% in *kivuguto,* whereas in single fermented milk of Lactococcus the inoculum is 100%. Meaning that the acidifying activity of 40% of Lactococcus in *kivuguto* is equally balanced by 60% of Leuconostocs. The V_m of *Leuconostoc pseudomesenteroides* is 0.17 g/L/h. Note that the lag phase is 4 h for *kivuguto* and Lactococcus milks, and 7 h for *Leuconostoc pseudomesenteroides* milk, meaning that Lactococcus is more active than Leuconostoc after the inoculation. Juillard et al. (1987) found the same result when culturing milk with mixed thermophile lactic acid bacteria. In addition, the lactic acid produced for mixed cultures was greater than that for single cultures (7.3 g/L for *kivuguto* and 7.2 g/L for Lactococcus milk and 7.0 g/L for Leuconostoc milk). Oliveira et al. (2001) found two folds the lactic acid produced by mixed cultures than the single cultured milks using Bifidobacteria cells. The cells count at the end of fermentation ranged to 3.3 10^9 to 4.2 10^9 cfu/mL for the three samples.

3.2. Rheological analyses

Small deformation oscillatory testing was applied for the evaluation of the *kivuguto* milk rheology. The G' and G" data evolution in time are presented in **figures 1, 2 and 3**. They show a growth up to 10 min of both G' and G". First, the experiment was applied with single strains of *kivuguto* milk, fermentative strains of CWBI-B1466 *Lactococcus lactis* and CWBI-B1470 *Leuconostoc pseudomesenteroides.* The results showed in two cases the G'>G" and never G">G', meaning that no liquid form is described by the two milks. Instead, the two milks showed an increase up to 10min of G' and G", with a high value of G' in milk of CWBI-B1470 *Leuconostoc pseudomesenteroides* than the one of CWBI-B1466 *Lactococcus lactis.* This behavior was also observed with the viscous rigidity of the two types of milk. Storage and loss moduli (G' and G" respectively) characterize well the level of solid-like (elastic) and liquid-like (viscous) character of a fermented milk.

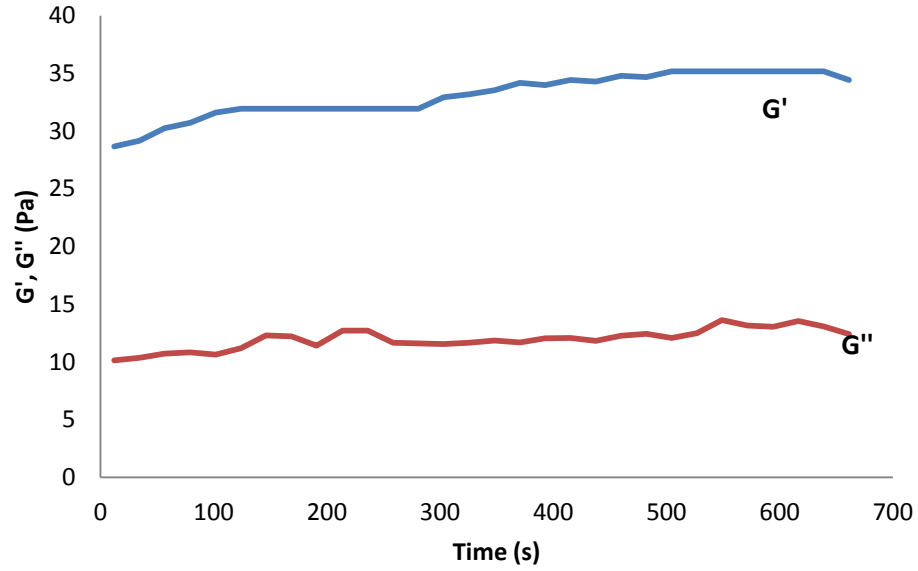

Figure 1: Elastic moduli and Loss moduli evolution of the strain CWBI-B1470 *Leuconostoc pseudomesenteroides* in fermented milk at pH 4.5

But the ratio of the two parameters tan δ=G''/G' is the best character to describe the viscoelastic property of a fermented milk. According to Kristo et al. (2003), the higher G' and lower the tan δ values, the more solid-like the character of the gel, and the firmer the gel.

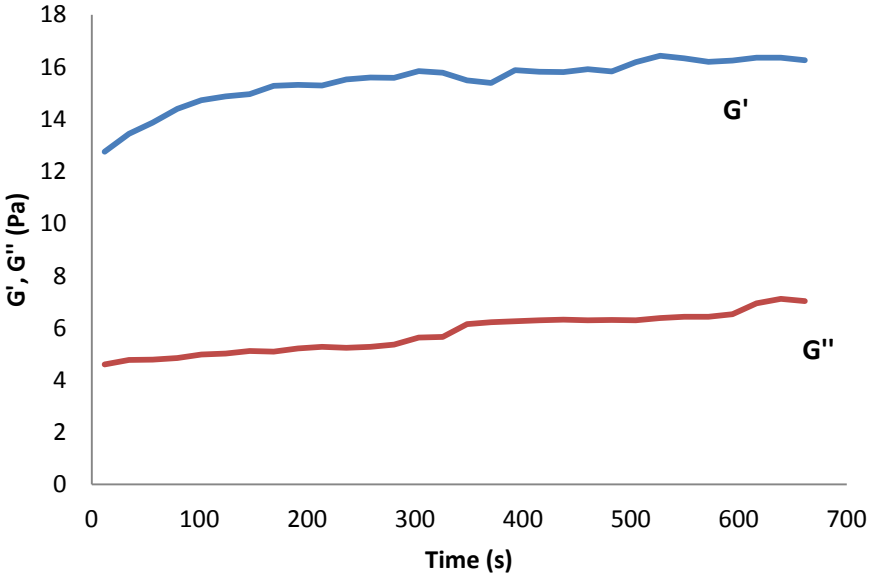

Figure 2: Elastic moduli and loss moduli evolution of the strain CWBI-B1466 *Lactococcus lactis* in fermented milk at pH 4.5

The data given by the two strains culturing alone the milk showed by calculation high value of tan δ in CWBI-B1470 *Leuconostoc pseudomesenteroides* milk than the one of CWBI-

B1466 *Lactococcus lactis* milk. Second, the experiment comprises the fermentation of a mixture of the two strains with a third nonfermentative strain CWBI-B1465 *Leuconostoc mesenteroides subsp. mesenteroides*, as the three strains characterize the *kivuguto* starter. The behavior of storage moduli and loss moduli in *kivuguto* comparatively in the two types of milk with single strains is that G'>G" and the *kivuguto* storage moduli G'<G' of CWBI-B1470 *Leuconostoc pseudomesenteroides*, but >G' of CWBI-B1466 *Lactococcus lactis* milk. This is the same for G". The data from these experimentations showed that the values of G' and G" of *kivuguto* seems slightly below to the one's of CWBI-B1470 *Leuconostoc pseudomesenteroides* and characterized the *kivugut*o as a viscoelastic milk.

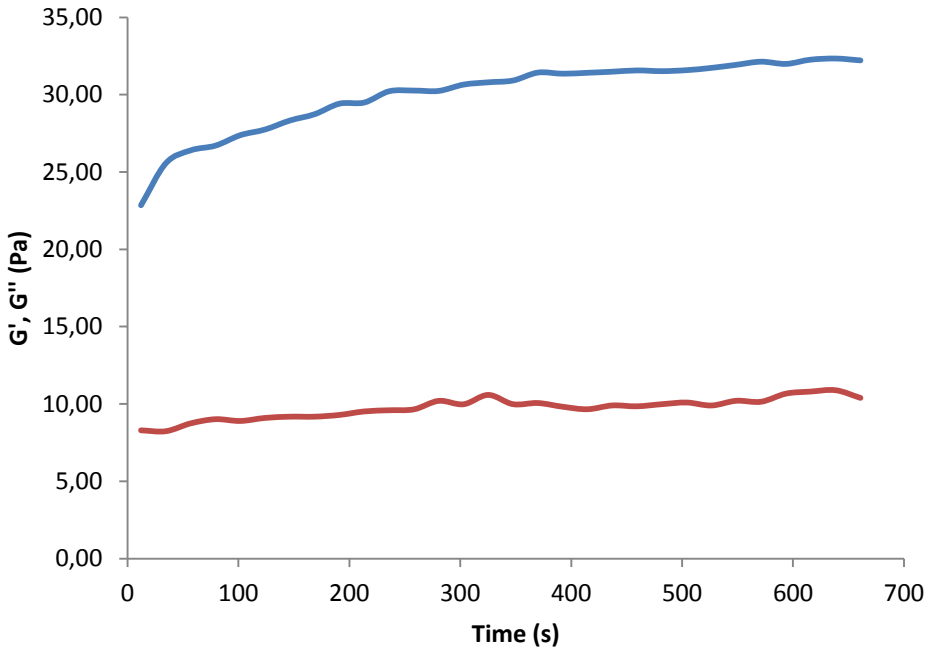

Figure 3: Elastic moduli and loss moduli evolution of *kivuguto* fermented milk at pH 4.5

It is known that the yogurt rheological properties depend essentially to the rate of total solids, to the milk composition (protein, salts), to the milk homogenization, to the type of culture, to acidity (pH), to proteolysis and to the heat treatment undergone by milk (Biliaderis et al., 1992; Heertje et al., 1985). This is in agreement with the starter cultures used, in the case of yogurt, which is similar to *kivuguto*. Previous studies showed that the lactic acid bacteria produce exopolysaccharides in milk, especially dextrans (Karenzi, 2012). Additionnally, the incorporation of cultures producing exopolysaccharides into dairy foods can provide viscosifying, stabilizing and water binding functions (Broadbent et al., 2003). The Leuconostocs are good producers of exopolysaccharides (EPS) especially dextrans, which increase the viscosity in milk. And the two leuconostocs composing the *kivuguto* starter

culture are EPS producers as underlined in previous studies. Leuconostocs also produce other EPS, like α-glucooligosaccharides (GOS) from maltose or isomaltose, which can be used as thickeners or texturizers in cultured milk or stabilizers (Sanchez et al., 2005; Vedamuthu et al., 1994). The glycolysis by the strain of Lactococcus reduced the pH and consequently the coagulation of milk by modification of the caseins structure. And best of the best, the CWBI-B1470 *Leuconostoc pseudomesenteroides* is involved in three catabolic pathways: acidification by lowering the pH, dextran production by increasing viscosity and by aroma generation through citrate metabolism, whilst the CWBI-B1465 *Leuconostoc mesenteroides subsp. mesenteroides* is only a dextran and aroma producer.

3.3. Proteolytic activity

The proteolysis of milk protein was carried out using a rapid and simple OPA-based spectrophotometric assay (Church et al., 1983). Measurements were performed at the inoculation and at the end of fermentation of each fermented milk. Data obtained and presented in **table 2** are the differences for each fermented milk.

Table 2. Proteolysis of milks fermented by *kivuguto* strains

Milk	Concentration of peptides/free amine acids at the fermentation time Tf (mg/L)
M1	3.04 ± 0.73
M2	3.11 ± 0.30
M3	5.45 ± 0.16
M4	< 0

M1 mixed culture; M2 Lactococcus culture, M3 Leuconostoc culture and M4 without culture

A sample of the sterile milk M4 (not inoculated) was also analyzed (result not shown). In this study, the hydrolysis of milk protein by the mixed cultures of *kivuguto* (Milk M1) released 3.04 mg/L of peptides/FAAs after the fermentation time, whereas the milks of single fermentations M2 (Lactococcus) and M3 (Leuconostoc) gave 3.11 and 5.45 mg/L respectively. The analysis of the proteolysis revealed greater production in mixed milk than in single fermented milks. This may be due to the third strain *Leuconostoc mesenteroides* which is not fermentative when cultured alone. These data showed low concentrations of peptides for all samples, meaning low proteolysis activity. This is very important because high proteolysis values are an indication of bitter peptides production. These values are very

similar to the ones reported by other authors in various fermented products. Thivierge (1999) found an increase of milk proteolysis ranging from 0.33 to 64.58 mg/L on 26 isolates of *Lactococcus spp.* More analyses allowed her to select only strains with proteolysis of 0.33 to 7.70 mg/L as presumptive cheese starter cultures. Canas et al. (2007) obtained an increase of 6.50 mg/L of total amino acids during spontaneous malolactic fermentation of wine by yeasts and *Oenococcus oeni*. Production of extra-cellular peptidases and proteases, which are secreted by some strains of *Oenococcus oeni* (Remize et al., 2005) could contribute to the increase. *Streptococcus thermophilus* and *Lactobacillus delbrueckii subsp. bulgaricus* have been reported to possess cell-surface proteinases. However, the free amino groups found in milk were very low compared with those of the sample made with protease addition (Phiromruk et al., 2009). Prior to perform analyses, Abdel Rahman et al. (2009) filtered the camel milk samples using Whatman filter paper and found about 60-fold of our results. Moreover, the amount of free amino acids was higher in the mixed starter cultures of *Lactobacillus bulgaricus* and *Streptococcus thermophilus* than in the corresponding single cultures. Fortina et al. (2007) obtained low proteolytic activities for most strains of *Lactococcus garvieae* species to be used as dairy starter culture. The mean value was 42.7 $g.L^{-1}$.

3.4. Aroma compounds analyses

Seven compounds were identified by comparison of their mass spectra with those described in three libraries, with data from the literature and with Kovats index (KI) of standard compounds run under similar conditions (Adams, 1995). Results (**table 3)** are reported together with their relative area and estimated concentrations: 3-methylbutan-1-ol, pentan-1-ol, acetic acid, methyl benzoate, furanmethan-2-ol, 1,7,7-trimethylbicyclo[2.2.1]hept-2yl acetate, furan-2(5)H-one were clearly identified. Other compounds were also detected but their signal/noise ratio and their very low concentrations prevent us from unambiguous identification. Two compounds (namely methyl benzoate and 1,7,7-trimethylbicyclo[2.2.1]hept-2yl acetate) belong to the sole UHT milk as they were found in 4 samples used for analyses, and are therefore not discussed in this work. The three strains produce aroma compounds: CWBI-B1466 *Lactococcus lactis* produced pentan-1-ol and 3-methylbutan-1-ol; CWBI-B1470 *Leuconostoc pseudomesenteroides* produced also pentan-1-ol and 3-methylbutan-1-ol.

Table 3. Headspace analyses of volatile compounds (VCs) of *kivuguto* milk

t_r [1]	CAS [2] number	IUPAC Name	Identification [3]	VCs in milk [4]				Sample RI [5]	Reference KI [6]	Relative area (%±SD, n=3)	Estimated concentration [7] (mg/kg±SD, n=3)	Observed mass spectrum [8] m/z (%) for >10%
				M1	M2	M3	M4					
8.46	**123-51-3**	**3-methylbutan-1-ol**	MS, STD, RI	+	+	+	-	1202	1204 [a]	11.53±0.64	0.051±0.003	**88(<1)**; 79(15), 69(54); 57(29); 55(100); 55(13); 45(11); 43(51); 41(62); 41(56); 38(25)
8.92	**71-41-0**	**pentan-1-ol**	MS, STD, RI	+	+	+	-	1226	1244 [b]	18.72±0.31	0.083±0.001	70(97); 56(29); 56(16); 55(100); 44(11); 43(52); 42(70); 40(71); 38(31)
14.26	**64-19-7**	**acetic acid**	MS, STD, RI	+	-	-	-	1473	1477 [c]	4.37±0.38	0.154±0.014	60(67); 45(94); 43(100); 41(19)
16.61	**93-58-3**	**methyl benzoate**	MS, STD, RI	+	+	+	+	1613	1635 [d]	3.99±0.005	0.079±0.001	**136(<1)**; 136(33) ; 105(100); 77(58); 51(21); 50(10); 42(14)
17.14	**98-00-0**	**furanmethan-2-ol**	MS, STD, RI	+	-	-	-	1650	1661 [e]	39.9±1.59	0.177±0.007	98(46); 94(10); 81(59); 69(30); 69(27); 54(12); 53(49); 51(18); 50(16); 42(11); 41(52); 41(42); 38(47); 38(14)
17.91	**92618-89-8**	**1,7,7-trimethylbicyclo[2.2.1]hept-2yl acetate**	MS, STD, RI	+	+	+	+	1699	1584 [f]	5.77±0.005	0.115±0.001	**196(<1)**; 137(14); 136(57); 121(52); 110(29); 109(30); 108(29); 106(12); 95(100); 94(12); 93(66); 92(20); 91(16); 83(14); 81(17); 79(12); 78(12); 77(12); 69(22); 67(10); 66(26); 55(14); 54(86); 53(14); 44(13); 42(13); 40(47); 38(15)
18.49	**497-23-4**	**furan-2(5H)-one**	MS, STD	+	-	-	-	1739	-	15.68±1.03	0.312±0.020	83(60); 54(100); 54(21); 39(21); 36(10)

[1] Retention time; [2] CAS number of compounds listed in order of elution from a VF-Wax. Source: CAS Scifinder® (Chemical Abstracts Service, Colombus, USA); [3] Identification methods: MS, comparison of mass spectra with those in NIST08, Wiley275 and PAL 600K libraries; RI, comparison of retention indices with those in literature; STD, comparison of retention time and mass spectra of available standards; [4] M1. Milk with 3 *kivuguto* strains; M2. Milk with *Lactococcus lactis;* M3. Milk with *Leuconostoc pseudomesenteroides*; M4. Raw milk without strains; With such a profile, the 3rd strain CWBI-B1465 *Leuconostoc mesenteroides* subsp.mesenteroides may have brought in the *kivuguto* milk acetic acid, furanmethan-2-ol and 2(5)H-furanone. [5]Retention indices on VF-Wax column experimentally determined using a saturated C7-C30 alcanes standard solution; [6] Kovats indices taken from literatures: **[a]** Fukami et al. (2002) (measured with a TC-Wax column); **[b]** Umano et al. (2002) (measured with a DB-Wax column); **[c]** Cullere et al. (2004) (measured on a DB-Wax column); **[d]** Ferreira et al. (2001) (measured on a DB-Wax column); **[e]** Wong & Bernhard (1988) (measured on a DB-Wax column); **[f]** Davies (1990) (measured on a Carbowax column); [7] Concentrations calculated in M1 milk; [8] EI, 70ev, source at 220°C.

CWBI-B1465 *Leuconostoc mesenteroides subsp. mesenteroides* may contribute with the rest of molecules found in *kivuguto*, i.e. acetic acid, furanmethan-2-ol, furan-2(5)H-one and consequently showed a high potential of flavoring strain as it has been selected for, when we were sure that it was not a fermentative strain, but might have a huge technological impact on *kivuguto* milk.

Our results showed that *kivuguto* is characterized by a typical profile of volatile components from various sources like acids, alcohol, lactone, esters, etc., resulting of the various metabolic pathways of its strains of Lactococcus and Leuconostoc. Considering the *kivuguto* flavor profile, we can suggest that the *kivuguto* selected strains have their own amino acid convertases which matches with their ability to produce unusual flavors (Ayad et al., 1999).

The esterification formed of 3-methylbutan-1-ol/pentan-1-ol from acetic acid gives respectively isoamyl acetate/amyl acetate (pentyl acetate), which smell like banana flavor. According to Arctander (1969), the acetic acid has an acid taste perceptible well below 1% in water. It has a vinegar, pungent aromatic note with a perception threshold of 22 to 54 ppm in water (Molimard et al., 1997; Boelens et al., 1983) and 3 to 7 in butter (Molimard et al., 1997; Urbach et al., 1972; Siek et al, 1969; Patton, 1964).

Also lactones are known as source of pleasant milk flavor. Gadaga et al. (2007) found in *amasi* milk, acetaldehyde, ethanol, acetone, 2-methyl propanal, 2-methylpropan-1-ol, 3-methyl butanal and 3-methylbutan-1-ol as important compounds in the flavor profile of the naturally fermented milk of Zimbabwe. Among these molecules, the most important were ethanol and 3-methylbutan-1-ol. Note that the *amasi* starter contains nine yeasts and four lactic acid bacteria. Our results are likely in accordance with Ayad et al. (1999) who reported that wild strains of *Lactococcus lactis* are able to produce flavors different from those produced by industrial strains mostly methyl alcohols and methyl aldehydes. 3-methylbutan-1-ol/pentan-1-ol can be derived from the reduction of the aldehydes formed via Strecker degradation from the amino acids: alanine, valine and leucine (Izco and Torre, 2000).

These free amino acids are catabolized via caseins proteolysis. Thereafter, the acetaldehyde oxidizes quickly into acetic acid in the presence of aldehyde dehydrogenase (Al-DH) by nicotinamide-adenine dinucleotide (NAD) (Hruskar et al., 2010). 3-methylbutan-1-ol/pentan-1-ol can also derive from lactose fermentation. Alonso & Fraga (2001) used the same method to analyze yogurt flavor compounds and obtained acetaldehyde, acetone, butanone, butan-2,3-dione (diacetyl), 3-hydroxybutanone (acetoin) and acetic acid. These compounds are the result of the catabolism of *Lactobacillus delbruecki subsp. bulgaricus* and *Streptococcus thermophilus* as strains fermenting yogurt.

Apart from the work on yeasts, there is little information on the production of furanones by microorganisms although there is a suggestion that some species of *Lactobacillus* can produce the two forms of furanones (Carter et al., 2000). Furanones are aroma found in Emmental cheese. According to Slaughter (1999), two of the food-derived furanones have antioxidant activity comparable to that of ascorbic acid.

3.5. Sensory analysis

The sensory properties of the samples were evaluated by panelists to find which sample was the closest to *kivuguto* milk. Samples were the milk made by selected strains of *kivuguto*, the yogurt and the commercial fermented milk sold in Rwanda. Panelists assumed to have consumed the three milks. The first set was composed by a sample of *kivuguto* with yogurt and the second set by *kivuguto* and fermented milk sold in Rwanda. Each set was assayed by the panel and each panelist tasted three cups distributed on a plate. In a randomized order, the panel of 8 subjects identified the *kivuguto* among other milks with significant differences of P=0.05 with the first set and P=0.01 for the second set. The results showed that the panel detected easily differences between samples. Moreover, they were able to recognize which sample was made by *kivuguto* starters.

4. CONCLUSION

The characterization of *kivuguto* milk and milks fermented by two strains of *kivuguto* starter in monoculture was studied on four technological properties: acidification, rheology, proteolysis and flavor compounds. The results of this study allowed the evaluation of the acidification level and counts of bacteria in milk made by *kivuguto* selected starters, as well as the viscoelastic properties. These properties showed how far the *kivuguto* rheology can be compared with other fermented milks, like yogurt, filmjolk, leben. The static headspace and the GC coupled to mass spectrometry is a suitable method for extraction and analysis of volatile compounds of fermented milks. The discrimination test by a sensory panel detected also differences of *kivuguto* milk against other milks. Our findings showed that the selection procedure made satisfactorily a starter culture for manufacturing the *kivuguto milk*.

