Physiological responses of bifidobacteria subjected to acid, cold and gastro-intestinal stress in organic and conventional milks

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TABLE OF CONTENTS

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ABBREVIATIONS LIST

ABY-type	Mixed probiotic culture (*Lactobacillus acidophilus*, *Bifidobacterium animalis* subsp. *lactis* and yogurt cultures)
ALA	α -linolenic acid (octadecatrienoic acid, C18:3)
ANOVA	Analyses of variance
ANVISA	Agência Nacional de Vigilância Sanitaria
AOAC	Association of Official Analytical Chemists
AOCS	American Oil Chemists' Society
ATCC15697	*Bifidobacterium longum* subsp. *infantis* strain ATCC 15697
ATP	Adenosine triphosphate
ATR	Acid Tolerance Response
B94	*Bifidobacterium animalis* subsp. *lactis* strain B94
BB12	*Bifidobacterium animalis* subsp. *lactis* strain BB12
BL04	*Bifidobacterium animalis* subsp. *lactis* strain BL04
$CaCO_3$	Calcium carbonate
cFDA	Carboxyfluorescein diacetate
CFU	Colony forming units
CLA	Conjugated linoleic acid (octadecadienoic acid, C18:2 *cis*-9, *trans*-11)
CLNA	Conjugated α-linolenic acid (octadecatrienoic acid C18:3 *cis*-9, *trans*-11, *cis*-15)
DHA	Docosahexaenoic acid
DIDGI	DIgesteur Dynamique Gastro-Intestinal
DPA	Docosapentaenoic acid
dpH/dt	Rate of acidification (upH/min)
DSM10140	*Bifidobacterium animalis* subsp. *lactis* strain DSM10140
EFSA	European Food Safety Authority
Eh	Oxidoreduction potential
Eh_0	Initial oxidoreduction potential
Eh_f	Final oxidoreduction potential
Eh_{max}	Maximum oxidoreduction potential
EPA	Eicosapentaenoic acid

EPS	Exopolysaccharides
FA	Fatty acids
FAME	Fatty acid methyl ester
FAO/WHO	Food and Agriculture Organization of the United Nations, World Health Organization
FOS	Fructo-oligosaccharides
GAD	Glutamate decarboxylases
GC	Guanine –Cytosine
GIT	Gastrointestinal tract
GLM	General linear model
GOS	Galacto-oligosaccharides
GRAS	Generally Recognize as Safe
H_2O_2	Hydrogen peroxide
HCl	Hydrogen chloride
HN019	*Bifidobacterium animalis* subsp. *lactis* strain HN019
IDF	International Dairy Federation
ISO	International Organization for Standarzation
KCl	Potassium chloride
KH_2PO_4	Potassium dihydrogen orthophosphate
$KHCO_3$	Potassium bicarbonate
KOH	Potassium hydroxide
LA	Linoleic acid (octadecadienoic acid, C18:2 *cis*-9,12)
LAB	Lactic acid bacteria
LB340	*Lactobacillus delbrueckii* subsp*. bulgaricus* strain LB340
LCFA	Long chain fatty acids (C16:0 to C18:3)
LC-PUFA	Long chain polyunsaturated fatty acids
MCFA	Medium chain fatty acids (C8:0 to C15:0)
$MgCl_2$	Magnesium chloride
MUFA	Monounsaturated fatty acids
n-3	Omega-3 fatty acids
n-6	Omega-6 fatty acids

Na_2CO_3	Sodium carbonate
NADH	Nicotinamide adenine dinucleotide (reduced form of NAD^+)
$NaHCO_3$	Sodium bicarbonate
$NaNO_3$	Sodium nitrate
NH_4Cl	Ammonium chloride
pHi	Intracellular pH
PUFA	Polyunsaturated fatty acids
RCA	Reinforced Clostridial Agar
rDNA	Ribosomal DNA
ROS	Reactive oxygen species
SCFA	Short chain fatty acids
SFA	Saturated fatty acids
SOD	Superoxide dismutase
TA040	*Streptococcus thermophilus* strain TA040
TAG	Triacylglycerol
tm	Time to attempt the maximum rate of acidification (in min)
$t_{pH4.5}$	Time to attempt pH 4.5 (minutes or hours)
TVA	*Trans*-vaccenic acid (octadecenoic acid, C18:1 *trans*-9)
UFA	Unsaturated fatty acids
UFA/SFA	Ratio between unsaturated and saturated fatty acids
Vm	Maximum acidification rate (upH/min)
XOS	Xylo-oligosaccharides

GENERAL INTRODUCTION

Probiotic fermented milks made from organic milk are of great interest for consumers as they combined advantages of probiotic cultures and organic milk. In a global market that may represent 67 billions $ en 2015[1], the development of these products requires increased knowledge about their specific characteristics, in terms of chemical and biological composition.