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Chapter 5

Characterization of CWBI-B1466 *Lactococcus lactis*, a strain selected in *kivuguto* milk

Le chapitre 5 traite de la production en fermenteur de CWBI-B1466 Lactococcus lactis, la souche la plus acidifiante du starter du kivuguto. Il étudie aussi sa conservabilité durant 90 jours pour évaluer la viabilité en stockage des cellules produites, lyophilisées, emballées sous-vide en sachets métallo-plastiques.

ABSTRACT

The strain CWBI-B1466 *Lactococcus lactis* has been previously selected from a traditional fermented milk of Rwanda as a starter culture. Sequence is deposited in GenBank under Accession Number JF313446. The report presents analyses on the biomass production, the freeze-drying process and the preservability during 90 days of storage. A pilot production of CWBI-B1466 *Lactococcus lactis* in a 20 L bioreactor in batch process was investigated. The preservability of this strain was analyzed after a freeze-drying process at three levels: by assessment of cells viability, by enumeration on agar medium, by the evaluation of oxidation of membrane cellular fatty acids and the survival rate throughout the shelf life of freeze-dried cells in storage. The final biomass was 1.96 10^{10} cfu.mL^{-1} in M17 medium and the growth rate was 0.83±0.007 h^{-1}. The conversion yield coefficient ($Y_{X/S}$) was 0.23 g.g^{-1}. The survival rate after lyophilization was 91.94 %; whilst the freeze-dried cells concentration was 2.66 10^{12} cells.g^{-1}. Seven cellular fatty acid (CFAs) of cell lipid extract were identified by GC/MS and stay stable during 90 days at 4°C and 20°C. The conventional plate counting exhibited 90.28 % and 83.33 of viable counts at 4 and 20°C respectively.

Key words: Batch, freeze-drying, preservation, *Lactococcus, kivuguto* milk, Arrhenius modeling, cellular fatty acid

1. INTRODUCTION

The production of fermented foods is based on the use of starter cultures, for instance lactic acid bacteria that initiate rapid acidification of the raw material (Leroy and De Vuyst**,** 2003). Milk has been produced since early times by fermentation process worldwide. Indeed, milk fermentation is a lactic fermentation process and lactic acid bacteria (LAB) are the main microorganisms involved in the fermentation. LAB played a major fermentative role in the development of aroma, texture and acidity of the product (Sodini et *al.*, 2002). LAB are nutritionally fastidious. They all require complex media for optimal growth (Teuber and Geis, 2006). They are considered of importance in regard to food and nutrition and an impressive number of microbial species and genera from that group are classified as probiotics (Holzapfel et *al.*, 2001). They are Gram-positive, none spore forming rods and cocci which produce lactic acid as a sole or major end product of fermentation (Dworkin *et al*., 2007). Important genera in dairy products are *Streptococcus, Enterococcus, Lactococcus, Leuconostoc, Pediococcus* and *Lactobacillus* (Desmazeaud, 1992). Strains belonging to the species *Lactococcus lactis* are the most important organisms in the manufacture of fermented dairy products (Samarzija *et al*., 2001), many cheeses (Gruyère, Cheddar, Colby, Emmental, Gouda, Brick, Limburger, Muenster**,** Parmesan, Romano, Provolone, Brie, Camembert, Blue, Gorgonzola and Mozzarella …), many types of fermented milk (amasi, zivda, dahi, filmjök, leben, …) and other dairy products. *Lactococcus lactis* strains usually occur in chains, they appear sometimes in single and paired forms. They are homofermentative and when added to milk, lactic acid (L form) often forms 95% of the end product. *Lactococcus lactis* is used in dairy technology as a starter culture with the major technological property of producing lactic acid from lactose fermentation, and so far of making the typical fermented milk when associated with other microorganisms. Apart from general demands for starter cultures from the view of safety, technological effectiveness and economics, numerous specific aspects have to be considered when selecting strains for the different food fermentations **(**Buckenhüskes, 1993). Therefore selection criteria for LAB depend on the functional property of the strain in substrate, in this case, the milk. But the first criterion that must meet the strains is the ability to grow in the medium of their utilization (Chamba *et al*., 1994). For a dairy starter culture, the acidification of milk is the major characteristic of a strain. The selected strain may allow the improvement and the control of the fermentation process, the formation of the desired organoleptic characteristics (Holzapfael, 1997), the rapid production of enough biomass in anindustrial medium. Indeed, a strain, even technologically interesting is of little interest if it

can not be produced in sufficient quantities (Chamba *et al*., 1994). The selection criteria depend also on the competitiveness, viability and survival and sometime on the antagonism with pathogens and microbial deterioration (Holzapfael, 1997).

A strain of *Lactococcus lactis* CWBI-B1466 was previously selected from *kivuguto* (Karenzi *et al*., 2012), a traditional fermented milk of Rwanda in order to use it as a starter culture. Some technological characteristics, especially the acidification, have been assessed and guided to its choice. But some experiments were still necessary to ascertain that this bacterium could be used as a starter culture. For that purpose, the production of that strain in M17 medium was assayed in batch production for biomass yield evaluation. The preservability was examined on freeze-dried cells during three months of storage under refrigeration and 20°C as well and viability analyses were carried out by tests on cell membrane fatty acids and by the classic plate counts on solid medium. *In fine*, the purpose of this work was to produce the selected strain and to ascertain its preservability.

2. MATERIALS AND METHODS

2.1. Bacterium and media

2.1.1. Bacterial strain

The strain CWBI-B1466 *Lactococcus lactis* has been isolated in *kivuguto,* a rwandese traditional milk. Phenotypic characterization has been performed by both preliminary tests and API biochemical tests according to BioMérieux (Marcy-l'Etoile, France) complemented by a genotypic characterization using the 16S rDNA PCR and 16S rDNA sequencing method. The strain is now preserved in CWBI Collection as CWBI-B1466 and registered to Genbank databases under accession number JF313446.

2.1.2. Media

Modified MRS agar (de Man *et al*., 1960) supplemented with 5.0 g $CaCO_3$ and 1.0 mL Tween 80 was prepared for viable count and for growing the bacterium taken from -80°C to obtain single colonies for inoculum preparation. A colony was purified once and incubated at 30°C and at pH 7.1 for 24 hours to inoculate M17 pre-culture broth. The composition of MRS is as following: 10.0 g peptone from casein; 10.0 g meat extract; 5.0 g yeast extract; 20.0 g

glucose; 2.0 g dipotassium hydrogen phosphate; 1.0 mL tween 80; 2.0 g di-ammonium hydrogen citrate; 5.0 g sodium acetate; 0.1 g magnesium sulfate; 0.1 g manganese sulfate; 16.0 g agar-agar and 1,000 mL distilled water.

MRS broth: a modified MRS for industrial production of *Lactococcus lactis subsp. lactis* by THT s.a containing 1.0 g soybean peptone; 5.0 g yeast peptone; 10.0 g yeast extract; 10.0 g meat extract; 0.2 g magnesium sulfate; 0.05 g manganese sulfate; 20.0 g glucose and 1,000 mL distilled water.

M17: a modified M17 (Terzaghi & Sandine, 1975) broth with 20 $g.L^{-1}$ of glucose was used as a pre-culture as well as a culture medium in batch and fed-batch. The medium contained also casein peptone 5.0 g, soybean peptone 5.0 g, yeast extract 2.5 g, beef extract 5.0 g, ascorbic acid 0.5 g, dipotassium glycerophosphate 19.0 g, 1M $MgSO_4.7H_20$ 0.25 g and distilled water 1,000 mL.

2.2. Fermentation and down-stream processes

2.2.1. Batch fermentation

All batch fermentations were carried out in triplicate in a 20 L bioreactor (Biolafitte, Poissy, France) with a 16 L working volume. 1 L of glucose solution of 20 $g.L^{-1}$ was added just before each fermentation. The regulation of the culture parameters (pH, temperature, alkali, etc.) was ensured by a direct control system (ABB). The culture in the bioreactor was held at 30°C and pH6.8 with a constant stirring speed of 100 rpm and no air flow was set. The foam level in the reactor was controlled by an antifoam probe placed at 10 cm from top of the vessel and by the addition of antifoam Tego KS911 (Goldschmidt, Germany). The inoculum for the 20 L bioreactor was prepared in M17 broth and autoclaved in a 1 L flask filled at half. It was prepared by inoculating some colonies of the bacterium grown on MRS agar plate.

2.2.2. Freeze-drying process and preservation

At the end of the fermentation, strains were harvested and centrifuged at 4°C and 4700 rpm for 20 min in a Sorvall® RC12BP™ Centrifuge (Thermo Scientific Inc., Massachusetts, USA). The fresh paste was diluted with 0.9% NaCl aqueous solution and mixed. The paste was supplemented with 2% of glycerol and 5% of maltodextrin as cryoprotectants, well malaxed and stored at -20°C before the freeze-drying process (Lyophilizator Liogamma,

Koeltechniek Louw B.V.B.A, Rotselaar, Belgium). The freeze-drying operation lasted in 20 hours and the pressure stay maintained at 0.6 mbar. Thereafter, samples of the freeze-dried cells were stored in metallo-plastic bags and sealed under vacuum pressure (Audion Elektro, Weesp, Netherlands). Then, the bags were stored at 4°C and 20°C for viability analyses after lyophilization (1 day), 15, 30, 60 and 90 days. This method was coupled to the enumeration on MRS agar. Tests were also made on bags stored at 30°C and 44°C for assessment of *Lactococcus* resistance.

2.3. Analytical methods

Optical density: cultures samples were collected aseptically after inoculation until the end of the growth phase. Biomass concentrations were determined from optical density (OD) measurements at 550 nm by a spectrophotometer (ThermoSpectronic, Madison, Wisconsin, USA).

Glucose consumption: sample cultures were collected for glucose analysis by an enzymatic method with an YSI 2700 Select analyzer (Yellow Spring Instruments, Ohio, USA). Glucose concentrations were also obtained from the KOH consumption and were used to confirm the values given by the glucose YSI 2700 Analyzer. Samples for analyses (OD, cell growth, glucose consumption) were collected each hour until the end of growth.

Determination of pH and alkali evolution: measurement of pH and KOH was carried out by a software connected to a pH probe Mettler Toledo InPro 2000/120/Pt100/9848) (Mettler-Toledo GmbH Im, Greifensee, Switzerland) and was regulated at a value of 6.8±0.1 by the addition of KOH 6N.

Growth kinetic: it was determined by the optical density coupled to cell enumeration or by dry matter determination. Cultures samples were collected aseptically after inoculation (To) and every 1 hour post-inoculation until the end of glucose in the culture. Culture sample of 1 mL was submitted to decimal dilutions in sterile peptone solution and MRS agar plates were performed to assess cell count. Plates were incubated at 30°C for 48 h. The specific growth rate was calculated in the growth exponential phase. For batch cultures, the growth kinetic parameters were calculated following the Monod equation:

$$\mu = \mu_m \frac{S}{S+Ks}$$

where μ is the specific growth rate and μ_m the maximum specific growth: $\mu = r_x / X$.

Dry matter determination: 1 g of paste with cryoprotectors before freeze-drying and 1 g of cells after freeze-drying were dried in a convection oven maintained at 105°C for 48 hours.

Survival rate in storage: The *survival rate* (*SR*) was calculated after lyophilization using the following formula:

$$\text{SR (yield \%)} = 100 \ \frac{Cp}{Dp} \cdot \frac{Din}{Cin} (*)$$

Where: - Cp is the concentration of powder (the outlet concentration in cfu.g^{-1})

- Dp the outlet dry matter (%)
- Cin the inlet concentration (the feed solution concentration in cfu.g^{-1})
- Din the inlet dry matter (%)

Extraction and analysis of fatty acids from cell membrane: The lipids were extracted following a protocol developed by Zelles (1999). Fatty acid fraction was extracted from 0.5 g freeze-dried cells according to the adapted method (Coulibaly et al., 2008). After two hours of extraction on a fixed speed rotator SB2 of Stuart®(Chelmsford, Essex, UK) from cell wall fractions of dried cells in 15 mL of ethanol-ether (3:1 v/v) mixture, the ethanol ether extracts were filtered on nylon membrane filters, Nylaflo™ of 0.2 µm pore size, with 47 mm of diameter (Pall life Sciences, Mexico, Mexico) and then evaporated on a Büchi Rotavapor®R-200 (Büchi Labortechnik AG, Flawil, Switzerland) coupled to a Büchi® Heating Bath B-490 (Büchi Labortechnik AG, Flawil, Switzerland) and concentrated under reduced pressure at 55-61°C. Fatty acid esters extract was then prepared from the concentrate with 14% (w/w) solution of boron trifluoride (0.5 mL) and 0.2 mL of n-hexane. The suspension was thereafter submitted to a trans-esterification process at 70°C in a water bath for 90 min; 0.5 mL of saturated NaCl, 0.2 mL of sulfuric acid (10%) and 4 mL of n-hexane were added. The fatty acids were extracted from the upper phase after 5 minutes. Gas chromatographic analysis of the fatty acid methyl esters (FAME) was carried out on a HP 6890 (Hewlett Packard) gas chromatograph equipped with a flame ionization detector at 250°C. A capillary column (30×0.25 mm, film thickness 0.25 µm) was used. The conditions were as follows: the carrier gas was helium (2.4 ml/min) and the injection volume was 1 µl. Injection was done at 250°C

in splitless mode for 1 min. The oven temperature was held at 50°C for 1 min, increased by 30°C/min to 150°C and then from 150°C to 240°C at 4°C/min with a final hold of 10 min at 240°C. Fatty acids methyl esters were identified by comparing their retention times with standard mixtures FAME MIX 47885U (Supelco, Bellefonte, USA). The relative fatty acid content was estimated as a relative percentage of the total peak area using a DP 700 integrator (Spectra physics). They are means of three independent experiments.

2.4. Statistical analyses

Productions in bioreactor were done in triplicate. The mean values and the standard deviation were obtained with three determinations. These data were then calculated using MS Excel software. CFU counts were transformed to their base 10 logarithms

3. RESULTS AND DISCUSSION

3.1. Kinetics of growth in 20 L fermentor and yields of freeze-drying.

The kinetic behavior of CWBI-B1466 *Lactococcus lactis*, GenBank database strain JF313446 was performed in M17 medium (batch) under controlled conditions of pH 6.8 and 30°C for biomass production. The growth parameters (μ, tg, $Y_{x/s}$) observed in the two processes are illustrated in **table 1**.

The agitation was fixed at 100 rpm because it was observed that the agitation affected the growth and the cell viability after freeze-drying of two *Lactococcus lactis* strains, especially when the agitation was higher than 150 rpm in 20 liters fermentor (Ziadi et al., 2008; Hamdi et al., 2000). The pre-culture was prepared in 500 mL in M17 broth during 8 hours under agitation before the inoculation of each 20 L fermentor with 16 L working volume.

Table 1. Growth parameters in 20 L fermentor of CWBI-B1466 *Lactococcus lactis* JF313446 in batch in M17 culture

Process	**Fermentation time** (h)	**Volume (Liters)**	**End of fermentation cells concentration** (cfu.mL^{-1})	[2] **μ** (h^{-1})	[3] **tg** (h)	[4] $\mathbf{Y_{x/s}}$ ($g.g^{-1}$)
Batch in M17	4h50	16	$1.96\ 10^{10}$	0.83	0.83	0.23

[1]**Cream**: the past diluted at the ratio of 3:1 with 2% of glycerol and 5% of maltodextrin; [2]**μ**: the specific growth rate; [3]**tg**: the generation time; [4]$\mathbf{Y_{x/s}}$: conversion yield coefficient

The inoculum concentration of 8 hours was around 9.92 log cfu.mL^{-1} ± 0.52. With such a pre-culture, the initial biomass concentration in the bioreactor was 8.54 log cfu.mL^{-1} in M17 batch culture. After ≈4h50, all glucose was exhausted (**figure 1**) and the corresponding yield of biomass on glucose was 0.23 in M17 batch. The end fermentation biomass was 10.29 log cfu.mL^{-1} ± 0.028.

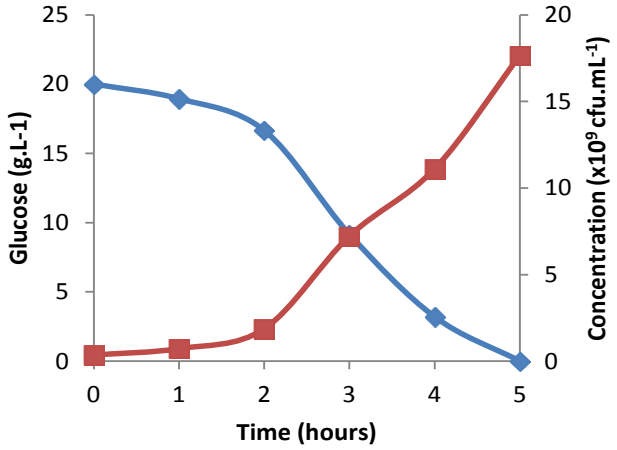

Figure 1. Growth kinetic and glucose consumption of CWBI-B1466 *Lactococcus lactis* JF313446 in batch processes

3.2. Viability of freeze-dried cells in storage

After freeze-drying **(table 2)**, the biomass concentration obtained in M17 batch production was 12.42 log cells.g^{-1} ± 0.141. This concentration is very high (≥10^{12} cells.g^{-1}). The glucose conversion was complete in the whole process resulting in exhausting it more quickly in the cultures. Freeze-dried concentrates are biological material and we use the equation (*) for viability estimation. During storage, the concentration varied slightly at 4°C and more at 20°C as illustrated in **figure 2** respectively 90.28 and 83.33%.

Table 2. Survival rates (%) and freeze-drying yields of CWBI-B1466 *Lactococcus lactis* JF313446 in batch M17 culture

Process	Before drying			After freeze-drying			Survival rate (%) at 4°C
	weight (g)	concentration (cfu.mL^{-1})	dry matter (%)	weight (g)	concentration (cfu.g^{-1})	dry matter (%)	
Batch in M17	293.3	0.8 10^{12}	25.5	70.3±1.53	2.7 10^{12}	89.8	93.3

The survival rates after freeze-drying were 93.3 and 83.3% of viability of viable cells at 4 and 20°C respectively. Thereafter, freeze-dried cells were studied in storage for the estimation of viability. Indeed, storage stability of lactic starter is a very important factor for its industrial use (Desmons et al., 1998). Viability and vitality of the starter culture in storage are required in order to ensure the quality of the product to be fermented.

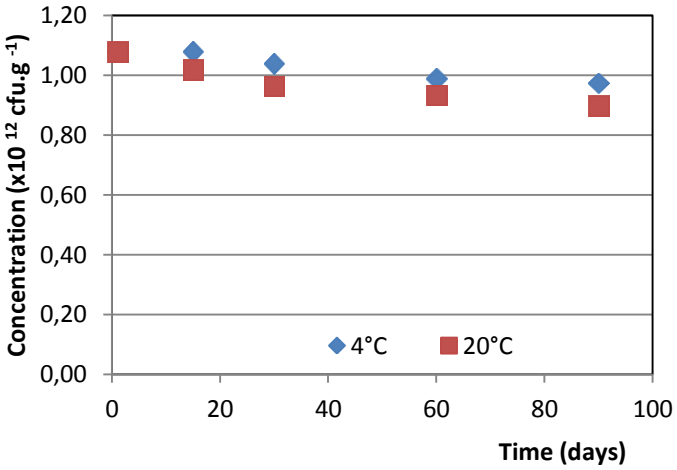

Figure 2. Cells concentration evolution during storage at 4°C and 20°C of CWBI-B1466 *Lactococcus lactis*, strain JF313446

3.3. Fatty acids from cell membrane

The cellular fatty acids (CFAs) composition in this study was apprehended for linking cell viability to conditions of storage during 90 days at two temperatures 4°C and 20°C. Each CFA relative percentage has been calculated from the total fatty acid methyl esters. The data are means of three independent experiments and the coefficients of variability were lower than 5%. Seven major fatty acids (CFAs) were found as illustrated on **figures 3 & 4**. There are meristic acid (C14); palmitic acid (C16); palmitoleic acid (C16:1); stearic acid (C18); oleic acid (C18:1); linoleic acid (C18:2t); linolenic acid (C18:3t); Among the 7 CFAs, palmitic acid and oleic acid represented more than 70 %. The ratio saturated fatty acids/unsaturated fatty acids stays stable on 90 days at 1.42 at 4°C and a very low variation was observed at 20°C with a ratio growing from 1.40 to 1.49. Lipid oxidation and survival during storage may be related (Teixeira et al., 1995; Teixeira et al., 1996). And the damage of cell membrane is due to the ratio saturated fatty acids/unsaturated fatty acids. This ratio depends on the type of cell. The structure of fatty acids has a hydrocarbon chain terminated

by a carboxylic group. The hydrocarbon chain also has two aspects. It is more saturated when there are only single bonds between carbons and unsaturated if one or more double bonds between two carbons. The higher the unsaturation, the lower the melting temperature of the fatty acid and higher the membrane fluidity that the fatty acid form.

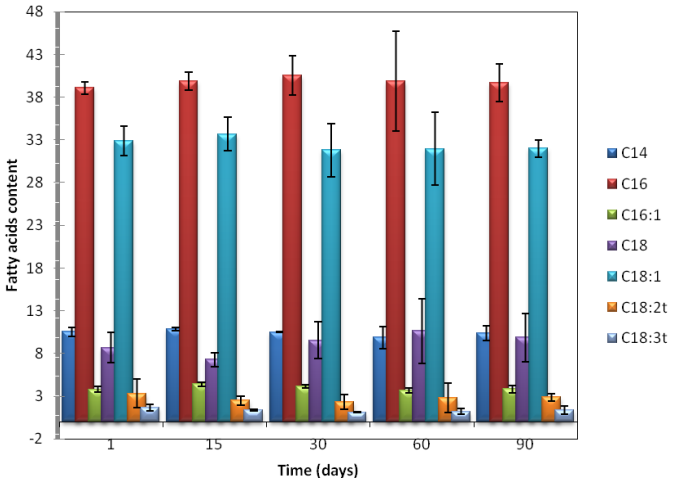

Figure 3. Cellular fatty acid profile of CWBI-B1466 during 90 days at 4°C
Meristic acid (C14); palmitic acid (C16); palmitoleic acid (C16:1); stearic acid (C18); oleic acid (C18:1); linoleic acid (C18:2t); linolenic acid (C18:3t); SFA **(**Saturated Fatty Acids): C14:0; C16: 0; C18:0; UFA **(**unsaturated fatty acids): C16:1; C18:1; C18:2t; C18:3t. Values are means of 3 experiments. Content in %.