The interest for the use of organic milk, as raw material during fermented milk production questions consumers who require new functional dairy products associated with sustainable technologies (Florence et al., 2009). Organic dairy products differ from those issued from conventional systems, as the handling of the animals is governed by the interdiction of use of synthetic livestock additives, genetically modified organisms, antibiotics and chemotherapeutic treatments (Toledo et al., 2002; Florence et al., 2009). Additionally, organic farming systems are characterized by specific rules for feed regimen that recommend the use of fiber rich diets and fresh matter (Bergamo et al., 2003). These differences in cow's diet impacts directly on chemical composition of milk are that differ by considering some bioactive unsaturated fatty acids, such as *trans*-vaccenic acid (TVA), conjugated linoleic acid (CLA) and α-linolenic acid (ALA) (Bergamo et al., 2003; Bisig et al., 2007; Prandini et al., 2007, 2009; Florence et al., 2009).

Besides this technical and biological information, the development of organic fermented products is an economic challenge as it permits the valorization of organic raw milk (Stolz et al., 2011), and enables the manufacture of fermented milks richer in bioactive fatty acids linked to beneficial health effects, such as decreasing risk of coronary heart disease and reducing incidence of some cancer types (Butler et al., 2011; O'Donnell et al., 2010).

[1] http://www.scientistlive.com/European-Food-Scientist/Technology/Yoghurt%27s_future_as_a_functional_food_assured/24379/

The health benefits of probiotic dairy products are the result of biologically active components that are present in fresh milk and of specific activities resulting from the action of probiotic bacteria (Vasiljevic & Shah, 2008). Probiotics have been defined in several ways, with the pioneering work of Tissier and Moro that was based on the Metchnikoff's theory of longevity (Tissier, 1906). Following recommendations of Food and Agriculture Organization of the United Nations, World Health Organization (FAO/WHO), probiotic are defined as "live microorganisms that, when administered in adequate amounts, confer a health benefit on the host" (FAO/WHO, 2002). Their benefits for human health have been recognized and explored for over a century. They include the bacterial survival through gut intestinal tract and their role for stimulating the immune system and for preventing microbial gastroenteritis (Hols et al., 2005; Foligne et al., 2007). In addition, CLA production by bifidobacteria is also considered as a health enhancing property (Oh et al., 2003).

Probiotic microorganisms used for functional food products include lactic acid bacteria (LAB), especially *Lactobacillus casei*, *L. acidophilus, L. johnsonii*, and *L. rhamnosus* (Vasiljevic & Shah, 2008) and bifidobacteria, such as *Bifidobacterium animalis* subsp. *lactis*, *B. longum*, *B. breve* (Borriello et al., 2003). Their selection for fermented milk production is based on technological criteria, such as genetic stability of the strains, high viability and survival during processing and storage, good sensory properties, high cold and acid tolerance, phage resistance and ability for large-scale production (Vasiljevic & Shah, 2008). In order to exert their functional properties in the host, sufficiently high concentrations of viable and active bacteria are required from inoculation throughout processing and storage, until consumption of the products. These requirements implicate that the bacteria may survive despite the stress conditions encountered in the fermented products, which involve low temperature of storage and acidic pH (Shah, 2000; Ramchandran & Shah,

2010). They also have to counteract the gastrointestinal stress conditions during their consumption, where they meet very acidic pH and bile salts (Gomes & Malcata, 1999). From many authors, the bacteria are affected by cold conditions during storage (4°C for at least three weeks) (Donkor et al., 2006), by low pH in the products (between pH 4 and pH 5) (Østlie et al., 2003), and by the gastrointestinal stress (pH 2 in the presence of pepsin, followed by bile salts in the presence of pancreatin) (Madureira et al., 2011). These stress strongly impact on bacterial concentrations that remain generally lower than recommended values (Vinderola et al., 2002). Consequently, the incorporation of bifidobacteria into fermented milk products is a big challenge in dairy industry.

The final bacterial concentrations at the end of fermentation can be improved by the strain association (Béal et al., 1999; Oliveira et al., 2001), by modification of fermentation temperature or final fermentation pH (Béal et al., 1999; Wang et al. 2005a; Shafiee et al. 2010), by use of specific raw materials such as organic milk instead of conventional milk (Florence et al., 2009), by exposition to moderate temperature during cooling after fermentation process (Dave & Shah, 1997; Panoff et al., 2000; Wang et al., 2005b) or by milk supplementation with beneficial molecules for growth (Oliveira et al., 2001; Akalin et al., 2007; Oliveira et al., 2009a).

Nevertheless, if the stress sensitivity to acidic conditions (Donkor et al., 2006; Mättö et al., 2006), to chilled storage (Donkor et al., 2007; Mortazavian et al., 2007) and to freezing (Foschino et al., 1995) of *Bifidobacterium* strains have still been tested, very few studies were conducted to understand the mechanisms involved in the bacterial response to a double stress, combining acid pH and low temperature. The physiological mechanisms that induce bacterial degradation are still poorly understood. Moreover, if biological adaptation to stress was still achieved, by submitting the cells to a moderate stress conditions before freezing, (Béal et al., 2001; Fonseca et al., 2001; Wang

et al., 2005b; Streit et al., 2008), this action was never applied to bacteria included in a fermented product. These adaptation phenomena induced a better resistance to the stress thus increasing survival. They were supported by biological changes at membrane and proteome levels (Lim et al., 2000; Wang et al., 2005a,b; Streit et al., 2008).

In this context, this PhD thesis aims first at characterizing the resistance of different strains of bifidobacteria to the double stress encountered in fermented organic and conventional milk products and to simulated gastrointestinal stress. The mechanisms involved in the physiological responses to these stress are characterized, at the membrane level, in order to improve knowledge on biological behavior. Finally, the application of various moderate stresses to favor cellular adaptation is conducted, in order to improve the resistance to acid, cold and gastro-intestinal stress.

The first part of this work focuses on the characterization of the growth of different *Bifidobacterium* strains in pure and mixed cultures, in conventional and organic milk, and of their resistance to double stress - cold and acid, suffered during storage of fermented milks at 4°C. The second step aims at identifying the membrane fatty acid composition of the bacteria in relation to the fatty acid composition of milk and to their behavior in fermented milk products. The third part of this work presents some physiological adaptations of the cells – by acting on culture and cooling conditions, thus allowing them to better withstand the deleterious effects of stress. Finally, the ability of *Bifidobacterium* cells to survive the stress encountered in the digestive tract and to adapt itself at the membrane level is studied by using a specific bioreactor that mimics the dynamic observed in the gastric and the duodenal compartments.

In order to attain these objectives, this manuscript is divided into six chapters: after a first part dedicated to a review of literature, five chapters summarize the results obtained in this study. They are presented in the form of

five articles, one of them being accepted. The corresponding material and methods, as well as the references are presented in details in each chapter.

Chapter 1 literature about fermented milk production (from raw material to chemical changes during fermentation and storage, reviews the environmental stress that influence viability and physiological changes of bifidobacteria in fermented milk products such as low temperature and pH, oxidative stress and gastrointestinal stress.

Chapter 2 deals with the characterization of the behavior of *Bifidobacterium animalis* subsp. *lactis* HN019 and of yogurt starters in mixed culture during fermentation in organic and conventional milks. They are related to the overall fatty acid composition of the milks, as well as *trans*-vaccenic, conjugated linoleic and α-linolenic acids relative contents. This Chapter is presented as the original article published in Food Chemistry in 2012.

Chapter 3 describes the survival of three *Bifidobacterium animalis* subsp. *lactis* strains in association with yogurt cultures during four weeks of storage at 4°C in organic and conventional fermented milks. Survival is related to the milk's contents in *trans*-vaccenic and α-linolenic fatty acids.

Chapter 4 reports the physiological responses of three *Bifidobacterium* strains during milk fermentation and storage of fermented product at 4°C for 21 days. Acidification and oxidoreduction activities during fermentation, carbohydrates, organic acids and fatty acids contents in fermented milks, as well as membrane fatty acid composition of *Bifidobacterium* cells are characterized and compared by considering organic and conventional milks.

Chapter 5 investigates the effect of moderate stress during fermented milk manufacture, by applying various fermentation pH and temperature and different cooling procedures, on the acidification activity, bacterial membrane fatty acids composition and survival of *Bifidobacterium animalis* subsp. *lactis* BB12 during chilled storage.

Chapter 6 allows characterizing the survival of *Bifidobacterium animalis* subsp. *lactis* BB12 to gastrointestinal stress simulated in a dynamic bioreactor DIDGI, such as acidic pH in the presence of pepsin (gastric conditions) followed by exposure to bile salts and pancreatin (duodenal conditions). Survival of the bifidobacteria is measured in organic and conventional fermented milks obtained under different manufacture conditions, and related to the membrane fatty acids of the bacteria.

Finally, a general conclusion summarizes the main results of this work concerning the survival and the physiological responses of different *Bifidobacterium* strains exposed to different environmental stress. The manuscript is concluded with key prospects envisaged for future research.

CHAPTER 1 – Review of Literature

1. Fermented milks production

Fermentation is one of oldest methods applied for food preservation, mainly intended to dairy and meat products. Milk supplementation with live and active microorganisms has been done for thousands of years, and fermented milk products are even mentioned in the Old Testament (Lerayer et al., 2009). In 1907, the Russian bacteriologist Metchnikoff noticed the longevity of Bulgarian people (life expectancy of 87 years) was associated with the large consumption of fermented milks (Vasiljevic & Shah, 2008). Thus, Metchnikoff attributed to *Bulgarican bacillus* the beneficial effects of the consumption of fermented milks (Tamime, 2002).

According to the definition provided by the Codex Standard, fermented milk is a dairy product obtained by acidification of milk which might have been manufactured from dairy products obtained with or without modification by the action of suitable microorganisms and resulting in reduction of pH with or without iso-electric precipitation. The starter microorganisms shall be viable, active and abundant in the product until the expiry date (Codex alimentarius, 2003).

Many different types of traditional and industrialized fermented milk products are manufactured throughout the world (Tamime et al., 2011). In nearly all civilization have developed some type of fermented milk product such as kefir, fermented cream, koumiss, buttermilk, acidophilus milk, bifidus milk, *Labneh, Gioddu*, *Tarag*, *Tuzlu, Shankleesh* and finally, the most consumed, yogurt (Tamime, 2002; Pashapour & Iou, 2006). From the technological point of view, fermented milks are presented into different forms to the final consumer:

set-type form, stirred-type, drinking-type, concentrated or dried (Tamime et al., 2011).