The figures 3 and 4 presented 7 CFAs identified in CWBI-B1466 cell membrane as meristic acid (C14); palmitic acid (C16); palmitoleic acid (C16:1); stearic acid (C18); oleic acid (C18:1); linoleic acid (C18:2t) and linolenic acid (C18:3t). The major components are the palmitic acid (C16: 0); oleic acid (C18:1) with more 70% and the meristic acid (C14) with about 10%. This is in agreement with the stability of CWBI-B1466 *Lactococcus lactis.* Teixeira et al. (1995) working on *Lactobacillus bulgaricus* found that this ratio was variable in time. When the content of the other cellular fatty acids (CFAs) was expressed as a ratio of each CFA and the C16:0 as this CFA remain stable more than other CFAs, the most stable ratios were observed on C14/C16, C16:1/C16, C18:1/C16, C18:2t/C16 (from 0.08 to 0.07 at 4°C and from 0.085 to 0.070 at 20°C), C18:3t/C16 (from 0.036 to 0.034 at 4°C and from 0.035 to 0.030 at 20°C). Those data showed that no significant variations ($p<0.05$) were observed at 4°C but less at 20°C. Low survival during storage was associated with a decrease in C18:2/C16:0 and C18:3/C16:0 (Teixeira et al., 1995). Coulibaly et al. (2008) showed that a higher decrease in C18:2/C16:0 was verified as 0% at 4°C and 19% at 20°C for *Lactobacillus plantarum* after 90 days storage, while the decrease of C18:3/C16:0 was 4.8% and 52.7% respectively.

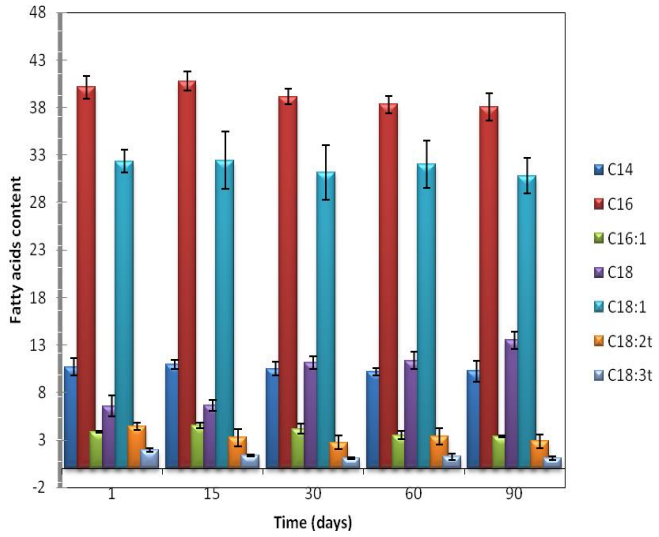

Figure 4. Cellular fatty acid profile of CWBI-B1466 during 90 days at 20°C
Meristic acid (C14); palmitic acid (C16); palmitoleic acid (C16:1); stearic acid (C18); oleic acid (C18:1); linoleic acid (C18:2t); linolenic acid (C18:3t); SFA **(**Saturated Fatty Acids): C14:0; C16: 0; C18:0; UFA **(**unsaturated fatty acids)**:** C16:1; C18:1; C18:2t; C18:3t. Values are means of 3 experiments. Content in %.

4. CONCLUSION

The strain CWBI-B1466 *Lactococcus lactis* production was tested in batch fermentation in M17 medium. The production in fermentor allowed obtaining a high concentration of biomass in batch process. The yields after freeze-drying showed that the culture conditions are satisfactorily in accordance with the physiology of the *Lactococcus* strain. The data obtained from the Cellular fatty acids analyses revealed that no oxidation was observed at 4°C and less at 20°C, which is also confirmed by the results given by plate counts. Ultimately, the strain CWBI-B1466 *Lactococcus lactis* exhibited good yields both in production and in preservation more at 4°C than at 20°C and can be therefore used as a starter culture.

5. ACKNOWLEDGEMENT

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Chapter 6

Characterization of CWBI-B1465 *Leuconostoc mesenteroides subsp. mesenteroides* isolated in *kivuguto* milk

*Le **chapitre 6** présente une étude de la conservabilité de la souche CWBI-B1465 Leuconostoc mesenteroides après sa production en fermenteur en batch et fed-batch, suivie par deux procédés de séchage: la lyophilisation et l'atomisation. En effet, pendant l'étape de screening, cette souche a montré une caractéristique particulière de résistance aux conditions très acides et aux hautes températures. Nous avons dès lors émis l'hypothèse qu'elle pourrait aussi supporter les conditions de séchage par atomisation, ce qui réduirait sensiblement son coup de production. Lors de cette étude, après calcul des rendements du process, des poudres lyophilisées et des poudres atomisées sont stockées sous-vide en sachets métallo-plastiques et conservées 90 jours. En parallèle du classique comptage sur milieu solide, la viabilité en stockage des poudres lyophilisées/atomisées a été estimée par l'analyse des acides gras membranaires, par l'analyse de l'oxydation par cytométrie de flux, par l'analyse de l'oxydation des protéines solubles et des carbonyles après rupture membranaire par sonication.*

*Les résultats constituent la charpente du **chapitre 6** et un article "**Freeze-drying versus spray-drying of CWBI-B1465 Leuconostoc mesenteroides subsp.mesenteroides isolated in kivuguto milk** " a été élaboré pour publication.*

Freeze-drying versus spray-drying of CWBI-B1465 *Leuconostoc mesenteroides subsp. mesenteroides* isolated in *kivuguto* milk

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ABSTRACT

The strain CWBI-B1465 *Leuconostoc mesenteroides* has been previously selected from the traditional *kivuguto,* a fermented milk of Rwanda. Strain is preserved in the CWBI Collection and the sequence is deposited in GenBank under Accession number JF313445. The paper reports the production and the preservation of that strain for dairy starter culture use. The production was carried out in a 20 L fermentor with 16 L working volume in batch process. A half of this was centrifuged. The cells were harvested, cryoprotected and *freeze-dried*. Freeze-dried powders were thereafter vacuum-sealed and stored for 90 days. The other half was also centrifuged. The cells were harvested, mixed with protectants and *spray-dried*. Spray-dried powders were thereafter vacuum-sealed and stored during 90 days. After the two drying processes, viability in storage at 4 and 20°C was examined on six levels: membrane fatty acids, flow cytometry, soluble proteins, carbonyl proteins and enumeration on agar MRS. The production was also made in 20 L fermentor with 16 L working volume in fed-batch process, only for optimal production assessment. The obtained results showed that CWBI-B1465 grows at 0.55-0.60 h^{-1} of specific growth rate, the generation time was 1.12 and 1.25 h in batch and fed-batch respectively. Its preservability exhibited low loss when it is stored at 4°C, and more if it is stored at 20°C. The most suitable drying process is freeze-drying. But the spray-drying is also very interesting and is recommended based on cost of the process.

Key words: Freeze-drying, spray-drying, *kivuguto*, *Leuconostoc mesenteroides*, batch fermentation, fed-batch fermentation, fatty acids, flow cytometry, soluble proteins, carbonyl proteins, down-stream process.

1. Introduction

Spray-drying can be used to produce large amounts of dairy ingredients relatively inexpensively, the spray-dried powders can be transported at a low cost and can be stored in a stable form for prolonged periods (Gardiner *et al*., 2000). Suitable strain selection necessitates consideration of three essential premises: encompassing general aspects (origin, identity, safety), technological aspects (growth properties during processing, survival during processing and storage) and stability of functional properties (Collins et al., 1998; Holzapfel and Schillinger, 2002; Stanton *et al.,* 2003). Spray-drying can produce stable powders of certain bacteria and yeast species; however, with the high temperatures involved in this process, the species require a certain level of thermotolerance (Golowczyc et al., 2010). The overall viability and subsequent oxidative damage affect primarily lipid membrane, proteins and DNA along the whole process. Polyunsaturated fatty acids and amino acid oxidation (primarily Pro, Arg, Lys and Thr) but also protein fragmentation and protein aggregation are the major damages for dried bacteria in storage. They are degraded into a variety of products (Grune *et al.* 2013; Coulibaly *et al.*, 2011; Møller *et al.*, 2011; Dalle-Donne *et al.* 2003; Yoon *et al.*, 2002; Fleming *et al.*, 1982) under many biochemical reactions. Among of them, the carbonylation is a chemical reaction that produces organic carbonyl compounds containing the C=O functional group such as aldehydes and ketones (Kalemba and Pukacka, 2014). Protein carbonylation was therefore examined as a potential cause for the loss of viability of dried bacteria. The reagent 2,4-dinitrophenylhydrazine (DNPH) reacts with the aldehyde or ketone carbonyl group and forms hydrazone derivatives (DNP), thus enabling spectrophotometric determination (Levine *et al.*,1994; Levine, 2002).

In this study, the strain CWBI-B1465 was investigated based on its ability to withstand the stresses associated with high temperature and acidity as it resists well to 55°C and pH2.5 (Karenzi *et al.,* 2012). In this regard, our hypothesis was that it could also withstand stresses of spray-drying. Therefore, the production in bioreactor, and the drying by freeze-drying in parallel to spray-drying was a suitable work allowing to understand the level of resistance of this strain. Data of this comparative study will allow us to know the most suitable process to be used for its preservation for dairy starter culture.

The aims of this study were therefore:

(i) to examine the yields of growth during production;

(ii) to evaluate the viability during freeze/spray-drying and storage

2. Material and Methods

2.1. Bacterial strains and cultures

From the CWBI Collection preserved at -80°C, the strain CWBI-B1465 *Leuconostoc mesenteroides subsp. mesenteroides* JF313445 was cultured on MRS agar and incubated for 36 hours at 30°C. Thereafter, it was inoculated in 500 mL of MRS broth as a pre-culture of a 20 L bioreactor (Biolafitte, Poissy, France).

2.2. Fermentation processes

2.2.1. Batch fermentation

All batch fermentations were carried out in triplicate in a 20 L bioreactor (Biolafitte, Poissy, France) with a 16 L working volume. 1 L of glucose solution of 20 g. L^{-1} was just added before fermentation. The regulation of the culture parameters (pH, temperature, alkali, etc.) was ensured by a direct control system (ABB). The culture in the bioreactor was held at 27°C and pH6.5, with a constant stirring speed of 100 rpm and with air flow. The foam level in the reactor was controlled by an antifoam probe placed at 10 cm from top of the vessel and by the addition of antifoam Tego KS911 (Goldschmidt, Germany). The inoculum for the bioreactor was prepared in MRS broth and autoclaved in a 1 L flask filled at half. It was prepared by inoculating some colonies of the bacterium grown on MRS agar plate. The growth kinetic parameters were calculated following the Monod equation:

$$\mu = \mu_m \frac{S}{S+Ks}$$

where μ is the specific growth rate: $\mu = r_x / X$, with **X** the biomass concentration (cells dry matter). The cell yield ($Y_{X/S}$) (g cells dry matter/g glucose) was calculated from plots of $X-X_0$ vs S-S0 (De Vuyst et al., 1996):

$$Y_{X/S} = \frac{X - X0}{S - S0}$$

Where X and Xo are the biomass concentrations at **t** time and the initial time $\mathbf{t_0}$ respectively; S and S0 the residual glucose ($g.L^{-1}$) at **t** time and $\mathbf{t_0}$ time.

2.2.2. Fed-batch fermentation

The fed-batch culture conditions are the same as the batch. The culture parameters differ only to the second glucose solution of 1 L added just before 7 hours. Fed-batch fermentation was conducted to increase biomass production.

2.3. Down-stream process

2.3.1. Concentration & protective agents addition

At the end of the fermentation, cultures were harvested and centrifuged at 4°C and 4700 rpm for 40 min in a Sorvall® RC12BP™ Centrifuge (Thermo Scientific Inc., Massachusetts, USA). Eight liters were concentrated by centrifugation for a further lyophilization and the other eight for atomization.

2.3.2. Freeze-drying

The fresh paste was weighted and diluted with one quarter of supernatant solution and mixed for paste recovery from the centrifuge pots. Thereafter, the cream obtained was supplemented with 2% of glycerol and 5% of maltodextrin as cryoprotectants, well malaxed and stored at -20°C before the freeze-drying process (Lyophilizator Liogamma, Koeltechniek Louw B.V.B.A, Rotselaar, Belgium). The freeze-drying operation lasted 20 hours and the pressure stayed at 0.6 mbar. Next, samples of the freeze-dried cells were vacuum- sealed in metallo-plastic bags. Then, the bags were stored at 4°C and 20°C for oxidation analyses, after lyophilization (1 day), 15, 30, 60 and 90 days. This method was complemented by enumeration on MRS agar.

2.3.3. Spray-drying

The cream was obtained as for the freeze-drying process. Once weighed, the supernatant 4-fold of the cream weight was first mixed with the protective agents under agitation during one hour for feed solution preparation. As protective agents, half of the cream weight made by casein pepton and the other half by maltodextrin were prepared. The feed solution was atomized using a GEA Niro spray-dryer (Sœborg, Denmark) under following conditions:

outlet air temperature 50°C, inlet air temperature 150°C and atomizing air pressure 4 bars. These conditions were obtained after many assays in search of the best yield. Powder was collected in a single cyclone separator in glass bottles and vacuum packed (Audion Elektro, Weesp, Netherlands) in metallo-plastic bags and stored at 4 and 20°C up to 90 days. The outlet temperature was maintained at 50-55°C to obtain powders with least moisture (5 %) and to control the flow rate.

Moisture content in freeze/spray-dried powders: The moisture content of dried powders was determined by oven drying at 105°C according to the International Dairy Federation Bulletin (IDF, 1993).

2.4. Analytical methods

2.4.1. Determination of the viability

After each drying process, the survival rate was calculated as follows:

$$\textbf{Survival rate}\ (\text{yield } \%) = 100 \quad \frac{\mathbf{Cp}}{\mathbf{Dp}} \cdot \frac{\mathbf{Din}}{\mathbf{Cin}} \quad ;$$

where: - Cp is the concentration of powder (the outlet concentration in $cfu.g^{-1}$)
- Dp the outlet dry matter (of powder) (%)
- Cin the inlet concentration (of cream) (in $cfu.g^{-1}$)
- Din the inlet dry matter (of cream) (%)

The concentration before and after freeze/spray-drying was determined as colony forming units (cfu) per g or per mL. Suitable decimal dilutions were prepared for enumeration and plated on MRS agar. After 90 days storage, the survival rate was calculated according to the relation:

$$\textbf{Survival rate}\ (\%) = \frac{\mathbf{N}}{\mathbf{No}},$$

with **N** the cells concentration ($cfu.g^{-1}$) of the freeze-dried samples at 90 days storage and **No** the cells concentration ($cfu.g^{-1}$) after freeze/spray-drying process.

2.4.2. Analysis of fatty acids extracted from cell membrane

The lipids were extracted following a protocol developed by Zelles (1999). Fatty acid fraction was extracted from 1 g freeze-dried cells according to the adapted method (Coulibaly *et al.*, 2008). After two hours of extraction on a fixed speed rotator SB2 of Stuart®(Chelmsford, Essex, UK) from cell wall fractions of dried cells in 15 mL of ethanol-ether (3:1 v/v) mixture, the ethanol ether extracts were filtered on nylon membrane filters, Nylaflo™ (Pall life Sciences, Mexico, Mexico) of 0.2 µm pore size, with 47 mm of diameter and then evaporated on a Büchi Rotavapor®R-200 (Büchi Labortechnik AG, Flawil, Switzerland) coupled to a Büchi® Heating Bath B-490 (Büchi Labortechnik AG, Flawil, Switzerland) and concentrated under reduced pressure at 55-61°C. Fatty acid esters extract was then prepared from the concentrate with 14% (w/w) solution of boron trifluoride (0.5 mL) and 0.2 mL of n-hexane. The suspension was thereafter submitted to a trans-esterification process at 70°C in a water bath for 90 min; 0.5 mL of saturated NaCl, 0.2 mL of sulfuric acid (10%) and 4 mL of n-hexane were added. The fatty acids were extracted from the upper phase after 5 minutes. Gas chromatographic analysis of the fatty acid methyl esters (FAME) was carried out on a HP 6890 (Hewlett Packard) gas chromatograph equipped with a flame ionization detector at 250 °C. A capillary column (30×0.25 mm, film thickness 0.25 µm) was used. The conditions were as follows: the carrier gas was helium (2.4 mL/min) and the injection volume was 1 µL. Injection was done at 250°C in splitless mode for 1 min. The oven temperature was held at 50°C for 1 min, increased by 30°C/min to 150°C and then from 150°C to 240°C at 4°C/min with a final hold of 10 min at 240°C. Fatty acids methyl esters were identified by comparing their retention times with standard mixtures FAME MIX 47885U (Supelco, Bellefonte, USA). The relative fatty acid content was estimated as a relative percentage of the total peak area using a DP 700 integrator (Spectra physics). Analyses were made in triplicate. All chemicals were analytical-grade reagents.

2.4.3. Flow cytometry analyses

The experiments were carried out on a BD FACSCalibur system composed with a FACSCalibur two lasers flow cytometer and a CellQuest software (BD Biosciences, San Jose, California, USA). For viability assessment of freeze/spray-dried cells, the carboxyfluorescein diacetate (cFDA) and the dye exclusion DNA binding probes propidium iodide (PI) were used

for a live and dead discrimination. 1 mL of cell suspension from freeze-dried cells of 10^9 cfu.g^{-1} washed successively in 1 mL of phosphate buffer solution (PBS) and centrifuged at 12500 rpm. Thereafter, it was stained with 10 µL of cFDA or PI and incubated at 37°C (Bunthof *et al.,* 2001; Rault *et al.,* 2007). Tubes were washed three times with PBS and analyzed on FACSCalibur two lasers flow cytometer. cFDA and PI were excited by a 488 nm laser, the signal was red from the cFDA in the FL-1 and that of PI in the FL-3. All experiments were repeated three times.

2.4.4. Soluble proteins

The amount of the cell soluble protein concentrations were analyzed by the method of Lowry *et al.* (1951) from 0.5 g freeze-dried cells after sonication (Bandelin Sonopuls HD 2070, Germany) using Fohlin reaction.

2.4.5. Carbonyl proteins

The determination of carbonyl compounds was obtained spectrophotometrically by derivatization of the carbonyl group with 2,4-dinitrophenylhydrazine (DNPH) using the labelling modified method of Levine (2002), which leads to the formation of a stable 2,4-dinitrophenyl (DNP) hydrazone product. Dried cells were lysed by sonication (Bandelin Sonopuls HD 2070, Germany) and centrifuged (1500 rpm, 10 min at 4°C). Thereafter, 0.2 ml was collected and incubated with 0.8 ml 0.2% DNPH in 2.5 M HCl for 1 h at room temperature. Then, the derivative protein contents were extracted with 10% (w/v) trichloroacetic acid, treated with ethanol/ethyl acetate 1:1 (v/v) and re-extracted with 10% trichloroacetic acid. The precipitated extract was dissolved in 6 M guanidine hydrochloride. A blank without the DNPH reagent but with 2 M HCl was treated in parallel following the same procedure. The protein carbonyl content of dried cells was calculated using a standard curve prepared from BSA standards. Carbonyl/protein contents were determined by absorption at 360 nm and expressed in nmol/mg (Kim *et al.,* 2002; Luqman and Rizvi, 2006).

2.4.6. Statistical Analysis

Productions were done in three bioreactors. The mean values and the standard deviation were

obtained from the triplicate. These data were then compared by Turkey's honestly significant difference and the level of significance was determined at $p<0.05$.

3. Results and Discussion

The strain CWBI-B1465 was produced in a 20 L bioreactor in batch and fed-batch processes for primarily the estimation of the yield of production. The working volume was 16 L. A half was thereafter dried by freeze-drying, whereas the other half was spray-dried. Results express a comparative study of growth parameters and conversion yield on the basis of two fermentation modes (batch and fed-batch) followed by two drying processes (freeze-drying and spray-drying). Secondly, the viability of the strain CWBI-B1465 in storage was examined and compared only on dried cells produced by batch mode. Here, a multiparameter analysis was applied for viability assessment.

3.1. Production in 20 L fermentor of CWBI-B1465 *Leuconostoc mesenteroides* JF313445

The growth was performed at 27°C and at pH 6.5 in MRS broth. The agitation was adjusted at 100 rpm. For batch process, the growth data were estimated with the end of the exponential phase at 7 hours. For fed-batch process, the second glucose was added after 6 hours and the exponential phase ended at 9 hours. A half liter pre-culture allowed a direct growth in fermentor without a significant lag phase. The cells concentrations as illustrated in **table 1** at the end of fermentation were 9.78 log cfu.ml^{-1} in batch, whilst in fed-batch the figure was 11.8 log cfu.ml^{-1}. That's meaning an increase of 20.66 % due to only the action of 320 g additional glucose dissolved in 1 L of distilled water and added in the fermentor for fed-batch process.

Correspondingly, the growth was 74 times in the first phase of fed-batch (at 6h) and 142 times at the end from the second inoculation. The specific growth rates (**μ**) were 0.61 and 0.55 h^{-1} in batch and fed-batch respectively and the generation time (**tg**) were 1.12 and 1.25 h in batch and fed-batch respectively. Manel *et al.* (2009) found about 0.9 h^{-1} of μ_{max} and 0.7 of **tg** for two Lactococcus strains produced in fed-batch in M17 medium. These data are in agreement with the growth behavior of the two genera, as it's well known that generally Lactococcus grows more quickly than Leuconostoc.

Table 1. Production in 20 L fermentor of CWBI-B1465 *Leuconostoc mesenteroides subsp. mesenteroides* JF313445.

Processes	Fermentation time (h)	End of fermentation cells concentration (log cfu.mL^{-1})	[1]μ (h^{-1})	[2]tg (h)	[3]$Y_{x/s}$ (g.g^{-1})	
Batch	7.33	9.78±0.17	0.61±0.01	1.12±0.02	0.155 ± 0.00	[4]**FD**
					0.152 ± 0.02	[5]**SD**
Fed-batch	9.50	11.08±0.05	0.55 ±0.07	1.25±0.02	0.16 ± 0.02	**FD**

[1]**μ**: the specific growth rate; [2]**tg**: the generation time; [3]$Y_{x/s}$: conversion yield coefficient; [4]**FD**: freeze-drying; [5]**SD**: spray- drying.

3.2. Viability of CWBI-B1465 *Leuconostoc mesenteroides* JF313445 to freeze-drying *vs* spray-drying.

Cells grown in a 20 L bioreactor (in batch and fed-batch) were harvested, concentrated by centrifugation and thereafter protected by addition of protective agents. A half was freeze-dried, whilest another half was spray-dried. Data of viable cells are presented in **table 2**. The batch process followed by freeze-drying showed a viable count of 11.90±1.04 log cfu.g^{-1}, while viable population resulted from the batch followed by spray-drying was 10.74±0.11 log cfu.g^{-1}. The two values represented respectively the survival rates (%) of 27.8 and 21.2which showed clearly how the freeze-drying mode (Yao *et al.*, 2009; Miyamoto-Shinohara *et al.*, 2000) proved effective in achieving high viable cells. However, the value obtained by spray-drying is very interesting in terms of the cost of the processes.