The main stages of fermented milk manufacture consist of choice of type of milk including specie, origin and system. Then, milk is standardized in relation to the solids non fatty (SNF) and fat level to guarantee legal standards. The milk basis can also be supplemented with growth factors, such as dietetic fibers, vitamins, minerals, cystein, caseins hydrolisate, whey powder and whey pretein concentrate, nevertheless this stage is considered optional (Tamime, 2002; Oliveira, 2009).

Subsequently, milk is homogenized in order to reduce the diameter of fat globules, to improve viscosity and consistency of the product and to decrease the syneresis of the gel. In general, homogenization process is done before the heat treatment. The thermal treatment of milk can be realized by different time and temperature combinations, such as 85℃ for 30 minutes, 90-95℃ for 5 minutes or 105℃ for 10 seconds. The main finality is the elimination of pathogenic microorganisms' presents in the milk. The heat treatment is followed by cooling until the temperature of fermentation (Oliveira, 2009; Tamime et al, 2011).

Afterward, the starter cultures are inoculated in milk which is incubated until reach the desired pH. Several methods of pH measurement and acidification activity are available (Cachon et al., 2002). The Cinac System (Ysebaert, Frépillon, France), method developed by Spinnler & Corrieu (1989) allows calculation of kinetic parameters, such as maximum acidification rate (Vm) and the time (Tm) at which Vm occurred from the measurement of pH decrease. Thanks to this system, data are recorded and displayed in real time on computer. The Cinac System is illustrated in Fig. 1.

Finally, the cooling step is applied after the end of fermentation in order to control the activity of microbial cultures in fermented milk (Tamime, 2002). At

this point, the addition of fruit into fermented milks is optional, and product is packaged and cold stored until consumption (Shafiee et al., 2010; Tamime et al., 2001). The basic requirements of the final product results from the composition and treatment of milk base, the specific starter culture association and the employed conditions during and after the fermentation process (Robinson et al., 2002).

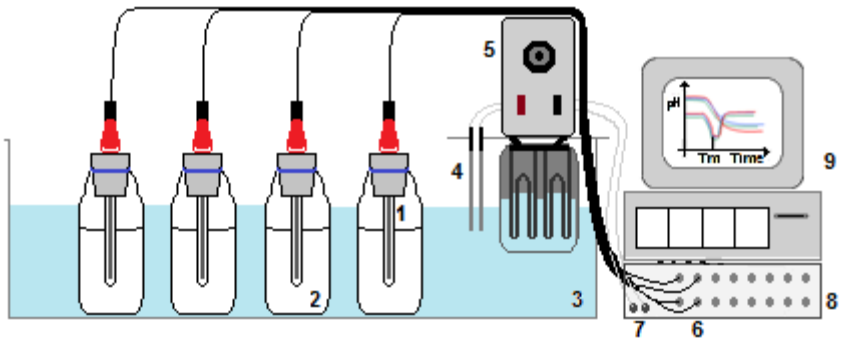

Fig. 1 Cinac System simplified diagram (adapted from Wang, 2005).

1 – pH electrode
2 – Erlenmeyer flask containing the inoculated milk
3 – Thermostatically controlled water bath
4 – Temperature sensor
5 – Heater resistance
6 – pH transmitter
7 – Temperature transmitter
8 – Electronic interface
9 – Microcomputer

1.1. Raw material

The specific use of milk and milk products within the general standards of fermented milk manufacture is one of strict aspects defined by the International Dairy Federation (IDF, 1992). Milk is complex mammalian fluid constituted from a liquid emulsion in which the continuous phase is formed by water soluble substances and the internal or discontinuous phase is composed by casein micelles and fat globules (Sgarbieri, 2005).

The chemical composition of any type of fresh milk varies over time depending on many factors as stage of lactation, age of the animal, breed, seasonal variations and animal diet (Florence et al., 2009; Tamime et al., 2011). Cow's milk is mainly composed of water, around 5.0% lactose, 3.5% protein, from 3.3 to 4.7% fat and 0.7% ash. In addition, milk contains vitamins, minerals and fatty acids that determine its nutritional quality as raw material to produce fermented milks and other dairy products (Penna, 2009).

The carbohydrate fraction of most mammalian is lactose (4-O-β-D-galactopyranosyl-D-glucopyranose). This carbohydrate is a substrate for lactic acid bacteria that transform into lactic acid, obtaining thus acidified products (Tamime et al., 2011).

Milk proteins include caseins, β-lactoglobulin, α-lactalbumin, immunoglobulins, lactoferrin and serum albumin (Ebringer et al., 2008). These proteins allow the conversion of milk into fermented milk products (Tamime et al., 2011).

Lipids in milk are formed by a complex lipids existing in microscopic globules of oil in water emulsion (Sgarbieri, 2005). They consist mostly of triglycerides (98%), phospholipids (~1%) and a small percentage of monoglycerides, di-glycerides, cholesterol esters and traces of fat-soluble vitamins (Hupertz et al., 2006). Additionally, a wide range of fatty acids have been identified in milk and dairy products, being approximately 65% of saturated fatty acids (SFA), 30% of monounsaturated fatty acids (MUFA), 4% of polyunsaturated fatty acids (PUFA) and 1% of unusual types of fatty acids (Miller et al., 2000).