Table 2. Effect of drying process on the viability of CWBI-B1465 *Leuconostoc mesenteroides* JF313445

Process	Before drying		After drying		Survival rate (%)	
	[1]Concentration (log cfu.mL^{-1})	Dry matter (%)	[2]Concentration (log cfu.g^{-1})	Dry matter (%)		
Batch	**11.90±1.04**	**24.58±0.52**	**11.43±0.31**	**91.88±1.29**	**27.8**	[3]**FD**
	10.74±0.11	**19.71±0.46**	**10.68±0.10**	**93.35±1.00**	**21.2**	[4]**SD**
Fed-batch	**11.44±0.25**	**22.42±0.86**	**11.08±0.05**	**91.65±0.45**	**23.9**	**FD**

[1]biomass before drying; [2]biomass after drying; [3]FD: freeze-drying; [4]SD: spray-drying

And comparing the batch and the fed-batch processes followed by the freeze-drying, it was also proven that the batch process seems to be the best as the fed-batch had only 23.89 % of viable free-dried cells. In all cases, the cells recovery after the drying process (FD or SD) was more than 96 % and the dry matters were >91 %. Even if there was a decrease in viability

after the drying process (Castro *et al.,* 1997), the survival rates after drying processes were somehow high. Coulibaly *et al.* (2009) found 18 % in the same conditions on another freeze-dried strain of *Leuconostoc mesenteroides*, meaning that our Leuconostoc is very resistant.

3.3. Viability of freeze/spray-dried cells in storage

During storage on 90 days, the enumeration showed a decrease in time with freeze- spray-dried cells produced by batch process. Powders stored at 4°C were reduced of 1.8 % for freeze-dried cells and 2.3 % for spray-dried cells. At 20°C, the decrease was very high with 46.0 % and 42.1 % respectively. These data showed again that freeze-drying is the best preservation technique, but the difference is very low. Viability assessment was also evaluated through the fatty acids extracted from cell membrane and the physiological state of cells by flow cytometry analyses. Analyses of the oxidation of cellular constituents were also carried out for cells viability estimation. For that issue, carbonyl proteins and soluble proteins contents were analyzed after sonication of dried powders stored for 90 days.

3.3.1. Fatty acids extracted from cell membrane

The cellular fatty acids (CFAs) composition in this study has been examined for linking cells viability to conditions of storage during 90 days at two temperatures: 4°C and 20°C. Freeze-dried cells (FD) and spray-dried (SD) cells were compared with regard to the fatty acids oxidation. As well known, the lipid oxidation of membrane fatty acids is deemed responsible for cell death during storage (Mputu-Kanyinda *et al.,* 2012; Andersen *et al.,* 1999; Teixeira *et al.,* 1996; Teixeira *et al.,* 1995). Each CFA relative percentage has been calculated from the total fatty acid methyl esters. The data are means of three independent experiments and the coefficients of variability were lower than 5% ($p<0.05$).

Seven major fatty acids (CFAs) were found as illustrated on **figures 1 & 2**. They are meristic acid (C14); palmitic acid (C16); palmitoleic acid (C16:1); stearic acid (C18); oleic acid (C18:1); linoleic acid (C18:2t); linolenic acid (C18:3t). Among the 7 CFAs, palmitic acid and palmitoleic acid represented more than 70 %. We have calculated the decrease of unsaturated fatty acids due to oxidation based on the ratio between each polyunsaturated fatty acid and the palmitic acid, since saturated fatty acids are not altered by oxidation. The ratio C14:0/C16:0; C18:0/C16:0; C18:2t/C16:0 stays unchanged for 90 days at 4°C and 20°C for freeze/spray-dried powders. The changes occurred on freeze-dried cells as following: at 4°C, the ratio

C18:1/C16:0 decreases for 2.2 %, whilst it decreases by 4.8 % at 20°C. At the two temperatures, the changes are very low so that the oxidation phenomenon does not affect the viability.

On spray-dried cells, decreases were observed on the ratio C16:1/C16:0. At 4°C, the ratio C18:1/C16:0 decreased by 3.0 % and by 4.1 at 20°C. Slight decrease was also present on the ratio C18:3t/C16:0 for 6.9 % at 20°C. Globally, it seems that the phenomenon of oxidation is very low and its value may be beared on the palmitoleic acid, the oleic acid and the linolenic acid in storage.

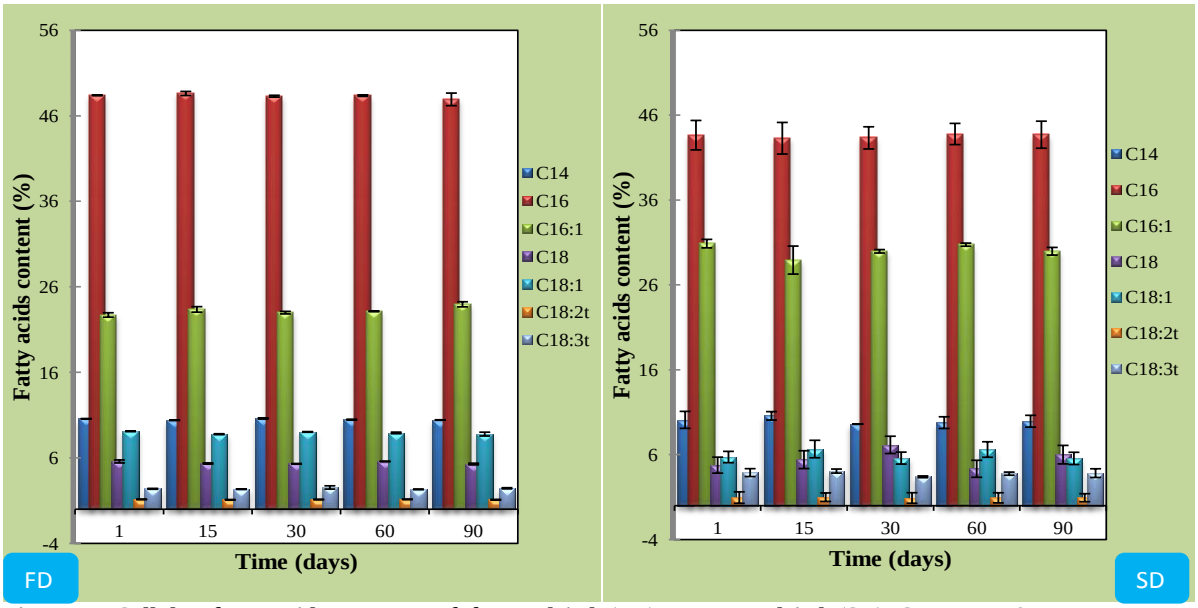


Figure 1. Cellular fatty acids contents of freeze-dried (FD) vs spray-dried (SD) CWBI-B1465 *Leuconostoc mesenteroides* during 90 days at 4°C. Meristic acid (C14); palmitic acid (C16); palmitoleic acid (C16:1); stearic acid (C18); oleic acid (C18:1); linoleic acid (C18:2t); linolenic acid (C18:3t); **SFA (**Saturated Fatty Acids)**:** C14:0; C16:0; C18:0; **UFA (**unsaturated fatty acids)**:** C16:1; C18:1; C18:2t; C18:3t. Values are means of 3 experiments. Content in %.

Our results disagreed with data obtained by Coulibaly *et al.* (2009) on another strain of *Leuconostoc mesenteroides*. He found decreases on the ratio C18:2/C16:0 and C18:3/C16:0. In general, CWBI-B1465 *Leuconostoc mesenteroides* stayed stable at 4°C for freeze-dried cells and spray-dried cells. But our results were consistent with our hypothesis that the strain CWBI-B1465 *Leuconostoc mesenteroides* is highly resistant.

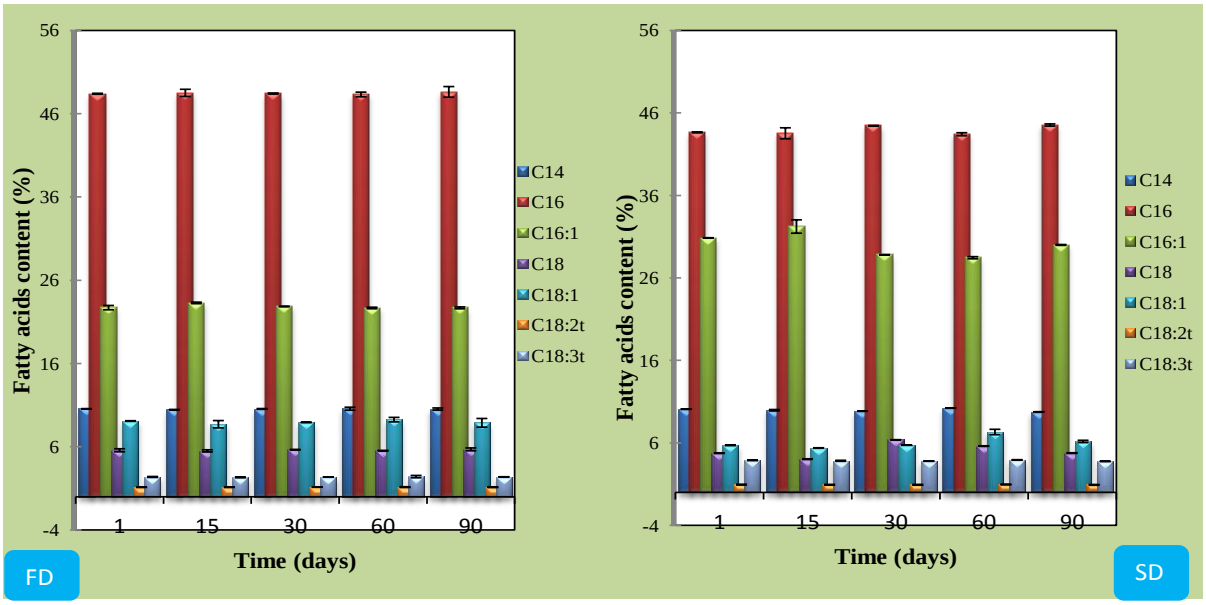


Figure 2. Cellular fatty acids contents of freeze-dried (FD) vs spray-dried (SD) CWBI-B1465 Leuconostoc mesenteroides during 90 days at 20°C. Meristic acid (C14); palmitic acid (C16); palmitoleic acid (C16:1); stearic acid (C18); oleic acid (C18:1); linoleic acid (C18:2t); linolenic acid (C18:3t); SFA (saturated fatty acids): C14:0; C16:0; C18:0; UFA (unsaturated fatty acids): C16:1; C18:1; C18:2t; C18:3t. Values are means of 3 experiments. Content in %.

3.3.2. Flow cytometry analyses

The flow cytometry analyses were applied for the assessment of the impact of the drying process on cells viability in storage. Prior to flow cytometry analyses on dried cells, cFDA and PI were tested for control on CWBI-B1465 *Leuconostoc mesenteroides* fresh cells supposed to be at 100% alive and on 100% fully heat-killed cells, as well as a multiparameter dot plot obtained after PI/cFDA double staining of their mixture (at a ratio of 1:1 vol/vol). These cells were not submitted to any drying process and were collected in exponential phase for 100 % living cells and a fraction was heat-killed for dead cells. Living cells and dead cells subpopulations were spatially separated in dot plots of FL_1 and FL_2; and relative percentages obtained on living cells stained with cFDA showed 99.11 % of viable cells, 0.01 % of dead cells and 0.74 of intermediate cells (viable but non cultivable); whereas living cells stained by PI gave 0.08 % alive cells, 9.49 % of dead cells, 0.11 % of intermediate cells and 90.32% of non stained cells (LL). Data obtained (**figure 3)** with fully dead cells were 0.0 % of dead cells, 0.0 % of intermediate cells; 99.99% of non-stained cells (LL) and 0.01 % living cells for cells stained with cFDA; whilst 99.98 % of dead cells, 0.0 % of intermediate cells and 0.0 % alive cells for cells stained with PI.

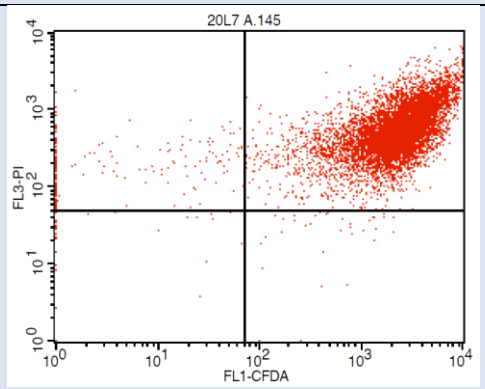
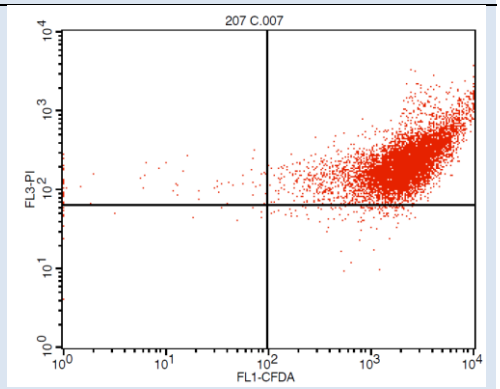
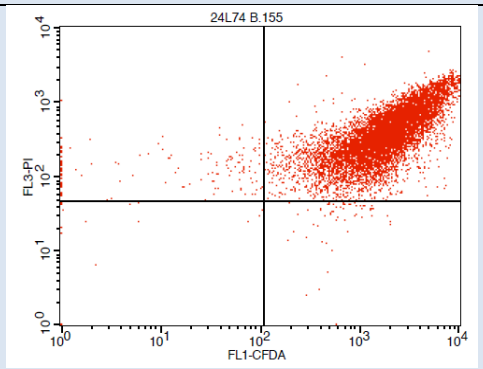
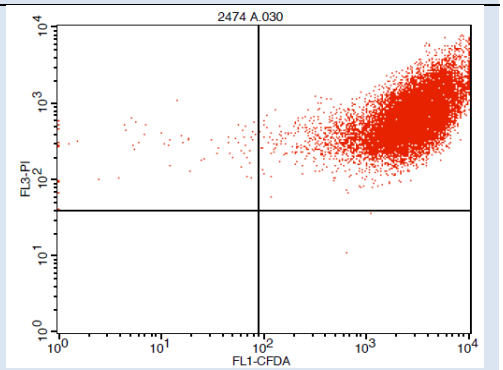
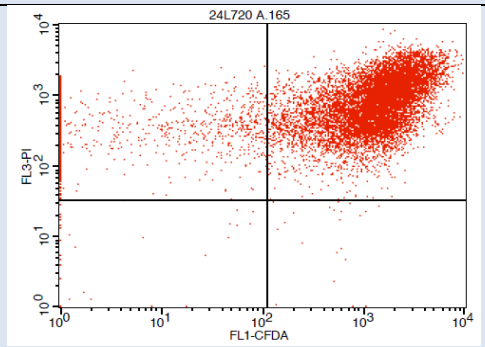
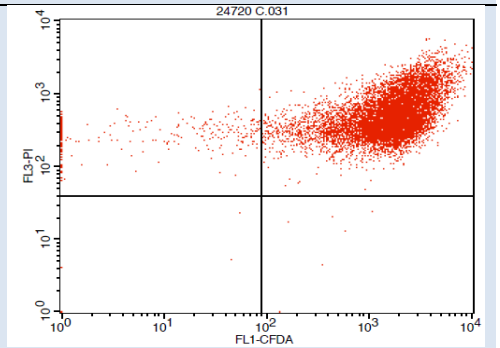
Time (days)	Storage Tempe-rature (°C)	Drying process	
		Freeze-drying	Spray-drying
1	4°C or 20°C		
90	4°C		
	20°C		

Figure 3: Flow cytograms of CWBI-B1465 *Leuconostoc mesenteroides* in storage. Cells were freeze-dried and spray-dried, stored at two temperatures 4 and 20°C during 90 days. Flow cytograms shown here were analysed at the initial time (day 1) and the end of storage time (day 90).

These findings enabled us the use of the double staining cFDA/PI for a live/dead discrimination, as live/dead assays with two differentially staining probes are attractive because detection is easier when all cells are labeled (Bunthof *et al.*, 2001). After staining with the fluorescent probes carboxyfluorescein diacetate (cFDA) and propidium iodide (PI), flow cytometry analyses were carried out at the first day and after 15, 30, 60, 90 days. A comparative overview of cell viability of freeze-dried cells versus spray-dried cells showed that for the two dried processes, the high percentage of cells shifts from alive cells to

intermediate cells (viable but not cultivable). Indeed, after the drying process, the physiological state of dried cells as illustrated by cytograms on figure 3 showed that relative percentages of freeze-dried/spray-dried cells were 3.05/3.37 % of dead cells, 95.24/97.13 % of intermediate cells, 1.29/0.84 % alive cells. These data showed that many cells are in an intermediate state but not dead as found in other previous studies (Mputu-Kanyinda *et al.*, 2012; Rault *et al.*, 2007). For spray-dried cells, the relative percentages stay stable at 4°C. The evolution occurred at 20°C as following: dead cells increased from 2.30 to 4.66 %, intermediate declined from 97.13 to 95.29 %, alive cells decreased from 0.46 to 0.03 %. These values seemed to be highly low and this strain exhibited a very good resistance in terms of the process yield.

From these observations, the freeze-drying process is the most suitable for preservation of CWBI-B1465 *Leuconostoc mesenteroides*. However, the differences are very low so that the spray-drying seems very interesting in regard to the process costs. The evolution in storage for 90 days showed stability at 4°C, whereas at 20°C, dead cells increased from 3.05 to 4.79 %, intermediate cells decreased from 95.24 % to 94.89 % and alive cells decreased also from 1.29 % to 0.16 %.

3.3.3. Soluble proteins (SP)

Analyses of protein contents in freeze/spray-dried powders stored at 4°C and 20°Cwas also carried out on 90 days. Measurements were made at day 1 (after the drying process), day 15, day 30, day 60 and day 90. Results of dried cells (**figure 4**) stored at 4°C showed that there is a slight decrease for freeze-dried cells 88 % to 87 % and from 88 % to 85% for spray-dried cells which is not significant in terms of the process yield.

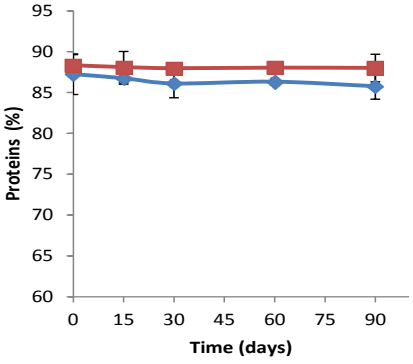


Figure 4. Soluble proteins content (%) during 90 days storage at 4°C of CWBI-B1465 *Leuconostoc mesenteroides* (■ freeze-dried and ♦ spray-dried).

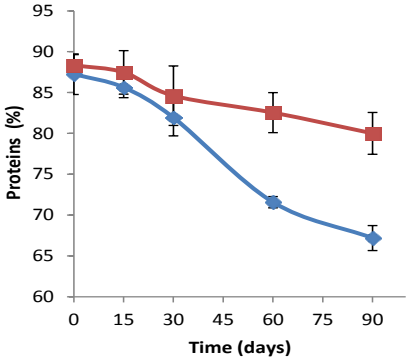


Figure 5. Soluble proteins content (%) during 90 days storage at 20°C of CWBI-B1465 *Leuconostoc mesenteroides* (■ freeze-dried and ♦ spray-dried).

However, for cells stored at 20°C (**figure 5**), the decrease of freeze-dried cells was in the range of 88 % to 80 %, whereas the range was very high from 88 % to 64.19 % for spray-dried cells. In all cases, the decrease is an indication of protein oxidation, which is expressed by protein insolubilisation. And the slope of this phenomenon showed that the insolubilisation is higher in freeze-dried cells than in spray-dried cells.

3.3.4. Carbonyl proteins contents (CP)

The carbonyl contents in freeze/spray-dried cells were compared as for soluble proteins. Freeze-dried cells have more carbonyl contents than spray-dried cells, about 3 folds. And we observed a slow growth during the storage at 4°C in the two cases. The growth was very high at 20°C storage also in both cases. An increase of carbonyl contents is an indication of the oxidation phenomenon.

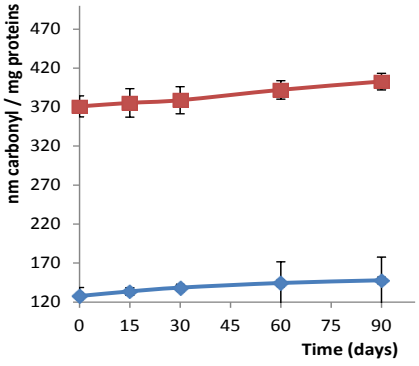


Figure 6. Protein carbonyl content (nmol/mg) during 90 days storage at 4°C of CWBI-B1465 *Leuconostoc mesenteroides* (♦freeze-dried and ■ spray-dried)

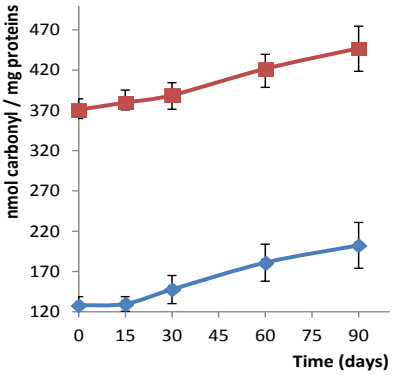


Figure 7. Protein carbonyl content (nmol/mg) during 90 days storage at 20°C of CWBI-B1465 *Leuconostoc mesenteroides* (♦ freeze-dried and ■ spray-dried)

4. Conclusion

The dried bacteria are subject to relative high viability loss from the concentration, and through the drying process as well as during storage. The strain CWBI-B1465 *Leuconostoc mesenteroides* can be produced in bioreactor in biomass concentration of 10^{10} cfu.g^{-1} by batch mode. In order to increase this biomass, the application of fed-batch mode is required. The generation time is therefore near 1.2 h, which stays low than Lactococcus or Lactobacillus strains. It is however in the range of growth characteristic of Leuconostoc strains. Cells produced can be either freeze-dried or spray-dried, as this strain showed good yields in both cases.

5. Acknowledgement

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Chapter 7

Production and Stability of *kivuguto* milk

Le chapitre sept traite de la production du lait formulé et étudie sa conservabilité. Elle vérifie en fait les propriétés du kivuguto étudiées au chapitre quatre lors de la fermentation pour évaluer leur stabilité. La post-acidification est suivie sur 36 jours, ainsi que la viabilité des cellules en stockage sous réfrigération. Sont aussi analysées l'évolution des propriétés rhéologiques, des composés volatils et de l'activité protéolytique.

*Les résultats constituent un article soumis pour publication et intitulé "**Manufacturing of kivuguto milk and stability in storage under refrigeration**".*

European Scientific Journal

Manufacturing of *kivuguto* milk and stability in storage under refrigeration

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ABSTRACT

The *kivuguto* milk was processed in a 20 liters bioreactor with three bacteria previously selected in *kivuguto* traditional milk. The aim of this work was the study of its stability during storage under refrigeration. Post-acidification, viability, proteolysis, flavor compounds as well as rheological characteristics were monitored for 36 days. The pH decreases from 4.54 to 4.45 and the titratable acidity grew from 73°D to 79°D. The final biomass after storage was 0.60 10^8 cfu.g^{-1} which is far higher than the recommended 10^6 cells before consumption. The proteolysis was at a range of 3.0 to 7.0 mg.L^{-1} of lysine equivalent, which is too low so that it can't produce bitter peptides. The evolution of flavor compounds in storage showed that no change found with 3-methylbutan-1-ol, acetic acid and furan-2(5H)-one, whilst pentan-1-ol and furanmethan-2-ol increased slightly upon 24 days' storage. The complex viscosity decreased from 4 - 5.3 Pas before storage to 2.9 - 4.0 Pas corresponding respectively to the ratio G"/G' of about 0.3-0.4 with a very low variation. These data allowed the good preservation of *kivuguto* milk at 4°C on 36 days.