Additionally, any type of milk contains a great variety of nutrients like vitamins and minerals, which are suitable for the growth of the starter cultures during dairy fermented products manufacture (Park et al., 2007).

Besides, milk owns various biologically active substances like immunoglobulin, enzymes, peptides, oligosaccharides, cytokines and long-chain unsaturated fatty acids (Ebringer et al., 2008). During heat processing of milk lactulose, a disaccharide composed of D-galactopyranose and D-fructofuranose is originated with important prebiotic properties (Gibson et al., 2004). This oligosaccharide stimulates the growth of probiotic bacteria including bifidobacteria and lactobacilli (Olano & Corzo, 2009; Oliveira et al., 2011). The long chain fatty acids with healthy effects are represented by *trans*-vaccenic acid (TVA), conjugated linoleic acid (CLA) and α-linolenic acid (ALA) (Palmquist & Jensen, 2007).

During the last years, organic movement has grown considerably and has gained increased interest worldwide (Fall et al., 2008). Products of organic system attract many consumers due to the idea of healthy sustainable food production attributed to this type of management (Toledo et al., 2002). Moreover, organic and conventional systems of milk production differ considerably in the handling of the animals, in the feeding of dairy cattle and in the interdiction of antibiotic treatments (Fall et al., 2008). All the standards and regulations of organic farming specify strict rules for milk production and, in particular, related to the animal diet (Molkentin & Giesemann, 2007).

The higher percentage of pasture or grass in organic milk production than in conventional milk over the year reflects on milk composition (Molkentin, 2009). Differences in diet among these two systems of dairy production have shown to affect the milk fatty acid composition that is mainly determined by the feed given to the animals (Bergamo et al., 2003; Ellis et al., 2006; Prandini et al., 2009).

According to some authors (Ellis et al., 2006; Fanti et al., 2008; Butler et al., 2011) the gross chemical composition between organic and conventional milks slightly differs. Nevertheless, organic products show distinct fatty acid

composition as compared to conventional products. This fatty acid composition was characterized by higher relative levels of some mono and polyunsaturated fatty acids (Prandini et al., 2009; Butler et al., 2011).

Within these fatty acids, organic milk possesses higher relative concentration in *trans*-vaccenic (TVA, C18:1 *trans*-11,) and α-linolenic fatty acids (ALA, C18:3 *cis*-9, 12, 15,) in comparison with conventional milks. Moreover, higher relative level of TVA in milk fat is related with an increase in conjugated linoleic acid (CLA, C18:2 *cis*-9, *trans*-11,) relative content, due to the action of Δ-9 desaturase in mammary gland. Superior percentages of CLA in milk fat have already been related to organic systems of dairy production (Prandini et al., 2009; Tudisco et al., 2010; Butler et al., 2011; Stergiadis et al., 2012). Nevertheless, the seasonal variation exerts effects on milk fat composition and also in the relative contents of CLA that became similar in the winter for both, organic and conventional milks (Molketin & Giesemenn, 2007; Fanti et al., 2008). As a consequence, higher relative contents in CLA might not be considered as a general rule to differentiate organic and conventional milks (Molketin, 2009).

Previous results allowed propose organic milk as raw material to produce probiotic fermented milks (Florence et al., 2009, 2012a,b), as the technological characteristics of the final product were considered acceptable (Florence, 2009). In addition, cultivability of bifidobacteria and lactic acid bacteria, as well as percentages of bioactive fatty acids were increased when using organic than conventional milk.

1.2. Starter cultures

Starter cultures or simply starters are preparations to assist the beginning of the fermentation process (Arora et al., 1991). Lactic acid bacteria (LAB), yeasts and moulds (or combination of these) are the microorganisms employed

in the manufacture of fermented milk products. The relevance of these microorganisms during fermented milk production includes its nature and fermentation temperature (Dellaglio et al., 2005; Tamime et al., 2011). Fermentation can be classified in lactic, yeast - lactic or mould – lactic according to the employed bacteria as well as therapeutic or probiotic type (Tamime, 2002). Lactic acid fermentation is responsible for the production of the majority fermented milk products (Vasiljevic & Shah, 2008; Tamime et al., 2011).

The employment of lactic acid bacteria in food technology was established in the end of the nineteenth century when Louis Pasteur (1857-1876) highlighted the role of these microorganisms in the lactic fermentation (Mofredj et al., 2007). After that, Metchnikoff recommended the use of LAB for reducing intestinal disorders (Gournier-Chateau et al., 1994).

Lactic acid bacteria are an extremely important group of starter cultures widely used by pharmaceutical, biomaterial, textile and food industries (Kim et al., 2012). LAB is as a group of cocci-bacilli Gram-positive microorganisms with different morphological, metabolically, taxonomical and physiological characteristics (Lerayer et al., 2009). LAB can be aerobic, aero tolerant, anaerobic, strictly anaerobic, non-spore forming and negative catalase bacteria which the main function is the production of lactic acid (Charteris et al., 1997; De Vuyst, 2000). Finally, lactic acid bacteria are considered “Generally Recognize as Safe” (GRAS).

A large variety of different species such as *Lactococcus*, *Lactobacillus*, *Streptococcus*, *Enterococcus* and probiotic organisms are included in the LAB group. Their importance is related to the preparation, alteration and hygiene of food products. These bacteria are traditionally employed in various fermented products like yogurt, cheese, fermented milk, dry sausage, salami and vegetable products (Hummel et al., 2007).

Lactic acid bacteria exhibit unique properties concerning growth and growth and metabolic rate, proteolytic activity and flavor production (De Vuyst, 2000). Moreover, they interact with their physico-chemical environment and alter its characteristics in a very complex manner (Cachon et al., 2002).

In general, *Lactobacillus*, *Lactococcus*, *Streptococcus* and *Leuconostoc* are the most employed lactic acid bacteria in the manufacturing of fermented dairy products (Tamime et al., 2011). The largest and best studied genus within the group of LAB is *Lactobacillus*. It contains large number of species that were isolated from various sources including plants, foods such as fermented products, as well as in the oral cavities like gastrointestinal tract, vaginas of humans and animals (De Angelis & Gobbetti, 2004; Walter, 2008).

In addition, bifidobacteria strains, grouped in Actinobacteria class based on their high GC (guanine –cytosine) in the DNA, are usually employed as starter cultures to produce fermented milks. Some *Bifidobacterium* strains share certain physiological and biochemical properties with typical LAB and some common ecological niches such as the gastrointestinal tract. Moreover, this microorganism is extremely important in the industry since it possess probiotic characteristics and can also act as a starter culture due to its technological properties during cheese maturation and fermented milk production (Meile et al., 2008; Vasiljevic & Shah, 2008).

According to Vinderola et al. (2002), the association of starter and probiotic bacteria exerts influence on technological characteristics of fermented milk. Some of them can be considered positives as the protosymbiotic relationship between *Streptococcus thermophilus* and *Lactobacillus bulgaricus*, or other antagonistic, like the inhibition of probiotic bacteria growth by the increasing production of organic acids by starter cultures (Donkor et al., 2006).

1.2.1. Yogurt cultures

Within the fermented dairy products, yogurt is one the most consumed. It is a coagulated dairy product obtained by the lactic acid fermentation of *Streptococcus thermophilus* and *Lactobacillus delbrueckii* subsp. *bulgaricus* (Guarner et al., 2005; Sarkar, 2008). The origin of the word yogurt is from the Turkish term “jugurt”, a traditional beverage in the Balkans and in the Middle East (Tamime & Deeth, 1980).

In some countries *Lactobacillus helveticus* and *Lactobacillus delbrueckii* subsp. *lactis* are mixed with the traditional yogurt starter cultures (Tamime, 2002). The terminology “probiotic yogurt” is not accepted by the totality of the scientific community. Nevertheless, some countries adopt this expression to indicate fermented milks obtained by the action of yogurt cultures and probiotic bacteria such as *Lactobacillus acidophilus*, *L. helveticus*, *L. johnsonii*, *L. rhamnosus*, *L. casei* subsp. *casei*, *L. paracasei* and *L. plantarum* and *Bifidobacterium animalis* subsp. *lactis*, *B. infantis*, *B. longum* and *B. bifidum* (Vasiljevic & Shah, 2008; Tamime et al., 2011). The term ‘yoghurt-like product’ is an alternative word for yogurt containing probiotic bacteria or when *L. bulgaricus* is replaced by other *Lactobacillus* species during milk fermentation (Guarner et al., 2005).

The Ministry of Agriculture, Livestock and Food (MAPA) in Brazil consider yogurt as dairy products submitted to lactic acid fermentation by the protosymbiotic action of *Streptococcus thermophilus* and *Lactobacillus delbrueckii* subsp. *bulgaricus*, with or without the addition of food ingredients, contributing to the characteristics of the final product (Ministério da Agricultura, Pecuária e Abastecimento, 2000). In France, to be called yogurt, fermented milk must be manufactured with the above lactic acid bacteria. In addition, these bacteria must be alive and abundant in the finished product at least 10^6 bacteria/g. In France, dairy products heat-treated after fermentation were

therefore not entitled to the appellation yogurt which is not the case in other countries (Spain and Germany). Moreover, according to French legislation the addition of other lactic acid bacteria and various ingredients such as gelatin, pectin and modified starch is also prohibit (CNIEL, 2006).

The employment of starter cultures in yogurt manufacture supposes some characteristics, such as, rapid acidification activity and great capacity to use carbohydrates in the raw material. In addition, starter cultures must be capable to produce lower post-acidification and elevate stability and cultivability throughout the period of shelf-life (Adolfsson et al., 2004; Ongol et al., 2007, Sarkar, 2008). Besides, other desirable criteria for yogurt cultures especially attributed to the probiotic ones are acid and bile tolerance (Liong & Shah, 2006) as well as survival through the human gastrointestinal tract (Mater et al., 2005).