Key Words: Manufacturing, Stability, *kivuguto* milk, Lactococcus, Leuconostoc

1. Introduction

Fermented milk was first produced around 10000 years ago (Tamime, 2002) and approximately 400 generic names are applied to traditional and industrialized products (Campbell-Patt, 1987; Kurmann *et al.,* 1987).

Nowadays, progress in the area of the development of defined starter cultures has been driven by an increased awareness of commercial food safety and the search for novel flavors, textures or potential health benefits (Wullschleger, 2013). In the dairy industries, the production of fermented milk results from the introduction in sterile milk of starter culture. Thereafter, the pH values of milk decreased during the manufacturing process, from the time it was inoculated with bacterial cultures to the time when it was manufactured (O'Neil *et al.,* 1979; Sokolinska *et al.,* 2004). Once coagulated at acid pH, fermented milks are cooled and kept cold until they are sold to consumers. During the cold storage, the biochemical composition of this fermented milk must stay stable for quality and consumer acceptance. These include the acidity, the rheological properties as well as the organoleptic features. All these characteristics depend on the viable cells and their balance in the fermented milk. According to Codex Alimentarius Standards (FAO & WHO, 2011), these starter microorganisms shall be viable, active and abundant in the product to the date of minimum durability. For instance, it is generally accepted that yogurt contains 10^7 cfu of viable bacteria (*Streptococcus thermophilus* and *Lactobacillus bulgaricus*) per ml (Chougrani *et al.,* 2009).

In dairy industries, it is well known that a slow acidification of fermented milk goes on even under refrigeration (Accolas *et al.,* 1977) for some microorganisms and a high rate of post-acidification affects the cells viability in storage. Studying the use of probiotic bacteria as starter culture, Demerdash and Otiabi (2008) found how an increase in the acidity of the product during storage adversely affects viability. Damin *et al.* (2008) showed that the fermented milk prepared with a coculture of *Streptococcus* and *Bifidobacterium lactis* gave the most constant rheological behavior and the best cell viability during cold storage. An important factor affecting the rheological behavior of the casein coagulum is the production of exopolysaccharides (EPS) by the starter culture. These polymers confer firmness and texture to the final product (Moreira *et al.,* 2000). According to Purohit *et al.* (2009), the molecular characteristics of exopolysaccharides and their interaction with milk proteins determine the rheological and sensorial characteristics of fermented milk. Along with the post-acidification, the starter cultures in fermented milk during storage bring about some changes in the proteolytic system. This system is involved in the aroma generation, but also in

the bitter peptides occurring during milk acidification and post-acidification. According to Law and Kolstad (1983), the proteolytic system of starter bacteria consists of proteinases, which catalyze the hydrolysis of native or denatured protein molecules, and peptidase which catalyze the degradation of the smaller peptides produced by proteinases action.

The present paper deals with the production and the behavior of the *kivuguto* formulated milk in storage under refrigeration. Three strains were previously selected from samples collected in *kivuguto*, a traditional fermented milk of Rwanda (Karenzi *et al.,* 2012). The evolution of the acidification, viable cells, proteolysis, rheology and volatile compounds analyzed after the fermentation is quantified using instruments for the estimation of the first alteration indicators. The work is a part of sequential analyses with the objective of industrial production of *kivuguto* milk.

2. Materials and Methods

2.1. Fermentations

The production of the three samples was made as following:

- *Kivuguto* milk sample: the pre-culture was inoculated at 10^6 cells.mL^{-1} of milk as a total initial concentration. This inoculum was used to culture at 4% (vol/vol) a 20 liters fermentor with 16 liters of Joyvalle sterile milk as a working volume (it means that we prepared 640 mL of pre-culture for 16 L). This initial inoculum was composed by the three selected strains as previously reported: CWBI-B1466 *Lactococcus lactis*, CWBI-B1465 *Leuconostoc mesenteroides* and CWBI-B1470 *Leuconostoc pseudomesenteroides* at a ratio respectively of 40%, 35% and 25%.
- For the two fermentative *kivuguto* strains, CWBI-B1466 *Lactococcus lactis* and CWBI-B1470 *Leuconostoc pseudomesenteroides*, samples were made by two cultures for each strain. The fermentations were performed in monoculture at 10^6 cells.g^{-1} for the pre-culture used to inoculate at 4% (vol/vol) a 20 liters fermentor with 16 liters of Joyvalle sterile milk as a working volume.

The pre-cultures of Joyvalle sterile milk were prepared using 10^6 freeze-dried cells for monocultures (*Lactococcus* milk or *Leuconostoc* milk). For *kivuguto* milk (mixed strains), the pre-culture was also inoculated by a total of 10^6 cells (40% of *Lactococcus*, 35% of *Leuconostoc mesenteroides* and 25% of *Leuconostoc pseudomesenteroides*). Indeed, after the freeze-drying process, 1 g of each powder concentration was estimated by plating on solid

medium and the corresponding quantity for pre-culture preparation was weighed. The cultures were carried out at 19°C which is the average fermentation temperature in Rwanda. The incubation time was determined by the end of the acidification, meaning at the pH ≈4.5-4.6. The experiment was conducted in triplicate.

2.2. Post-acidification

Milk samples were stored at 4°C for 36 days in head-space (HS) vial (Filter Service, Eupen, Belgium) sealed hermetically with a polytetrafluoroethylene-coated rubber septum and an aluminum cap (Filter Service, Eupen, Belgium.The pH (pHmeter WTW pH351i, Weilheim, Germany) was measured and the titratable acidity (°D) was measured after incubation time (Tf) by titrating a 10 ml sample with NaOH (1/9N) using phenolphthalein as an indicator (Afnor, 1980). Measurements were also made after 12, 24 and 36 days.

2.3. Viability

After suitable dilutions, the enumeration of *kivuguto* milk strains was performed on MRS agar incubated at 30°C for 48 h. Viable cells counts were expressed as colony forming units per gram ($cfu.g^{-1}$) of milk. Four counts were carried out to determine the number of bacteria during storage: just after fermentation (at Tf), after 12, 24 and 36 days.

2.4. Rheological data evolution

The rheological parameters complex viscosity, elastic modulus (G') and loss modulus (G") of the milk samples were estimated at 10°C using a high resolution Bohlin CVO 120 rotational rheometer (Malvern Instruments, Worcestershire, UK). The measuring geometry employed was a rotating upper cone and a fixed lower plate (α=4°, $\varnothing$=40 mm). The oscillation frequency was 1.0 Hz, and the shear stress was 1Pa, which was found to be within the linear viscoelastic region of fermented milk samples according to Stern *et al.* (2008). Three replicates were applied.

2.5. Proteolysis

The proteolytic activity of the tested strains in the three milk samples stored at 4°C

was determined using the o-phthaldialdehyde (OPA) method (Church *et al.,* 1987). This method is based on the reaction of OPA and β-mercaptoethanol with the α-amino groups released during hydrolysis of milk proteins. The results were calculated from a standard curve obtained from dilution of leucine in distilled water and expressed in leucine equivalent ($mg.l^{-1}$) (Sigma Aldrich, Diegem, Belgium) of milk. Analyses were made in triplicate four times during the storage: day 1, day 12, day 24 and day 36.

2.6. Flavor stability

Flavor compounds were studied on 24 days by static headspace sampling and GC/MS analysis.

- **Headspace sample preparation**

Headspace (HS) samples were prepared manually. A milk sample (10 g) was introduced in a 20-mL HS vial (Filter Service, Eupen, Belgium) sealed hermetically with a polytetrafluoroethylene-coated rubber septum and an aluminum cap (Filter Service, Eupen, Belgium). The samples were kept at 4°C before analysis for a short time and those stored longer were put at −20°C and put at 4°C the day before analysis. Samples were equilibrated for 65 min at 70°C prior to analysis, and the volatile compounds trapped in the headspace region of the vial (2000 µL) were taken with a micro syringe (Filter Service, Eupen, Belgium) and analyzed by GC using direct gas injection.

- **Gas chromatography**

Milk samples volatiles (2000 µL) were injected into an Agilent Technologies 7890A GC System (Agilent Technologies, Santa Clara, CA, USA) equipped with a flame ionization detector (FID) and a 30-m x 250-µm x 0.25-µm VF-WAX polar column (Agilent Technologies, Santa Clara, CA, USA) was used for the study. Helium was used as the carrier gas, at a flow rate of 1.5 mL min^{-1}, and the splitless mode was used. The following temperature program was used: 50°C for 6 min, increased to 180°C for 5 min at a rate of 8°C min^{-1}, and held for 10 min at 15°C min^{-1} from 180 to 250°C. The injector and detector temperatures were 220 and 250°C, respectively.

- **Mass spectrometry analysis**

The volatile compounds were identified by mass spectrometry using an Agilent Technologies 5875C with Triple-Axis Detector coupled to 6890 GC System (Agilent Technologies, Santa Clara, CA, USA). The MS was carried out in EI mode, with an ionization potential of 70 eV, an ionization current of 2 A, an ion source temperature of 200°C, a resolution of 1000 and a mass range 30 to 450 m/z.

- **Chemical identification**

Compounds were identified by comparing recorded mass spectra with the Willey 275L mass spectra Library (Scientific Instrument Service, Ringoes, NJ, USA), the NIST MS Library (NIST, Gaithersburg, MD, USA), the PAL 600K Mass spectral Library (Palissade Corporation, Ithaca, NY, USA), and those in literature, as well as comparison of their retention times with authentic standards of saturated *n*-alcanes standard solution (C_7-C_{30} alkanes) (Sigma Aldrich, Diegem, Belgium), as external references under the same chromatographic conditions, allowing calculation of Kovats index (Kovats, 1987) of the separated volatile compounds (Harris, 1987).

- **Standard solutions and quantification**

Aqueous solutions of acetic acid, pentan-1-ol, and methyl benzoate as an external standards were prepared from high purity chemicals (>99%) purchased from Sigma-Aldrich (Diegem, Belgium). The mass of 40 µL of each standard was accurately measured and diluted in 100 mL in double-distilled water and thereafter mixed at a ratio of 1:1. The prepared solution was hermetically sealed in 20-mL headspace vials at -20°C until they were used. The compounds were quantified by external standard technique as previously described.

2.7. Statistical analyses

Results were analyzed by using analysis of variance to determine significant differences ($P \leq 0.05$) between mean values from three independent experiments.

3. Results

3.1. Post-acidification

Fermented milks were made in three types: with mixed *kivuguto* strains, with single cultures of CWBI-B1466 Lactococcus and with single culture of CWBI-B1470 *Leuconostoc*. The results of *kivuguto* milk titratable acidity are presented in **fig.1.**

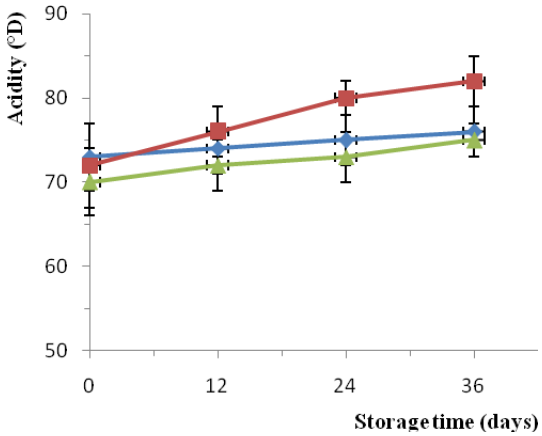

Figure 1. Evolution of titratable acidity in storage (at 4°C) of milk fermented by *kivuguto* selected strains (♦*kivuguto* milk, ■Lactococcus cultured milk, ▲Leuconostoc cultured milk)

For *kivuguto* milk, the acidity varied in storage from 73 to 76 °D. For *Lactococcus* milk, the titrable acidity evolution in storage was in the range of 72 to 82°D, whereas for *Leuconostoc* milk, the acidity changed from 70°D to 75°D. During the storage time, the pH of *kivuguto* milk changed slightly from 4.54 to 4.45 as illustrated on **fig. 2.** *Lactoccus* milk pH varied from 4.56 to 4.38, whilst the *Leuconostoc* milk pH decreased from 4.58 to 4.46.

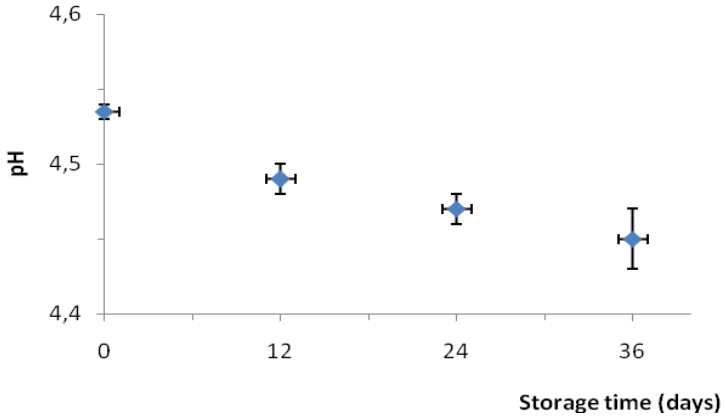

Figure 2. Evolution of pH of *kivuguto* milk during 36 days in cold storage (4°C).

3.2. Viability

During 36 days of cold storage, the viable cells in *kivuguto* milk decreased from 38.5 10^9 cfu.g^{-1} to 0.59 10^8 cfu.g^{-1} as shown in **table 1**.

Table 1. Evolution of cells viability of ****kivuguto* **fermented milk storage at 4°C

	Cells concentration (10^8 cfu.g^{-1})			
	Before storage (at Tf)**	**Day 12**	**Day 24**	**Day 36**
***kivuguto* milk**	38.5	35.0	2.7	0.6

*milk with mixed strains, ** Tf: end of fermentation

3.3. Rheological data evolution

The evolution of rheological properties complex viscosity, elastic modulus (G') and viscous modulus (G") as a function of time at 10°C during storage of *kivuguto* milk were followed on 36 days using a high-resolution Bohlin CVO 120 rotational rheometer (Malvern Instruments, Worcestershire, UK). Results are presented in **fig. 3, 4, 5 and 6** respectively at the end of fermentation (time Tf), at day 12, at day 24 and at day 36.

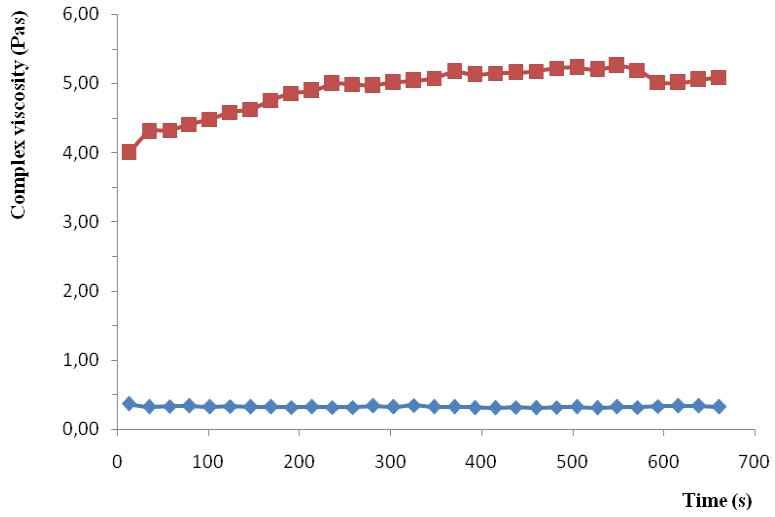

Figure 3. Viscosity of *kivuguto* after fermentation (■ complex viscosity in Pas; ♦ tan δ as the ratio G"/G').

At Tf, the ratio G"/G′ expressed as tan δ varies from 0.3-0.4 and the complex viscosity changes from 4 to 5.3 Pas, as shown on **fig. 3.**

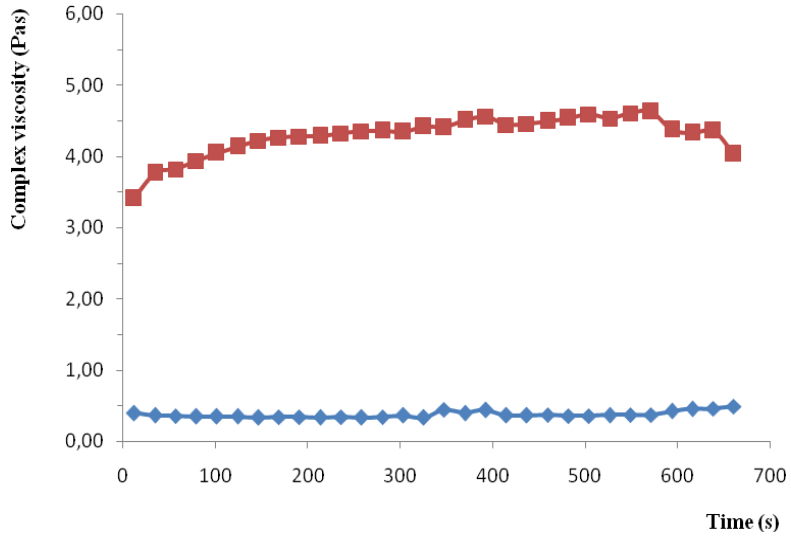

Figure 4. Viscosity of *kivuguto* after 12 days in storage (4°C) **(**■ complex viscosity in Pas; ♦ tan δ as the ratio G"/G').

After 12 days in storage, tan δ varies from 0.3-0.5 and the complex viscosity changes from 3.4 to 4.6 Pas, as shown on **fig. 4.**

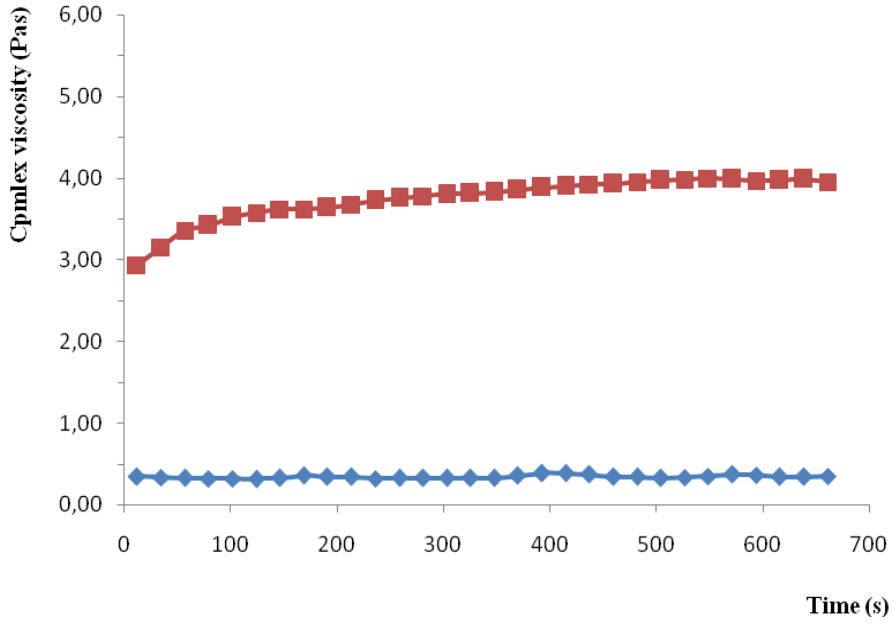

Figure 5. Viscosity of *kivuguto* after 24 days in storage (4°C) **(**■ complex viscosity in Pas; ♦ tan δ as the ratio G"/G')

After 24 days in storage, tan δ varies from 0.3-0.4 and the complex viscosity changes from 2.9 to 4.0 Pas, as shown on **fig. 5**.

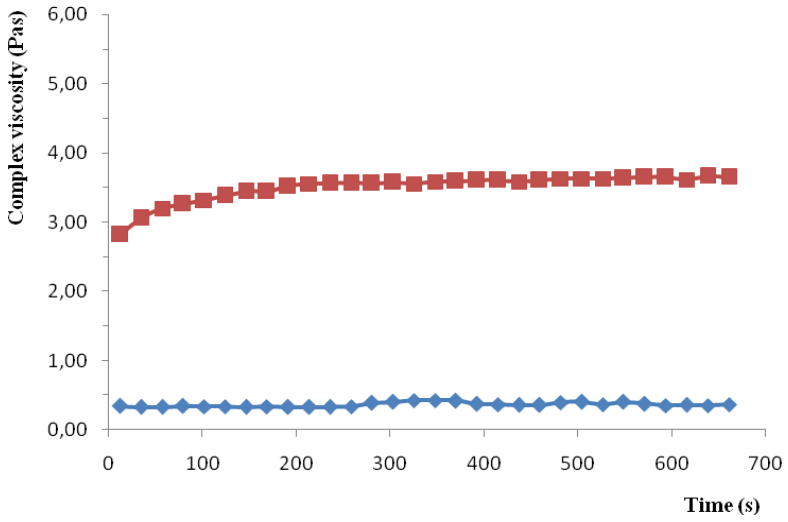

Figure 6. Viscosity of *kivuguto* after 36 days in storage (4°C) (■ complex viscosity in Pas; ♦ tan δ as the ratio G"/G').

After 36 days in storage, tan δ varies from 0.3-0.4 and the complex viscosity changes from 2.9 to 4.0 Pas, as shown on **fig.6**.

3.4. Proteolysis

The proteolytic activity of *kivuguto* strains in the 3 samples showed an increase more in single cultured milks than in mixed cultured milk as summarized in **table 2.**

Table 2. Proteolysis evolution of *kivuguto* milk & *kivuguto* strains milks in storage at 4°C

Milk	**Concentration of peptides/free amine acids in storage (mg/L)**			
	Before storage (at Tf)*	**Day 12**	**Day 24**	**Day 36**
Kivuguto	3.0 ± 0.7	4.9 ± 0.4	5.6 ± 1.0	7.0 ± 1.0
Lactococcus milk	3.1 ± 0.3	4.6 ±1.1	20.0 ± 0.1	39.4 ± 6.2
Leuconostoc milk	5.5 ± 0.2	21.3 ± 3.5	31.9 ± 3.4	42.8 ± 3.5
Sterile Joyvalle**	< 0	< 0	< 0	< 0

*Tf end of fermentation, **Sterile Joyvalle is the milk used for fermentation. Here, it was not inoculated

The increase of proteolysis in *kivuguto* milk was 2 fold after 36 days, 13 fold in *Lactococcus* cultured milk and varied from 5.5 to 42.8 in Leuconostoc cultured milk.

3.5. Aroma compounds stability

The volatile compounds (VCs) profile of *kivuguto* milk were extracted by headspace and identified by mass spectrometry using an Agilent Technologies 5875C coupled to 6890 GC System. It was composed of five main molecules of: 3-methylbutan-1-ol, pentan-1-ol, acetic acid, 3-methylbutan-1-ol and furan-2(5H)-one. The evolution of the 5 volatile compounds previously characterized was studied on 24 days for stability assessment. As illustrated in **table 3**, furan-2(5H)-one, acetic acid and 3-methylbutan-1-ol stayed stable on the period of storage, whereas pentan-1-ol carried out an increase of 18.6 % on 24 days. The VC furanmethan-2-ol content grew quickly up to 14.9% after 12 days and stays stable until 24 days.

Table 3. Evolution of VCs in *kivuguto* milk in storage on 24 days.

t_r [1]	CAS [2] Number	IUPAC Name	Identification [3]	VCs in *kivuguto* milk (%) [4]			Sample RI [5]	Reference RI [6]
				0	Day 12	Day 24		
8.46	123-51-3	3-methylbutan-1-ol	MS, STD, RI	11.5±0.6	13.58±1.4	12.95±0.4	1202	1204 [a]
8.92	71-41-0	pentan-1-ol	MS, STD, RI	18.7±0.3	21.98±0.3	22.21±0.1	1226	1244 [b]
14.26	64-19-7	acetic acid	MS, STD, RI	4.4±0.4	4.13±0.4	5.0±0.6	1473	1477 [c]
16.61	93-58-3	methyl benzoate	MS, STD, RI	4.0±0.0	- [7]	- [7]	1613	1635 [d]
17.14	98-00-0	furanmethan-2-ol	MS, STD, RI	39.9±1.6	45.9±0.6	44.6±0.3	1650	1661 [e]
17.91	92618-89-8	1,7,7-trimethylbicyclo [2.2.1]hept-2yl acetate	MS, STD, RI	5.8±0.0	- [7]	- [7]	1699	1584 [f]
18.49	497-23-4	furan-2(5H)-one	MS, STD	15.7±1.0	14.5±0.1	15.2±0.4	1739	- [8]

[1]Retention time; [2]CAS number of compounds listed in order of elution from a VF-Wax. Source: CAS Scifinder® (Chemical Abstracts Service, Colombus, USA); [3]Identification methods: MS, comparison of mass spectra with those in Nist 08, Wiley275 and PAL 600K libraries; RI, comparison of retention indices with those in literature; STD, comparison of retention time and mass spectra of available standards; [4] *kivuguto* milk relative contents (%): milk with 3 *kivuguto*'s strains; [5]Retention indices on VF-Wax column experimentally determined using a saturated C7-C30 alcanes standard solution; [6]Kovats indices taken from literatures: [a]Fukami *et al.* (2002) (measured with a TC-Wax column); [b]Umano *et al.* (2002) (measured with a DB-Wax column); [c]Cullere *et al.* (2004) (measured on a DB-Wax column); [d] Ferreira *et al.* (2001) (measured on a DB-Wax column); [e]Wong & Bernhard (1988) (measured on a DB-Wax column); [f] Davies (1990) (measured on a Carbowax column); [7]not calculated as it do not come from starters used; [8]not found.

4. Discussion

In this study, using three strains selected in traditional *kivuguto* [18], three fermented milks were processed: one with mixed strains (*kivuguto* formulated milk), two with two *kivuguto* fermentative strains in monoculture. The investigation was followed with 5 parameters sustaining the quality of fermented milk. By monitoring the change of these parameters in our samples in storage under refrigeration, our objective was to predict the level at which they can be unacceptable for consumption. These parameters are:

(i) the acidification as its evolution bring about many changes both in taste and in viable starters in milk, and we measured along the storage time the pH and the titratable acidity (°D);

(ii) the viability as living biomass is very important for evaluating the quality of a dairy product, for instance, the IDF (1992) suggests for the minimum level for bacteria in probiotic milk a range of 10^6 to10^7 cfu.mL^{-1} in order to produce therapeutic benefits (Moayednia *et al.,* 2009);

(iii) the proteolysis as a beneficial property at a certain level, unless, it is responsible of flavor defects;

(iv) the rheology as consistence and firmness variations leads to negative properties such as syneresis and atypical texture. It is obvious that the rheological properties influence the texture which in turn affects the sensory perception and ultimately the acceptance of a product by the consumer (Aichinger *et al.,* 2003) and;

(v) the aroma and flavor attributes whose stability in milk guides a favorable effect on customer's decision until the final day of storage. And this was complemented by a casual sensory evaluation made for detection of any appearance of noticeable bitterness or off-flavor at each stage of storage.

The post-acidification was estimated by pH and titratable acidity. The evolution of acidity of *kivuguto* milk showed insignificant changes in pH as presented on **fig. 2**. The decrease from 4.54 to 4.45 is almost tolerated by the majority of local *kivuguto* customers. Even fresh fermented milks can reach these values or more during manufacturing (Sokolinska *et al.,* 2004; O'Neil *et al.,* 1979). At the same time, the titratable acidity increases slightly up to 79°D in some samples, which represents 8.2 %. This value is very low compared with other data in literature. For instance, titratable acidity increased on average by 22.3% in the yogurts and by 14.9% in the yogurt-related products during storage (Kneifel *et al.,* 1993).

For the *Lactococcus* cultured milk, it reached 85°D. This is in agreement with its acidifying capacity, as the best acidifier among the three strains of *kivuguto* starter. But the titratable acidity of *kivuguto* strains is very low compared to those of yogurt. Indeed, the titratable acidity of yogurt reaches 101.5°D (Chougrani *et al.,* 2009; Salvador and Fiszman, 2004; Gueimonde *et al.,* 2003). And it is in the range of wild *Streptococcus thermophilus* and *Lactobacillus delbrueckii* subsp.*bulgaricus* isolated by Soomro and Masud (2008) in *dahi*. Indeed, they found values ranging from 69°D to 77°D in milk fermented by each strain.

The explanation is only based on the capacity of *Lactococcus* to grow in milk. It needs only 7 amino acids to grow, when *Leuconosto*c needs 9 amino acids. Generally, *Lactoccus* is a good acidifier. Specifically, it acidifies milk more than *Leuconostoc*.This is due to its capacity to hydrolyze milk caseins using its extracellular proteases. *Lactococcus* also has endocellular proteinases which allow it the degradation of the peptides formed during caseins catabolism.

The growth rate of *Lactococcus* is very important in dairy technology as it reduces quickly the lactosis in lactic acid, and subsequently reduces the pH with the formation of the curd. The counts of bacteria in the fresh *kivuguto* milk were about 3.9 10^9 cfu.ml^{-1}, and the count at the end of storage was 5.9 10^7 cfu.ml^{-1}.

After 24 days, viable counts were 2.7 10^8 cfu.ml^{-1}, which is far from the recommended level of 10^6 cells during shelf life in storage. Akalin *et al.* (2004) found the highest viable number of bifidobacteria of 3.6-2.3 10^7 cfu.g^{-1} in the milk containing *B. animalis* and fructooligosaccharides and viability of *B. longum* in yogurt containing fructooligosaccharides remained above 10^6 cfu.g^{-1} for up to 21 days.

The rheological parameters evolution in storage were followed using a high-resolution Bohlin CVO 120 rotational rheometer (Malvern Instruments, Worcestershire, UK) coupled to a computer, allowing a real time recording of data during analyses: the time, temperature, frequency, phase angle, complex modulus, elastic modulus G', viscous modulus G", complex viscosity, shear stress, strain. The visco-elastic data of the samples were characterized on 600 s (10 min) using a couple of two parameters: tan δ and the complex viscosity. From the data of elastic modulus G' and viscous modulus G", the ratio G"/G' or tan δ was calculated and plotted with the complex viscosity against time. The initial complex viscosity was between 4 and 5.3 Pas, whilst tan δ was about 0.3. At day 12, the complex viscosity varies from 3.7 to 4.6 Pas and tan δ increases about 0.3 to 0.5. The decrease of viscosity was also observed at day 24 at a range of 2.9 to 4.0 Pas, corresponding to tan δ of 0.3 to 0.4. Only on 24 days, a correlation with the pH decline from 4.54 to 4.45 can explain the observed decrease of complex viscosity. Correspondingly, the live biomass began a big drop from 35.0 10^8 cfu.g^{-1} at day 12 to 2.7 10^8 cfu.g^{-1} at day 24, until to reach, 0.6 10^8 cfu.g^{-1} at day 36. At this level, the cell lysis didn't bring bitter peptides in milk as confirmed by routine tastes along the storage period and confirmed by instrumental analyses. Note that the general flavor is a result of the equilibrium of small peptides and other molecules released in the milk during the acidification and the storage as well. Interestingly, tan δ at day 36 was about 0.3 to 0.40. This value shows a stability comparatively to the variation at day 24. The reduction of tan δ is subsequent to the increase of G' and this make the milk more firm according to Kristo *et al.* (2003). This fact is in agreement with the equilibrium of the three strains involved in *kivuguto* fermentation in storage. At low pH, CWBI-B1470 *Leuconostoc pseudomesenteroides* and CWBI-B1466 *Lactococcus* begin to decline, whilst CWBI-B1465 *Leuconostoc mesenteroides* resists well. This strain also produce exopolysaccharides, bearing the firmness up to day 36 in the milk. It seems that its activity is not fully stopped during storage. However, the low variation of pH,

the titratable acidity and the viable cells in *kivuguto* milk is in agreement with the usual behavior of the strains found in *kivuguto* milk and their association in milk.

Extending the correlation to proteolysis and aroma compounds, the equilibrium encountered in *kivuguto* milk versus *Lactococcus* cultured milk and *Leuconostoc* cultured milk revealed the importance of the strain CWBI-B1465 *Leuconostoc mesenteroides.* In *kivuguto* milk, the proteolysis (table 2) varies from 3.0 to 7.0 $mg.L^{-1}$, which is very low comparatively to *Lactococcus* cultured milk and *Leuconostoc* cultured milk, with high values reaching about 40 $mg.L^{-1}$. It seems that the third strain of *kivuguto* milk, the strain CWBI-B1465 *Leuconostoc mesenteroides,* is a good stabilizer throughout the storage time. This is also highlighted by its capacity in aroma compounds stability in *kivuguto* milk. Over three weeks' storage, the variability in aroma compounds (table 3) was also very low for the 5 volatile compounds (VCs) identified. In a previous study, we have shown that this strain produce the majority of *kivuguto* VCs.

5. Conclusion

This work showed the behavior of *kivuguto* milk in storage with regard to post-acidification, viability, rheology, proteolysis and flavor profile stability. The interaction of the three strains forming the *kivuguto* starter culture revealed the activity of each strain. However, it is paramount to pursue a distinctive work focusing only to the interaction of these bacteria in milk fermentation process.

The presence of *Leuconostoc* strains in the *kivuguto* starter culture revealed the importance of this genus in dairy industry. On overall basis, the present work has shown the importance of a good association during the selection of starter cultures, which allowed a stable milk upon the storage period.

6. Acknowledgements

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Chapitre 8

Discussion et Conclusion générales

*Le **chapitre 8** discute globalement les résultats obtenus et présente une conclusion générale de ce travail. Il esquisse ensuite les perspectives de cette recherche.*

Chapitre 8

Discussion et Conclusion générales

8.1. Discussion générale

8.1.1. Problématique et contexte de la recherche

Le présent travail porte sur la production industrielle du *kivuguto,* lait caillé artisanal du Rwanda. Il aura fallu, à cet effet, mettre au point un *ferment lactique,* à partir des isolats du *kivuguto* artisanal. Sur le marché local, divers laits abusivement appelés *kivuguto* existent, car, en langue locale, ce mot signifie lait fermenté. Le *kivuguto* est consommé aussi bien en milieux urbains qu'à la campagne. Dans la seule ville de Kigali, plus de 1500 kiosques à lait servent de lieux de consommation. On y vend aussi du lait frais, du thé, du café et autres denrées. Forts de cette niche inestimable de marché, des industriels ont à ce jour installé environ 10 laiteries. La tendance est encore plus développée en Ouganda, en Tanzanie, au Burundi et au Kenya, voire dans toute la région qui compte, pour le moment, plus de 130 millions d'habitants. Avec l'urbanisation galopante, la production artisanale n'est plus à la hauteur de la demande. Malgré les variantes du vrai *kivuguto,* les populations préfèrent consommer leur lait traditionnel.

La très jeune unité des Biotechnologies de l'Université Nationale du Rwanda se devait donc de faire face à la problématique ainsi posée. A cet effet, la stratégie a été de mener la collecte des échantillons loin des milieux urbains, en vue d'éviter les ferments utilisés dans les industries laitières. L'isolement des micro-organismes impliqués dans le processus fermentaire, leur purification et leur caractérisation, ont permis d'obtenir des souches pures. Celles-ci devaient constituer la base de l'étude de mise au point du starter du *kivuguto*. Rappelons que la stratégie globale de recherche adoptée est fondée sur quatre thématiques:

- La sélection microbienne dans l'environnement naturel du *kivuguto;*
- Les propriétés technologiques des micro-organismes sélectionnées;

- La production et la conservation de ces micro-organismes;
- La production et la stabilité sous réfrigération du *kivuguto.*

8.1.1.1. Le lait fermenté

Le lait est un produit des mammifères femelles. Chaque espèce produit un lait parfaitement adapté aux besoins de son petit, avant la consommation des aliments. Seul l'homme continue d'en consommer après, et même à l'âge adulte. Il est très riche en nutriments utiles pour le veau au point de doubler son poids à la naissance en 47 jours, le bébé en 4 à 5 semaines, le raton en 6 jours. Mais le lait s'altère très vite, s'il n'est pas conservé dans de bonnes conditions. Il est consommé frais, ou après fermentation (Gningue *et al.,* 1991) dans la plupart des pays du sud. Dans ce dernier cas, il donne une variété de produits dérivés, comme le lait fermenté, le fromage, le beurre, etc., grâce à l'action des micro-organismes. Certaines variétés de fromage peuvent se conserver jusqu'à 5 ans (Kongo, 2013), alors que le lait frais peut à peine dépasser quelques heures.

Dans les pays tropicaux, éviter l'altération du lait constitue un défi majeur, car les conditions de conservation sont très coûteuses. La seule façon accessible d'économiser le surplus de production est la fermentation, qui aide à la conservation du lait par la suppression de l'altération et des micro-organismes pathogènes (Mokua, 2004). Elle est aussi pratiquée partout dans le monde, car les produits laitiers fermentés sont très variés et très riches en saveurs et en arômes agréables. Le lait fermenté traditionnel reste très populaire (Abdelgadir, 2001; Gningue *et al.,* 1991; Caicike *et al.,* 2010; Gonfa *et al.,* 2001; Savadogo *et al.;* 2004). Il est consommé à la fois comme nourriture et comme boisson (O'Mahony, 1988; Akabanda *et al.,* 2010; Motarjemi and Nout, 1995; Gonfa *et al.,* 2001). Les produits laitiers fermentés jouent un rôle multiple pour les populations rurales africaines. Le beurre, par exemple, est utilisé comme cosmétique (Gonfa *et al.,* 2001; Karenzi *et al.,* 2013).

Dans la plupart des pays africains, la vache tient un rôle de premier plan dans la vie économique, sociale et culturelle. Les Massai du Kenya et de Tanzanie ne vivent, pratiquement, que du seul lait comme aliment et comme boisson, pour l'essentiel de leur vie. Un Massai consomme, en moyenne, 3 à 5 litres de lait par jour (Isono *et al.,* 1994). L'élevage du bétail constitue leur activité principale. En Afrique de l'est (Tanzanie, Kenya, Ethiopie, Ouganda, Somalie, Soudan, Sud-Soudan, Rwanda, Burundi, Est du Congo), le lait, plus particulièrement le lait fermenté, constitue le petit-déjeuner prisé par la majorité de la

population. Des kiosques à lait sont éparpillés dans toutes les agglomérations urbaines. Très tôt dans la matinée, nombre de personnes se bousculent pour aller en prendre en moyenne un demi-litre ou plus, selon les moyens. En milieu rural, seul le manque de moyen empêche les gens de consommer ce produit tant apprécié par la population, et les gens travaillent durement pour s'acheter leur première vache.

Sans jouir d'une vénération comme on en observe en Inde, la vache constitue, au Rwanda, un véritable foyer culturel. Mais si la vache rwandaise est très respectée, elle n'est en aucun cas sacrée (Torrens, 2004). Il n'existe surtout pas, dans les traditions du Rwanda ancien, de divinisation de la vache, comme on a pu en constater en Inde ou en Egypte. Hathor, la Déesse-vache du Panthéon égyptien, porte le disque solaire entre deux larges cornes, qui ne sont pas sans rappeler celles des Ankolé du Rwanda (Torrens, 2004), du Burundi, de l'Ouganda.

Photo: Torrens (2004)

Selon Sebasoni (2000), la vache est le don et le contre-don par excellence: il faut donner une vache pour avoir son épouse. On reçoit, de ses parents, une vache, quand on vient leur montrer le fruit du mariage. Il faut donner une vache pour sceller une amitié. Il faut donner une vache comme cadeau de bienvenue. Elle est notamment appréciée pour son lait, sa viande, son sang, sa prestance, son importance culturelle, la beauté de ses cornes ou la couleur de sa robe (Singiza, 2011). Et de tous temps, l'élevage a joué un rôle très important pour les ménages rwandais. Lorsque la monnaie n'existait pas, les vaches avaient un rôle économique essentiel: elles constituaient en effet la monnaie la plus forte. Au XIVe siècle, des troupeaux suivaient les armées partant pour la guerre (Burette and Mbanzamihigo, 2009).

Fidèle à sa tradition pastorale, le Rwanda a vu ainsi, au cours des siècles, l'élevage bovin tenir une place importante dans la vie économique et socio-culturelle. Perchées entre 1.000 et 4.500 m d'altitude, avec une température moyenne annuelle de 19°C, une pluviométrie moyenne et un réseau de rivières et de ruisseaux qui sillonnent toute l'étendue du territoire, une multitude de lacs, ses « mille collines» et ses flancs constituent, du nord au sud, et de l'est à l'ouest, d'excellents prairies et pâturages, biotope idéal pour le bétail. La consommation du lait et de ses dérivés constitue, pour une part essentielle, une source en protéines animales. Le lait

fermenté était produit de façon traditionnelle jusqu'en 1984, date de la première laiterie moderne. Aujourd'hui, on compte plusieurs laiteries utilisant toujours des ferments importés. Mais le lait fermenté artisanalement reste très populaire (Schutte, 2013; Mathara *et al*., 2004; McMaster *et al*., 2005). Il est préparé par une fermentation spontanée, ou au moyen d'une culture de la précédente production (Harun-ur-Rashid et *al*., 2007, Shuangquan et *al*., 2004, Gonfa et *al*., 2001; Abdelgadir et *al*., 2001; Mutukumira, 1995, Beukes et *al*., 2001, Isono *et al.*, 1994), comme la plupart des laits naturellement fermentés dans le monde. Des études de laits naturels fermentés ont été menées dans plusieurs pays du monde: le yaourt en Bulgarie (Metchnikoff, 1908), le kéfir en Russie, le *didah* en Indonésie (Yodoamijoyo *et al.*, 1983; Hosono *et al.*, 1989), l'*amasi* au Zimbabwe (Feresu and Muzondo, 1990, Mutukumira *et al*., 1995; Gadaga *et al*., 1999), le *leben* au Maroc et en Tunisie (Hamama, 1992), le *zabady* en Egypte (El-Samragy, 1988), le *filmjölk* en Suède, le *dahi* indien (Bourre, 2010), le *maas* en Afrique du sud (Keller and Jordan, 1990; Beukes *et al.*, 2001); l'*ergo* et l'*ititu* en Ethiopie (Gonfa et al., 2001; O'Connor *et al*., 1993; O'Mahony, 1988); le lait fermenté des Fulani au Burkina Faso (Savadogo *et al*., 2004), le *nunu* au Ghana (Akabanda *et al.*, 2010), le *M'banik* au Sénégal (Gningue *et al.*, 1991), etc. Cependant, peu d'études ont abouti à la formulation biochimique des starters issus de ces laits fermentés.

8.1.1.2. Les ferments en technologie laitière

Les starters lactiques sont des préparations microbiennes actives intentionnellement ajoutées dans les produits à base de lait, pour la fabrication de produits laitiers (Chandan *et al.*, 2008), en vue d'une standardisation de leurs propriétés sensorielles, nutritionnelles et hygiéniques (Djemali and Guellouz, 2003). Les starters lactiques sont essentiellement constitués de bactéries lactiques et de bactéries non lactiques, de levures et de moisissures. Après les levures (Streit, 2008), les bactéries lactiques représentent le deuxième plus grand marché de biomasse. Alors que, dans les pays développés, les fermentations sont en majorité contrôlées lors de la fabrication de produits laitiers, elles sont, pour la plupart, spontanées, encore aujourd'hui, dans les pays en voie de développement. Le besoin de standardisation reste donc manifeste. Et la production de ferments à partir de micro-organismes isolés dans la très grande diversité de produits laitiers épars dans le monde est l'ultime solution pour la fabrication industrielle de ces produits.

8.1.2. Approche méthodologique de la recherche

L'objet de cette recherche est un lait fermenté artisanalement. D'une flore microbienne, se développe un écosystème, où des *réactions biochimiques* déterminent les caractéristiques et la qualité du produit fini. La mise en œuvre de la production en laiterie de ce lait fermenté doit tenir compte de cette flore microbienne et des *propriétés technologiques*, pour formuler un *starter lactique*. La stratégie adoptée agit sur l'isolement des micro-organismes impliqués dans la fermentation par la taxonomie phénotypique et moléculaire. Elle part donc de la méthode culture dépendante. Cette technique présente l'avantage d'utiliser des milieux sélectifs de synthèse permettant le développement microbien.

La pertinence de cette méthode est en soi exigée par l'étape suivante, qui consiste, en fait, à la production, en fermenteur des starters sélectionnés, par l'utilisation de milieux de culture. De ces milieux de culture, la propagation des cellules sélectionnées devient aisée. La technique permet, dès lors, de contourner les difficultés liées aux populations microbiennes viables non cultivables. Ces dernières ont été révélées par Lonvaud-Funel et Joyeux (1982), Froudière et *al*. (1990). Elle présente néanmoins le désavantage de la dépendance de l'état physiologique des populations. En effet, une souche réagit différemment aux tests phénotypiques selon le stade de croissance et les conditions de culture. En outre, des souches viables mais non cultivables ne sont pas prises en compte. Cependant, durant les dernières décennies, des milieux de culture performants ont été développés. Cela limite remarquablement la sous-estimation microbienne due aux méthodes de cultures dépendantes au détriment des méthodes d'identification moléculaire appliquées directement sur l'échantillon. Ces dernières méthodes viennent, par la suite, compléter les méthodes phénotypiques sur des isolats qui ont montré un fort potentiel technologique. Le grand mérite de cette approche est qu'elle limite sensiblement les coûts d'analyses moléculaires d'un grand nombre d'isolats issus de plusieurs milieux de culture. En effet, après des analyses d'acidification du lait et de croissance pour le screening des souches plus performantes, on procède aux essais-pilotes de formulation du starter, avant les analyses technologiques proprement dites. Ces essais-pilotes utilisent des isolats pré-sélectionnés en mélange de souches pour fermenter le lait. Ensuite, les laits présentant de grands défauts caractéristiques sont répertoriés, et les souches utilisées sont carrément éliminées très tôt. La méthode procède donc par élimination des souches, au lieu d'utiliser un grand nombre de souches pour choisir les plus performantes sur un grand nombre de paramètres technologiques. Il reste un nombre restreint de souches utilisées, dont les défauts

sont étudiés par la suite en stockage sous réfrigération. Les laits obtenus sont alors conservés à 4°C pendant 21 jours, délai légal correspondant à la date limite de conservation (DLC). Le défaut éventuel d'une souche devrait se révéler pendant cette période. Cette méthode limite les analyses classiques de comparaison de souches pour évaluer leurs potentialités. Enfin, une analyse instrumentale du lait formulé permet l'évaluation des principales propriétés technologiques. Ces tests sont effectués pour valider les résultats obtenus antérieurement. Ils permettent aussi une meilleure caractérisation de l'acidification, la viabilité cellulaire, la protéolyse, la production des arômes et la rhéologie, aussi bien en fin de fermentation qu'en stockage sous réfrigération. L'autre action de notre approche se focalise sur la production en fermenteur par la quantification du rendement de la production et de la viabilité en stockage des souches séchées. Cette série d'expérimentations requiert l'application de méthodes analytiques variées.