The lactic acid bacterium *Streptococcus thermophilus* is the major component of dairy starter cultures used for the manufacture of yogurt. The principal function of *S. thermophilus* growing in milk is to provide rapid acidification as a consequence of the production of lactic acid (Arioli et al., 2007). This specie is a facultative anaerobe, with homofermentative metabolism (producing L (+) lactate, acetaldehyde and diacetyl from lactose in milk), cytochrome oxidases and catalase positive (Robinson et al., 2002).

Lactobacillus delbrueckii subsp. *bulgaricus* is presented as non-flagellated rods joined in long chains or as coccobacilli forms. It can be aerotolerant, anaerobic strictly or strictly fermentative. In addition, bacteria from this specie is considered acid tolerant, with homofermentative metabolism, producing L-lactic acid as the principal metabolic end product (Dellaglio et al., 1992). Some authors have reported that *L. delbrueckii* subsp. *bulgaricus* is a greater acetaldehyde producer than *S.thermophilus* (Ott et al., 1997), whereas others have suggested the contrary (Chavez et al., 2002).

These two lactic acid bacteria exhibit an interaction during production of yogurt. In the beginning, a more rapid initial growth phase is performed by *S. thermophilus* followed by growth of *L. bulgaricus,* which favors acidification activity. More specifically, *Lactobacillus* produces enzymes to degrade casein, releasing aminoacids such as methionine, threonine and valine. These peptides act as growth factors for *S. thermophilus*, which, in turn liberates formic acid and CO_2, providing improved development of *L. bulgaricus* (Oliveira et al., 2009a,b). These facts indicate a mutually favorable relationship between these two microorganisms (Sarkar, 2008; Oliveira et al., 2012).

Table 1 Main characteristics of selected yogurts strains employed on the production of probiotic fermented milks (Adapted from Oliveira et al., 2009b)

Characteristics	*Lactobacillus delbrueckii* subsp. *bulgaricus* LB340	*Streptococcus thermophilus* TA040
Lactose fermentation	+	+
Lactic acid isomers	D	L
Acid tolerance	–	+
Pepsin resistance	–	+
Bile salts tolerance	–	+++
Pancreatin resistance	–	–
Adhesion to intestinal mucosa	–	Caco-2 +++ H1-29 ++
Health effects	• Inhibits the angiotensin-converting enzyme (ACE) • Presents good antioxidant activity • Displays good immunomodulatory activity (Qian et al., 2011)	Influences Immune regulation by the increase in induction of IL-10 (*in vitro*) (Lammers et al., 2003)

– Absent or undetectable levels
+ Fair
++ Good
+++ Excellent

1.2.2. Probiotic cultures

The term probiotic derives from two Greek words, "pros" and "bios", which means "for life", contrary to the term antibiotic, which means" against" life. It was first introduced by (Lilly & Stillwell, 1965) to describe substances secreted by a microorganism which stimulates the growth of another. Since then, several definitions have been established for probiotics, depending on their effects on host health (Vasiljevic & Shah, 2008).

The definition of probiotics has changed over the last years, and the most accepted definition is based on work of ILSI Europe (International Life Sciences

Institute) and the FAO/WHO group (FAO/WHO, 2001, 2002) that probiotics are "live microorganisms which when administered in adequate amounts confer a health benefit on the host". In Brazil, the National Agency for Sanitary Vigilance, (ANVISA) consider probiotics as live microorganisms that can improve the intestinal microbial balance, producing beneficial health effects of the individual (Agência Nacional de Vigilância Sanitaria, 2002). Nevertheless, these definitions conflicts with the results on the effects of the same strain administered in either viable or non-viable forms (Aureli et al., 2011; Bogsan et al., 2012).

The European Food Safety Authority (EFSA, 2011) indicates that some specific bacterial strains have shown proven actions that cannot be applied for all of the available strains in the marketplace. Recently, EFSA (2011) published a guidance document on scientific requirements for health claims related to gut and immune function. According to them, the claimed effect should be sufficiently defined and be measurable. EFSA requires that probiotic strains might be identified clearly and deposited in public culture collections. Moreover, EFSA also indicates that a strain-specific approach on health claims, stating that probiotic properties are “strain and species specific” (Salminen & Van Loveren, 2012).

Most of the microorganisms currently applied as probiotics belong to the *Lactobacillus* and *Bifidobacterium* genera; however, some other species such as *Lactococcus lactis* and *Saccharomyces boulardii* are also used (Vasiljevic & Shah, 2008; Sánchez et al., 2009). From *Lactobacillus* genus, *Lactobacillus acidophilus*, *L. rhamnosus*, *L. casei*, *L. johnsonii*, *L. reuteri, L. plantarum*, and *L. fermentum* have been isolated from the human intestine (Mitsuoka, 1992) and applied in food products due to their safety and functional properties (Vasiljevic & Shah, 2008). The production of functional products containing bifidobacteria strains has increased due to their beneficial health effects on the host (Martinez-Villaluenga & Gomez, 2007). The most common species of

Bifidobacterium used in foods and food complements are *Bifidobacterium animalis* subsp. *lactis, B. longum*, *B. bifidum*, *B. adolescentis, B. breve*, and *B. infantis* (Saxelin, 2008; Vasiljevic & Shah, 2008). As probiotic health benefits are strain specific, some general health effects are listed in the Table 2.