8.1.2.1. Taxonomie phénotypique et moléculaire microbienne

Nos échantillons ont été collectés dans deux fermes paysannes, loin des contacts avec les laits modernes fermentés, dans la Province du Nord, District de Musanze, Secteur Rwankeri, et dans la Province du Sud, District de Huye, Secteur de Muyange. L'échantillonnage a été réalisé dans le strict respect de la Norme FIL 122C, 1996. La norme FIL 122C concerne la préparation des échantillons et des dilutions en vue de l'examen microbiologique. Celle-ci a sa jumelle mise au point par ISO, qui reprend toutes les étapes de la norme FIL.

Huit milieux de culture ont été utilisés pour l'isolement. Le choix de ces milieux avait, pour cibles, les grands groupes microbiens les plus fréquents dans le processus de fermentation laitière. Une partie couvrait les microbes totaux, et des milieux plus sélectifs ciblaient les micro-organismes, de façon plus ou moins précise. A cet effet, on ajoutait, dans un milieu donné, des réactifs ayant un pouvoir sélectif ou, tout simplement, on modifiait les conditions de culture (le pH ou la température d'incubation), le tout en reconnaissant qu'aucun milieu ne peut être 100% sélectif, surtout avec des souches sauvages. Et dans l'ensemble, l'expérience de la FIL a guidé le choix opéré (FIL, 1997) avec sa norme FIL 147A, en matière de dénombrement sélectif de groupes microbiens dans les produits laitiers (Ninane, 2008).

L'expérience de la CWBI, en technologie microbienne, a été un plus dans ce choix, surtout pour la différentiation plus ciblée. Par exemple, l'ajout du raffinose, en lieu et place du glucose dans le Rogosa, pour la préparation de Rogosa raffinose, est une adaptation de

Gningue *et al.* (1991) pour la détection de *Lactobacillus plantarum*. Pour différencier les levures et bactéries, la morphologie des isolats a subi des tests microscopiques et biochimiques usuels. Et pour mettre en évidence les bactéries lactiques, les isolats ont subi la réaction à la catalase et la coloration de Gram. Et en complément, la méthode des tests API 50 CH (BioMérieux, Marcy-l'Etoile, France), qui est la plus classique des méthodes biochimiques a permis le groupement des bactéries lactiques en fonction de leur affinité à la consommation des carbohydrates fermentescibles (Boyd *et al.,* 2005). Les micro-organismes ont été testés sur 49 sucres de la galerie API 50 CH, et ainsi groupés en profils biochimiques. Le profil biochimique est comparé à des références standard, pour arriver à une identification (Hall *et al.,* 2001; Holzapfel et Stiles, 1997; Nishikawa et Kohgo, 1968). Les références sont rassemblées dans une base de données associée au logiciel APILAB PLUS V3.2.2 (BioMérieux, Marcy-l'Etoile, France). Créé en 1970, le système API s'est distingué dans l'identification microbienne en miniaturisant et en standardisant les techniques conventionnelles. Il associe une galerie de tests biochimiques et une base de données. Au terme de cette étape, une souchothèque a été constituée et conservée à -80°C de laquelle des souches pures de chaque profil ont été évaluées par des premiers tests technologiques, notamment l'acidification, la croissance en milieux synthétiques, la résistance aux conditions extrêmes de l'environnement (acidité, NaCl, température) et aux essais-pilotes en laboratoire de mélange de souches dans le lait. Malheureusement, si le système API est utile en complément des méthodes morphologiques, son pouvoir discriminant reste néanmoins très limité. En effet, il n'arrive pas toujours à distinguer tous les groupes bactériens jusqu'au genre (Bill *et al.,* 1992; Klinger, 1992), mais il parvient à grouper des souches bactériennes en profils biochimiques. Ainsi, des souches présentant potentiellement des qualités recherchées et incomplètement identifiées sont analysées aux tests moléculaires.

L'extraction de l'ADN, sa quantification et la PCR ont été réalisées suivant le protocole du fabricant Promega (Madison, WI, USA), qui fournit, par ailleurs, un kit de réactifs utilisés à cet effet. La zone-cible était le gène de l'ARNr 16S. En effet, les bactéries lactiques font partie des phyla bactériens qui ont été distingués d'après l'analyse des signatures oligonucléotidiques des ARNr 16S (Woese *et al.,* 1985). Et il existe aujourd'hui une convention générale d'utiliser la séquence d'ADN comme une référence standard pour déterminer la phylogénie et la taxonomie moderne doit se baser sur la phylogénie (Wayne *et al.,* 1987). La PCR était suivie du séquençage afin de déterminer la séquence. Et par le BLAST (Basic local alignment search tool) développé par Altschul *et al.* (1990) de la NCBI,

cette séquence a été comparée aux autres séquences de la base de données de Genbank/EMBL en vue d'une identification complète. Nous avons constaté que l'ADNr 16S distinguait jusqu'à l'espèce *Lactococcus lactis*, mais il n'est pas assez discriminatif pour nos souches de *Leuconostoc* pour les distinguer jusqu'à l'espèce. Notre approche a poussé encore plus loin dans la recherche d'un autre gène plus discriminatif. Dès lors, le gène cible était la zone intergénique 16S-23S ARNr qui a été en mesure de déterminer les séquences obtenues à un pourcentage satisfaisant.

8.1.2.2. Evaluation des paramètres technologiques

Les propriétés technologiques sont la caractéristique fondamentale en technologie laitière.
L'étude de ces caractéristiques détermine donc le choix des souches à sélectionner. Le paramètre étudié en premier lieu est l'acidification, car c'est à partir d'elle que découlent tout le procédé et toutes les autres propriétés. Lors d'une caractérisation technologique préliminaire effectuée pendant le screening, nous avons soumis nos isolats à l'acidification du lait et à la croissance en milieu synthétique. Toute souche utilisée en starter doit respecter deux critères pour être sélectionnable:

- elle doit être acidifiante et/ou au moins résister aux conditions acides du lait fermenté;
- elle doit être productible en fermenteur, c'est-à-dire qu'on peut facilement la propager en milieu synthétique.

Quatre paramètres ont été examinés pour l'évaluation des potentialités technologiques proprement dites au cours de cette recherche: acidification, rhéologie, protéolyse et arômes.

- **Acidification**

L'acidification constitue le caractère le plus important dans la sélection pour un starter lactique. En technologie de fermentation, c'est toujours par des souches acidifiantes que commence tout le processus de fermentation du lait. En effet, elles déclenchent la dégradation de caséines par les protéinases de l'enveloppe cellulaire. Les peptides libérés sont récupérés par ces cellules et dégradés ensuite par l'action des peptidases, qui ont plusieurs spécificités (Kunji *et al.,* 1996), et cela favorise la croissance cellulaire. Grâce à cette croissance, le lactose est fortement dégradé, diminuant ainsi le pH, en augmentant l'acidité qui va accélérer la coagulation. Il s'ensuit l'activation de la synérèse du caillé et la solubilisation du calcium

micellaire. L'acidité participe directement ou indirectement à la formation des arômes du lait fermenté. L'acidification peut être mesurée par quatre paramètres:

- la cinétique d'acidification qui détermine la vitesse d'acidification
- l'acidité ou pH final mesurée au moyen d'un pH-mètre
- la quantité d'acide lactique produit. Cette quantité est exprimée souvent en acidité Dornic qui résulte de l'acidité naturelle du lait donnée par ses protéines et minéraux. A cette dernière, s'ajoute l'acidité développée par la flore des ferments lactiques.
- la post-acidification qui caractérise l'acidité après fermentation ou l'acidité du lait sous réfrigération.

Nous avons mesuré l'acidification par l'acidité Dornic et le pH aussi bien en fin de fermentation qu'en post-acidification.

- **Rhéologie**

L'étude de ce paramètr**e** peut se faire par les analyses des exopolysaccharides produits dans le lait fermenté par les starters. En effet, certaines souches de bactéries lactiques sont capables de synthétiser des exopolysaccharides, des glucanes (dextranes), des fructosanes (levanes) ou des polysaccharides plus complexes qui constituent la capsule cellulaire (Leveau *et al.*, 1994). Mais les exopolysaccharides couvrent une infime partie des propriétés rhéologiques des laits fermentés. L'étude de ces propriétés peut se faire aussi par l'analyse de la texture, de la viscosité ou de la rhéologie. Souvent, la texture du lait fermenté se mesure par pénétrométrie au moyen d'un analyseur de texture. Les propriétés rhéologiques couvrent un domaine plus vaste, car traitant de l'écoulement des matériaux et de la déformation causée par une contrainte caractéristique des laits fermentés rhéofluidifiants. Nos échantillons présentaient une structure visco-élastique, c-à-d qu'ils possédent à la fois les propriétés visqueuses des liquides et élastiques des solides. Des tests rhéologiques à l'aide d'un rhéomètre étaient les mieux adaptés à ce type de fluide. Le rhéomètre est équipé d'un module à cylindres coaxiaux avec un rotor cylindrique. Les mesures peuvent se faire en mode harmonique ou par balayage en contrainte. Les paramètres mesurés sont ceux du modèle d'Hershel Burkley qui caractérisent bien les liquides rhéofluidifiants. On peut mesurer la viscosité complexe, le module de conservation ou module élastique G′, le module de perte ou module visqueux G", l'angle delta δ ou ratio G" sur G′. Au cours de ce travail, les tests rhéologiques ont été privilégiés au détriment de la viscosité produite par les exopolysaccharides (EPS).

- **Protéolyse**

Les starters lactiques possèdent un grand potentiel protéolytique qui leur permet de combler un déficit naturel en composés azotés. Ces enzymes protéolytiques sont des protéinases et peptidases pariétales, membranaires et cytoplasmiques. Les protéinases extracellulaires sont capables d'hydrolyser l'α et la β-caséine en peptides de haut poids moléculaire (Rattray & Fox, 1999). Les peptidases interviennent dès lors pour leur dégradation, libérant ainsi des acides aminés nécessaires à la croissance cellulaire. La protéolyse peut être bénéfique pour les produits laitiers quand elle est modérée. Une cellule à très forte activité protéolytique libère des peptides amers, qui altèrent les propriétés organoleptiques du produit laitier. Plusieurs méthodes sont utilisées pour l'évaluation de l'activité protéolytique. La mise en évidence de la production de l'ammoniac par les starters, le dosage de la fraction azotée soluble par la méthode Kjeldahl, ou d'enzymes spécifiques de la protéolyse, ou encore de peptides formés par HPLC sont des méthodes couramment utilisées pour caractériser l'activité protéolytique. Mais la technique la plus utilisée et la plus simple à mettre en œuvre est la méthode de Church *et al.* (1983). Elle est basée sur la réaction d'o-phthaldialdéhyde (OPA) et du β-mercaptoéthanol avec les groupements aminés libérés pendant l'hydrolyse des protéines du lait. Ces groupements forment un complexe qui absorbe fortement à 340 nm qu'on mesure par spectrophotométrie. Cette méthode a été privilégiée lors de nos analyses.

- **Les arômes**

Les composés volatils ont été analysés par la méthode statique d'espace de tête, couplée à la chromatographie en phase gazeuse (GC) et à l'identification par spectrométrie de masse (MS). La méthode est couramment utilisée pour l'analyse des yaourts (Alonso et Fraga, 2001).

8.1.2.3. Quantification et rendement de la production microbienne

Les souches sélectionnées ont été produites en fermenteur de 20 L en batch et/ou en fed-batch, en utilisant des milieux de propagation MRS pour les leuconostocs et M17 pour *Lactococcus* afin d'évaluer la quantité de biomasse produite. Nous avons ensuite concentré par centrifugation. Enfin, nous avons ajouté les cryoprotecteurs et procédé à la lyophilisation pour obtenir des poudres. La souche CWBI-B1465 a été aussi atomisée, car elle possède une

grande résistance à haute température.

8.1.2.4. Evolution de l'acidification et de la qualité du lait sous réfrigération

La post-acidification caractérise l'acidité du lait sous réfrigération. Nous avons mesuré l'évolution de l'acidification en stockage (4°C) par l'acidité Dornic (°D) et le pH. Ensuite, la qualité du lait en stockage a été suivie par la mesure de la protéolyse, des composés volatils, des propriétés rhéologiques et de la viabilité cellulaire.

8.1.3. Diversité microbienne des laits fermentés

Au cours de ce travail, la caractérisation microbienne a été abordée sur sept souches, toutes des bactéries lactiques: deux lactocoques et cinq leuconostocs. Après des essais de formulation de lait fermenté, trois souches se sont révélées plus aptes à entrer dans le starter proposé: CWBI-B1466 *Lactococcus lactis* comme acidifiant, CWBI-B1465 *Leuconostoc mesenteroides subsp.mesenteroides* comme aromatisant et CWBI-B1470 *Leuconostoc pseudomesenteroides* comme aromatisant et acidifiant.

Le dénombrement a montré la domination de souches *Lactococcus* sur les milieux sélectifs de ce genre et l'identification phénotypique l'a confirm**é**. Certes, un très grand nombre de lactocoques pousse sur le milieu M17 (Terzaghi & Sandine, 1975) utilisé pour l'isolement des lactocoques totaux, mais le milieu au jus de tomate avec 7.55 $\log_{10}$ ufc.ml^{-1} a prouvé que la majeure partie de ces lactocoques étaient en fait des *Lactococcus lactis*. Par contre, la domination observée en comptage de *Lactobacillus* sur le milieu Rogosa (Rogosa *et al.,* 1951) avec 7.63 $\log_{10}$ ufc.ml^{-1} a été invalidée par l'identification phénotypique et moléculaire, qui a montré que c'était bien des leuconostocs qui ont poussé sur Rogosa, au lieu des lactobacilles. Ces espèces se sont montrées très envahissantes, car elles ont même poussé en conditions particulières de Rogosa-raffinose (6.60 $\log_{10}$ ufc.ml^{-1}) à pH 5.4, 30°C en anaérobiose et de MRS (6.60 $\log_{10}$ ufc.ml^{-1}) à pH 6.1-6.4, 37°C en aérobiose. Ceci a été confirmé par un test de vérification qui a prouvé que les souches de *Leuconostoc* spp. poussent bel et bien sur ces milieux. Le dénombrement effectué sur le milieu de Mayeux (Mayeux *et al.,* 1962) a montré, sans conteste, que c'étaient des Leuconostoc produisant du dextrane seuls qui se développaient sur ce milieu. Néanmoins, il n'est pas assez riche pour obtenir un grand nombre de cellules (6.04 $\log_{10}$ ufc.ml^{-1}). Dans la littérature, plusieurs milieux ont été utilisés pour la

sélection microbienne dans le lait fermenté. Schutt (2013) a conduit une étude de dénombrement des laits fermentés suivants: *omashikwa* de Namibie, masse du Mozambique et *chekapmkaika* d'Ouganda, deux laits fermentés commerciaux *chambiko* du Malawi, et *omaere* de Namibie. Les plus grandes valeurs obtenues pour l'isolement des bactéries lactiques (LAB) étaient 5.26 $\log_{10}$ ufc.ml^{-1} sur le milieu KCA+V (potassium carboxymethyl cellulose agar avec 30 µg.ml^{-1} de vancomycine) pour le chambiko, 6.36 $\log_{10}$ ufc.ml^{-1} sur KCA+TTC (triphenyltetrazolium chloride) pour l'omaere, 6.79 $\log_{10}$ ufc.ml^{-1} sur KCA+TTC pour l'*omashikwa* et 3.30 $\log_{10}$ ufc.ml^{-1} sur MRS+C (C pour 100 µg.ml^{-1} de cycloheximide) pour *chekapmkaika*. Comparativement, les milieux utilisés par Schutt (2013) semblent peu riches en nutriments pour les micro-organismes cibles, avec un maximum de 6.0 $\log_{10}$ ufc.ml^{-1}. En fait, le milieu KCA pour l'isolement des lactocoques n'a pas d'extrait de viande, ce qui lui crée un déficit en acides aminés et en facteurs de croissance tant utiles pour le développement microbien. En outre, les nutriments vecteurs d'ions minéraux indispensables Mg^{2+} et Mn^{2+} ne figurent pas non plus dans la composition de ce milieu. Les dénombrements réalisés sur les autres laits fermentés d'Afrique de l'est sont supérieurs aux nôtres. Mathara *et al.* (2004) ont trouvé des valeurs moyennes de 8.0 $\log_{10}$ ufc.ml^{-1} au Kenya. Abdelgadir *et al.* (2001) ont trouvé presque les mêmes résultats, en plus des levures allant jusqu'à 7.6 $\log_{10}$ ufc.ml^{-1}. En Tanzanie, Isono et al. (1994) ont compté des bactéries lactiques de 7.0 à 9.0 $\log_{10}$ ufc.ml^{-1} dans le lait fermenté des Maasaï. Il convient de noter que Mathara *et al.* (2004) ont utilisé, dans leurs cultures, du MRS, M17 et du Rogosa, alors que Abdelgadir *et al.* (2001) ont utilisé MRS pour les lactobacilles, M17 pour les lactocoques et PDA (potato dextrose agar) pour les levures.

Nos dénombrements sont cependant similaires à ceux qui ont été faits dans certaines régions du Tibet (Caicike *et al.* (2010), quoique les comptages faits dans d'autres régions du Tibet soient légèrement supérieurs. La variabilité des concentrations microbiennes est due d'abord aux milieux de culture utilisés. Les populations faibles observées dans les laits d'Afrique australe seraient dues à l'exigence en nutriments des bactéries lactiques. Les conditions de culture peuvent aussi expliquer les différences observées. En cherchant, a priori, un groupe microbien donné, les milieux ont été modifiés au gré de chaque étude. Il est cependant très difficile d'affirmer que la région ou l'environnement serait à l'origine de cette variabilité. D'autre part, les conditions de transport des échantillons vers l'Europe peuvent avoir joué une grande influence sur les chiffres. Les identifications ont donné aussi des différences énormes quant à la diversité microbienne observée. Si, dans nos échantillons, *Lactococcus* est l'espèce

dominante, il représente la minorité des bactéries lactiques du lait du nord de la Tanzanie et du Kenya (14%), *Lactobacillus* restant le genre dominant à 55% (Mathara *et al.*, 2004), bien moins nombreux que le genre *Enterococcus* (25%). Le genre *Leuconostoc* semble aussi très peu représenté ailleurs, alors que dans le lait *kivuguto* il est bien caractéristique, et même dans des conditions de culture extrêmes. Le profil microbien du nunu du Ghana semble suivre presque le même schéma que celui du *kule naoto* du Kenya: *Lactobacillus* (53.52%), *Leuconostoc* (15.49%), *Lactococcus* (9.86%), *Enterococcus* (15.49%) and *Streptococcus* (2.82%) (Akabanda *et al.*, 2010). Schutt (2013) a identifié, dans le lait *chambiko* du Malawi, trois espèces dominantes de *Lactobacillus casei*, *Lb. paracasei* et *Lb. paracasei subsp.paracasei,* alors que dans *omaere* de Namibie, deux espèces de *Lactococcus lactis subsp.lactis* et *Lactococcus lactis subsp.cremoris* étaient identifiées. Il importe de noter que ces deux laits fermentés sont des laits commerciaux. Dans le lait fermenté traditionnel *omashikwa* de Namibie, Schutt (2013) a identifié *Lactobacillus helveticus*, *Lactobacillus kefiri*, *Lactobacillus casei*, *Lactobacillus rhamnosus* and *Lactobacillus paracasei*, ainsi que les genres Lactococcus, Leuconostoc and Enterococcus. Et dans le lait fermenté traditionnel *masse* du Mozambique, trois coques ont été identifiées: Lactococcus, Leuconostoc et Enterococcus. Les Leuconostoc étaient *Leuconostoc pseudomesenteroides*, *Leuconostoc lactis* et *Leuconostoc garlicum.* Seul *Lactobacillus helveticus* a été identifié dans le lait *chekapmkaika* d'Ouganda. Dans le *maziwa lala*, un autre lait fermenté du Kenya, Miyamoto *et al.* (1989) ont isolé *Lactobacillus plantarum, Lactobacillus curvatus, Lactococcus lactis subsp. lactis* et *Leuconostoc mesenteroides subsp. mesenteroides*. L'étude du *rob* du Soudan a montré la domination de *Lactobacillus fermentum* (34.5%), *Streptococcus salivarius* (30%), *Lactococcus lactis subsp. lactis* (26.4%) et *Lactobacillus acidophilus* (8.5%), ainsi qu'un grand nombre de levures dont *Candida kefyr* (22%) et *Saccharomyces cerevisiae* (78%) (Abdelgadir *et al.*, 2001). Cette diversité des populations levuriennes est également remarquée dans l'*amasi* du Zimbabwe, et dans le *nunu* du Ghana (Akabanda *et al.*, 2010), avec des espèces comme *Saccharomyces cerevisiae* 35.42%), *Saccharomyces pastorianus* (4.17%), *Candida kefyr* (33.33%), *Yarrowia lipolytica* (4.17%), *Candida stellata* (14.58%), *Kluyveromyces maxianus* (4.17%), *Zygosaccharomyces bisporus* (2.08%) and *Zygosaccharomyces rouxii* (2.08%). Les levures caractérisent aussi les laits fermentés du Caucase, comme le koumiss, l'ayran et le kéfir notamment *Candida friedrichii, Candida inconspicua*, *Candida maris*, *Candida tenuis*, *Pichia fermentans*, *Saccharomyces cerevisiae*, *Saccharomyces unisporus*, *Torasporula delbrueckii*, *Yarrowia lipolytica* et

Zygosaccharomyces sp. (Ottogalli *et al.*, 1973; Angulo *et al.*, 1993; Pintado *et al.*, 1996; Lin *et al.*, 1999; Barnett *et al.*, 2000; Simova *et al.*, 2002; Witthuhn *et al.*, 2004).

8.1.4. Les propriétés technologiques et leur stabilité

La sélection microbienne dans le lait fermenté, pour la formulation du starter, débute par la constitution d'une souchothèque, qui est constituée de souches isolées du produit en question. L'étape la plus importante concerne la caractérisation des isolats les plus aptes à entrer dans le processus de fermentation du lait. Quatre paramètres ont été analysés pendant cette étude: l'acidification, la rhéologie, les arômes et la protéolyse. Les propriétés technologiques sont des caractéristiques fondamentales en technologie laitière Elles constituent des critères ciblés par les sélectionneurs. Les souches les plus actives se distinguent par une rapide et immédiate formation de l'acide lactique au début de la fermentation, entraînant plusieurs réactions cataboliques, avec la diminution du pH de 6.6 à 4.6 au point isoélectrique des caséines. Les changements opérés pendant ce processus impliquent la dégradation des protéines du lait en acides aminés utiles pour les cellules en pleine augmentation de biomasse, caractéristique de l'acidification même. Ces phénomènes sont à l'origine de la formation de la saveur acide et de l'arôme caractéristique du lait fermenté. Des changements physico-chimiques concernent la texture, qui devient plus visqueuse. Au cours de notre étude, les analyses d'acidification ont été réalisées en trois étapes: lors du screening, pour comparer l'activité acidifiante des souches provenant des mêmes profils biochimiques suite aux tests API; lors des mélanges de souches, pour tester les diverses formulations, et lors d'analyses proprement dites en fioles de 3 litres, et en fermenteur de 20 litres, pour mesurer l'acidité du lait formulé, en fin de fermentation et en stockage sous réfrigération, pendant 36 jours, pour un suivi de la post-acidification. Des tests d'acidification du lait par des isolats issus de nos échantillons ont permis de mettre en évidence des souches acidifiantes à 30°C en fioles et à 19°C en fioles et en fermenteur. Deux groupes microbiens ont réagi différemment à ce paramètre. Les lactocoques se sont montrés plus rapides, en acidifiant le lait à partir de 4 heures et les leuconostocs à partir de 7 heures. A 19°C, la fermentation s'achevait après 14 h d'inoculation pour le *kivuguto* formulé. Les souches du *kivuguto* sont donc peu acidifiantes par rapport à celles du yaourt par exemple. En effet, les bactéries mésophiles offrent des profils cinétiques d'acidification plats par rapport à ceux des bactéries thermophiles (yaourt) qui sont pointus (Corrieu et Luquet, 2008). La rhéologie est une caractéristique qui détermine la qualité du lait

fermenté. Les mesures ont été faites en fin de fermentation et un suivi tous les 12 jours en stockage sous réfrigération. Cette caractéristique résulte tout d'abord de la teneur de la matière sèche totale du lait (Kristo *et al.,* 2003). En effet, la matière sèche totale affecte la fermeté et de la viscosité du lait fermenté (Biliaderis, Khan et Blank, 1992; Tamime et Robinson, 1985). Par ailleurs, lors de la dégradation du lactose, certains starters produisent des exopolysaccharides (EPS) qui vont jouer un rôle important sur la rhéologie en fin de fermentation et même lors de la conservation du lait sous-réfrigération. Dès lors, les variations entre les cellules productrices d'EPS vont être à l'origine des variétés rhéologiques des laits fermentés. Selon Purohit *et al.* (2009), ces variations peuvent être dues à des différences des EPS produits et leur interaction avec le réseau de protéines et partant à l'activité protéolytique résultant du catabolisme des caséines.

Le starter formulé a montré une fermeté plus importante du lait en fin qu'au début du stockage, et aucun phénomène de synérèse n'a été observé. Et avec deux espèces de *Leuconostoc* productrices de dextrane, des modules de viscosité et d'élasticité ont montré des valeurs intermédiaires pour la culture mixte (starter formulé) entre le lait fermenté par une monoculture de *Leuconostoc* et celui fermenté par une monoculture de *Lactococcus*. Cinq composés volatils majeurs ont été identifiés dans le lait *kivuguto*: 3-methylbutan-1-ol, pentan-1-ol, acetic acid, furanmethan-2-ol, furan-2(5)H-one . Et ce profil aromatique caractérise la typicité même du *kivuguto*. Utilisant la même méthode avec une légère modification, Alonso & Fraga (2001) ont montré que le yaourt se caractérise par cinq composés majeurs dont seul l'acide acétique est présent dans le profil aromatique du *kivuguto*. Rappelons que le starter du yaourt est constitué de *Streptococcus thermophilus* et *Lactobacillus delbrueckii subsp. bulgaricus*. Mais, Cheng (2010) rapporte la présence dans le yaourt de 1-pentanol, du furanmethan-2-ol et de 3-methylbutan-1-ol, mais en très petites concentrations, composés trouvés dans le *kivuguto* comme composés principaux. Le starter du kéfir (*Lactobacillus delbrueckii subsp. bulgaricus* + *Lactobacillus helveticus* +*Lactococcus lactis subsp. lactis* +*Streptococcus thermophilus*+*Saccharomyces cerevisiae*) produit l'acétaldéhyde, l'acétone, le 2-butanone, l'ethyl acétate, l'éthanol et le CO_2 comme composés volatils selon Beshkova *et al.* (2003). Il apparaît tout à fait clair que le profil aromatique est déterminé par le profil microbien de chaque type de lait fermenté, créant ainsi la typicité caractéristique. Par exemple, l'étude de Beshkova *et al.* (2003) montre que *Lactobacillus helveticus* est responsable de la production du 2-butanone ou que l'éthanol est produit par *Saccharomyces cerevisiae.*

8.1.5. La production des starters et leur conservabilité

Ce travail avait pour objectif la production industrielle du *kivuguto*, ce qui passe inévitablement par la mise au point d'un starter. La production d'un starter stable dans le temps nécessite une sélection de souches performantes, une optimisation de leur production et une bonne conservation pour préserver le plus longtemps possible les propriétés véhiculant l'identité du lait étudié.

Au cours de cette étude, après une étape de sélection réalisée par des essais pilotes en fioles, les starters sélectionnés ont été produits en fermenteur de 20 L. Cette partie couvrait des essais de screening pour comparer les souches issues de même profil biochimique. Cela a permis d'obtenir les souches les plus intéressantes en termes d'acidification et de croissance en milieux MRS et M17. Les souches sélectionnées pouvaient donc bien avoir une bonne production de biomasse. Les rendements obtenus sont très intéressants. Il restait alors à étudier leur conservabilité. Les techniques utilisées aujourd'hui recourent au séchage. La lyophilisation est la technologie la plus utilisée aujourd'hui. Nos starters sont lyophilisables avec des rendements en fin de séchage très élevés. Une souche qui a montré une très bonne résistance à de hautes températures a même été séchée par atomisation, qui est une autre technologie de séchage, pas très utilisée en raison du fait qu'elle exige de hautes températures. La viabilité des starters a ensuite été étudiée en stockage sur une période de trois mois. La mise en évidence de ce phénomène a été évaluée par une série de techniques, car les pertes pendant le stockage sont énormes. La perméabilité membranaire de nos starters a été étudiée par l'oxydation des lipides membranaires des cellules, par la cytométrie de flux.

En effet, les techniques de séchage et le stockage dans le temps s'accompagnent du changement de la structure des lipides membranaires (Teixeira *et al.,* 1996 ; Coulibaly *et al.,* 2010), cause de mort cellulaire. Le phénomène de mortalité de cellules en stockage a été aussi abordé par l'étude de l'oxydation des protéines solubles et des carbonyles, car la formation ou la diminution de ces molécules traduisent l'oxydation et donc la mortalité cellulaire. Ces techniques étaient bien entendu menées en parallèle avec les méthodes classiques de comptage sur milieu solide. Il ressort globalement de ce travail que les souches sélectionnées répondent bien à leurs critères de choix pour constituer le starter du *kivuguto* moderne ayant de très fortes similitudes ($p=0.01$ et $p=0.05$) avec le *kivuguto* artisanal.

8.2. Conclusion générale et Perspectives

8.2.1 Conclusion générale

L'objectif de ce travail de recherche était la production industrielle maitrisée du *kivuguto,* un lait artisanal fermenté. Cela revient à la caractérisation de la microflore impliquée dans le procédé de sa fermentation et à la formulation d'un starter. Deux approches ont été initiées, pour atteindre cet objectif. La première a consisté en une étude méthodologique pour isoler, purifier, identifier les micro-organismes du *kivuguto*. Elle a été complétée par une recherche sur les procédés de production et de conservation du starter. Une fois cette partie terminée, une approche expérimentale nous a permis d'aborder ce travail en suivant quatre voies: la sélection des micro-organismes; *une* étude technologique et la formulation du starter; la production et la conservation du starter; la production et la stabilité du *kivuguto* au moyen du starter formulé.

Sélection des micro-organismes du *kivuguto*

Un échantillonnage dans deux fermes du Rwanda nous a permis d'obtenir 390 isolats, qui ont constitué un pool microbien à la base de cette étude. Des méthodes testées classiquement et mises au point par la Fédération Internationale de Laiterie (FIL-IDF) ont été utilisées afin d'isoler et purifier, en utilisant des milieux de culture adaptés, les micro-organismes supposés être dans le lait fermenté. Un dénombrement et des tests technologiques préliminaires ont aidé à observer l'organisation microbienne de ce lait fermenté. Ensuite, une identification phénotypique a séparé ces micro-organismes en profils biochimiques. Ici, des observations morphologiques, la coloration Gram et le test à la catalase ont été adoptés comme méthodes de screening bien utilisées pour des isolats lactiques observés sur frottis. En complément, ces tests ont été appuyés par des tests API, utilisant les galeries développées par BioMérieux s.a., en vue de grouper les souches pures en profils biochimiques.

Par la suite, des tests de comparaison de souches du même profil ont montré quelles souches étaient aptes à entrer dans la composition du starter. Ces tests consistaient à inoculer du lait stérile et des milieux MRS et/ou M17 avec chaque souche du profil. De cette étape, sept souches ont été pré-sélectionnées. Nous avons, dès lors, procédé à une identification complète au moyen des techniques moléculaires basées sur l'analyse de la séquence du gène codant,

pour l'ARNr 16S, et/ou de la séquence du gène codant pour la zone intergénique (ITS) 16S-23S de l'ARNr pour des souches non complètement identifiées par la 16S ARNr.

De nos jours, cette approche polyphasique est prônée par tous les laboratoires, pour faire une bonne caractérisation microbienne d'un substrat donné, vu que le pouvoir discriminant d'une seule analyse est généralement limité. Quoique fiables à un certain groupe microbien, les tests API seuls ont aussi des limites, qui ont été remarquées sur nos isolats. Les souches de lactocoques ont été bien identifiées, du moins jusqu'à l'espèce, mais d'énormes erreurs ont été observées sur les leuconostocs. L'utilisation des amorces pour cibler l'ARNr 16S a donc été requise. Elle a permis de confirmer l'identification des tests API des lactocoques, mais elle n'a pas validé celle des leuconostocs, du moins jusqu'au genre. Une autre zone plus discriminative a été alors ciblée. Quoiqu'elle soit très courte par rapport à la 16S ARNr, l'ITS 16S-23S ARNr différencie bien les leuconostocs jusqu'à l'espèce. Notre caractérisation a ainsi mis en évidence la flore microbienne du *kivuguto* comme étant composée de *Lactococcus lactis*, *Leuconostoc mesenteroides* et *Leuconostoc pseudomesenteroides*.

Etude technologique et formulation du starter du *kivuguto*

Après le dénombrement, l'identification et les premiers essais des paramètres technologiques, des essais-pilotes de mélange de souches ont été réalisés. Il était question de suivre la compatibilité entre souches, l'acidification rapide et complète du lait, la qualité organoleptique des laits fermentés et de la stabilité des laits fermentés sur une période d'au moins trois semaines. L'originalité de cette approche est qu'elle requiert moins d'analyses que les analyses technologiques proprement dites, en suivant la stabilité des propriétés technologiques du lait, pendant le stockage du lait sous réfrigération. Elle permet, en plus, d'éliminer très tôt les souches qui développent des défauts technologiques majeurs sans devoir procéder aux analyses souvent très coûteuses.

Production et conservation du starter du *kivuguto*

Les souches sélectionnées pour formuler le lait *kivuguto* ont ensuite été produites en fermenteur, car une souche qui donne peu de biomasse est technologiquement incapable de faire un starter. Elles ont été lyophilisées et/ou atomisées, après ajout d'agents protecteurs des cellules. Une étude de conservation de leurs poudres a été menée sur trois mois. Les

rendements de production et de conservation ont montré que la souche de *Lactococcus* donnait un excellent rendement de biomasse (10^{12} cfu.g^{-1}) et de viabilité en stockage >90%. Quant aux deux souches de leuconostocs (*Leuconostoc mesenteroides* et *Leuconostoc pseudomesenteroides*), leurs productions en biomasse étaient de l'ordre de 10^{12} et 10^{11} cfu.g^{-1} respectivement et leurs viabilités en stockage >90%. En outre, la souche *Leu. mesenteroides* présentait un bon rendement, aussi bien par lyophilisation que par atomisation. Il ressort, de ces résultats, que de bonnes conditions de culture, suivies d'une concentration avec ajout de glycérol et de maltodextrine, ainsi que du séchage par lyophilisation, garantissent un starter de trois souches de bonne qualité.

Production et conservation du *kivuguto* au moyen du starter formulé

L'étape de formulation terminée, un ajustement de la composition de chaque souche du starter a été fait, suivi de la production du *kivuguto* en bioréacteur. L'objectif était d'évaluer et de caractériser technologiquement le lait fermenté formulé, et sa stabilité sous réfrigération. Enfin, un test de validation a permis de comparer la similitude entre le *kivuguto* formulé et le *kivuguto* artisanal.

Globalement, les résultats de cette étude proposent un ferment lactique fait d'une combinaison de trois souches clairement sélectionnées. Ce ferment permet dès lors la standardisation d'un procédé moderne de fabrication du *kivuguto*, qui respecte les normes internationales.

8.2.2. Perspectives

L'analyse des résultats de cette étude ouvre des perspectives pour de futurs travaux sur le lait fermenté *kivuguto*. Il suffit de voir la littérature déjà produite sur le yaourt, pour comprendre le travail qui se profile pour une meilleure maîtrise de la production de *kivuguto*.

Pour la *sélection*, il convient de reconnaître que l'échantillonnage a porté uniquement sur deux fermes et sur deux saisons de l'année. Les applications des micro-organismes lactiques étant variées, un travail plus large de collecte des souches et des écosystèmes microbiens de l'environnement du Rwanda s'impose, afin de valoriser les nombreuses propriétés fonctionnelles et métaboliques. Pour ce faire, la création d'une collection de cultures microbiennes est une nécessité, afin de préserver cette biodiversité, surtout qu'il n'y a pas de

doute quant à l'impact de la détérioration des conditions climatiques sur la survie de ces écosystèmes. Ce travail de sélection peut aboutir à l'obtention de souches plus performantes que celles de notre étude par exemple, sous l'aspect de l'acidification, de la texture, du développement d'arôme, de la protéolyse. D'autres travaux de recherche ayant des objectifs spécifiques peuvent même être entrepris: recherche des souches ayant des propriétés probiotiques, des souches pouvant être utilisées comme vaccins vivants, en les modifiant comme vecteurs d'antigènes, ou encore des souches productrices de bactériocines ou d'autres métabolites, etc.

Pour les *propriétés technologiques*, notre étude était limitée dans le temps. Il serait encore très intéressant de faire une investigation plus poussée de certains caractères de chaque souche du starter. L'activité acidifiante de *Lactococcus* est très intéressante, mais son activité bactériocinique n'a pas été abordée. La souche *Leuconostoc mesenteroides* est très résistante et productrice de dextrane dont on sait que les applications sont très nombreuses, notamment en médecine hospitalière pour le remplacement de plasma et pour le dosage des lipoprotéines du sang, pour la fabrication de larmes artificielles, etc. Un travail très intéressant serait d'étudier l'impact de ses propriétés sur la conservation de la souche et/ou du lait en stockage, mais aussi d'analyser son interaction avec les deux autres souches. La souche *Leuconostoc pseudomesenteroides* est une souche acidifiante et productrice de dextrane également et n'a fait l'objet que de peu d'études.

Pour la *production et la conservation*, afin d'optimiser le rendement de nos souches, il serait intéressant d'effectuer une recherche sur le séchage sur lit fluidisé, car ce procédé coûte moins cher. Ensuite, il serait très intéressant d'aborder le mécanisme de viabilité cellulaire des cellules lyophilisées en stockage par d'autres outils analytiques (RMN) ou par d'autres cibles que l'ADN, comme l'ARN par exemple.

Pour la *valorisation de ces résultats*, il est vivement recommandé de mettre à disposition le starter formulé à un industriel pour la production du *kivuguto* en laiterie.

Enfin, pour *le développement et la conservation du patrimoine microbien*, il convient de développer un programme global des biotechnologies sur toute l'Afrique. Et pour chaque pays concerné, il est nécessaire de développer les biotechnologies pour être en phase avec le monde du XXI^{ème} siècle, car une fois de plus, il ne faut pas manquer ce rendez-vous de l'humanité.

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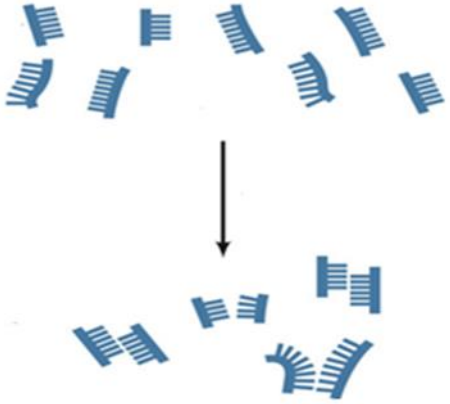
Annexe 1

Major functions of starter cultures in milk fermentations (Mullan, 2005)

Function	Role/Mechanism
Acid production	gel formation
	whey expulsion (syneresis)
	preservation
	flavour development
Flavour compound production	formation of diacetyl and acetaldehyde
Preservation	lowering of pH and redox potential
	production of antibiotic substances (bacteriocins): e.g. nisin
	production of hydrogen peroxide
	formation of D-leucine
	production of lactate/lactic acid
	acetate formation
Gas formation	eyehole formation
	production of openness to facilitate 'blue veining'
Stabilizer formation	body and viscosity improvement
	increase cheese yield
	result in reduced use of milk powder in yoghurt manufacture
Lactose utilization	reduce potential for gas and off-flavour development
	make products more acceptable to the lactose intolerant
Lowering of redox potential	preservation
	aids flavour development

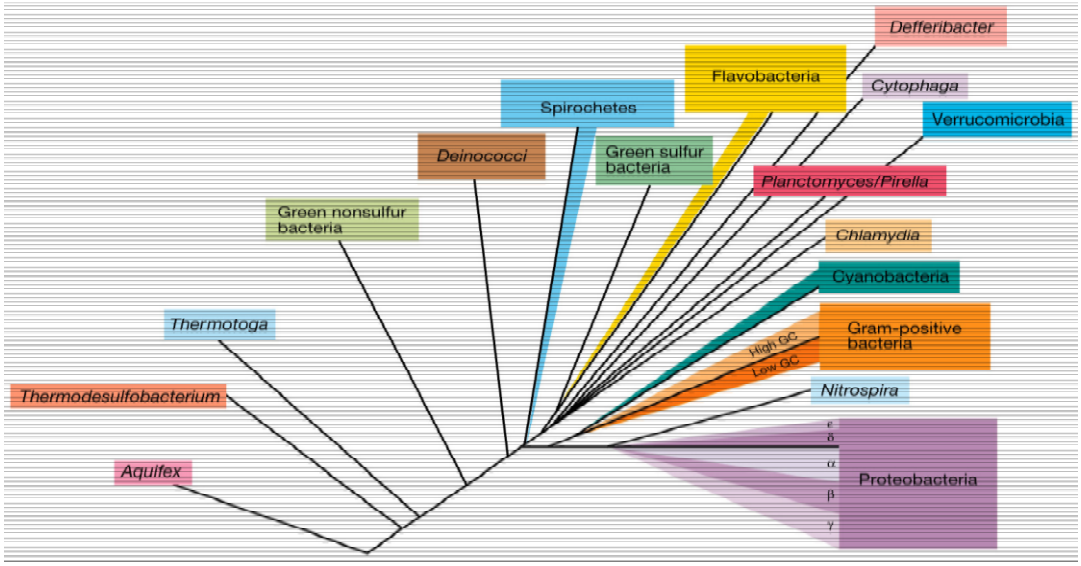
Annexe 2

DNA reassociation. The example shows that the degree of reassociation is 100%, i.e. the similarity between the two strains. Strains with reannealing values of ≥70% are considered to be the same species.



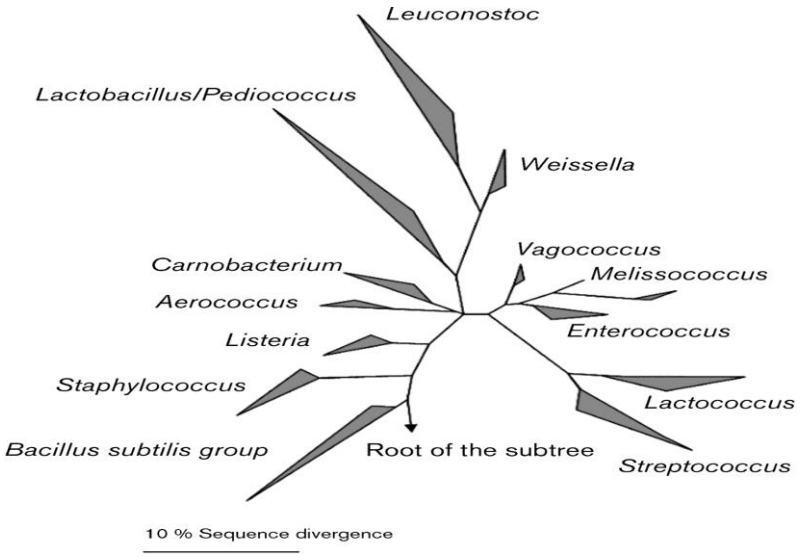
Annexe 3

Phylogenetic position of lactic acid bacteria (LAB) in relation with major lineages (phyla) of bacteria based on 16S ribosomal RNA sequence comparisons. LAB are Gram+ with low % GC content and Bifidobacteria are also Gram+ with high % GC content (RDP, 2013).



Annexe 4

16S rRNA dendrogram of phylogenetic position of Lactobacillus/ Pediococcus species, adapted of Fisher and Phillips (2009).



Annexe 5

Phylogenetic positions based on neighbour-joining of the 16S rRNA gene sequence of a strain *Brevibacterium picturae* LMG 22061T (Heyrman, 2004), representative of the isolates from the genus *Brevibacterium*. Species involved in cheese ripening are *Brevibacterium aurantiacum* (Leclercq-Perlat et al., 2007), *Brevibacterium casei* (Dolci et al., 2009) and *Brevibacterium linens* (Albert et al., 1944).