The essential technological characteristics of probiotics in the development of dairy products are based in supposition more than rigorous science (Sanders, 2008). These attributes are related to the GRAS criteria, desired viability during processing and storage, good sensory properties, genetic stability, tolerance to cold, gastric acid and bile salts, adhesion to mucosal surface phage resistance, ability for large-scale production, moderate resistance to aerobic conditions and antagonistic activity towards gastrointestinal pathogens, such as *Helicobacter pylori* and *Candida albicans* (Vasiljevic & Shah, 2008; Sánchez et al., 2009). Other criteria widely suggested for probiotics applications stipulate that probiotics for human use must be of "human origin". However, some strains that are not isolated from humans have shown to be effective probiotics, such as *Bifidobacterium animalis* subsp. *lactis* which contradicts this requirement (Sanders, 2008).

Table 2 Beneficial effects of probiotic microorganisms

Health effect	Reference
Action on constipation	Marteau et al. (2002); Aureli et al. (2011)
Alleviation of atopic dermatitis and eczema symptoms in children	Kankaanpää et al. (1998); Gueimonde et al. (2006)
Alleviation of lactose intolerance	Vasiljevic & Shah (2008); Sánchez et al. (2009).
Hypocholesterolemic effect	Begley, Hill, & Gahan (2006); Liong & Shah (2006)
Inhibition of *Helicobacter pylori* and intestinal pathogens	Marteau et al. (1997); Alakomi et al. (2000)
Potential role in the treatment of obesity and metabolic syndrome	Lee et al. (2006); Bogsan et al. (2011)
Modulation of intestinal microbiota	Marteau et al. (1997); Isolauri et al. (2001)
Modulation of Immune response	Isoulari et al. (2001); Paturi, Phillips, & Kailasapathy, (2008)
Prevention and control of oral infections	Ahola et al. (2002); Hatakka et al. (2007)
Prevention of Inflammatory Bowel Disease	Ruiz et al. (2005); Zhang et al. (2005)
Prevention of urogenital infection	Reid & Bruce (2003); Hütt et al. (2006)
Prevention of rotavirus nosocomial infection in children	Marteau et al. (2002); Sazawal et al. (2006)
Reduction of symptoms of diarrheal diseases (antibiotic associated and traveler's diarrhea)	Mattila-Sandholm et al. (2002); Sazawal et al. (2006)
Reduction in respiratory infection in children	Hatakka et al. (2001)

In order to produce the health benefits, a sufficient number of viable probiotic microorganisms must be present throughout the entire shelf life of the product (Sánchez et al., 2009). Moreover, the dose of probiotics ingested is a key factor to obtain high concentrations in the various compartments of the gastrointestinal tract (Laparra & Sanz, 2010). Therefore, the recommended levels of probiotics ranges from 10^6 colony forming units (CFU) per gram (Kurman & Rasic, 1991) to over 10^7 and 10^8 CFU per gram of product (Sanders,

2008; Vasiljevic & Shah, 2008) with no general agreement for the scientific community. In addition, probiotic strains have to overcome biological barriers present in the gastrointestinal tract (GIT) to exert their health-promoting effects. For this reason, several strategies are investigated to improve probiotic survival, specificity and efficacy. Most of them, related to stress adaptation, encapsulation and gene modification (Sánchez et al., 2009).

1.2.3. Bifidobacteria

The genus *Bifidobacterium* was isolated and described over a century ago by Tissier 1899-1900 and named *Bacillus bifidus* (Tissier, 1906). These microorganisms are Gram-positive, anaerobic, non-gas producing, non-spore-forming, non-motile, non-filamentous, catalase negative and their morphology may assume different shapes such as bifid, irregular V and Y-shaped rods resembling branches that occur singly, in chains or clumps (Lee & O'Sullivan, 2010; Oliveira et al., 2012).

During the last two decades, bifidobacteria taxonomy has highly developed by the use of comparative analysis of the 16S rDNA gene (Ventura et al., 2001; Zomer et al., 2009) and housekeeping genes (Ventura et al., 2004, 2005; Zomer et al., 2009). Currently, *Bifidobacterium* belongs to the subclass Actinobacteridae of the phylum Actinobacteria, order Bifidobacteriales and family Bifidobacteriaceae. These bacteria possess high-GC-content (55 to 67%) (Ventura et al., 2007; Lee & O'Sullivan, 2010) and comprise 37 species with over than 93% identity of their 16S rDNA (Turroni et al., 2011). The genus *Bifidobacterium* is mainly represented by *B. longum*, *B. animalis*, *B. breve, B. pseudolongum,* and *B. thermoacidophilum* which are further subdivided into subspecies (Miyake et al., 1998; Turroni et al., 2011).

Bifidobacterium species are common inhabitants of the GIT of mammals and other animals, but they can be encountered in other different ecological

niches (Ventura et al., 2007), such as oral cavity (Lee & O'Sullivan, 2010). They are one of the first colonizers of GIT of newborns and represent the prevailing genus of gut bacterial population of healthy breastfed infants (Borba et al., 2003; Penders et al., 2005). In adults, the intestinal microbiota is composed of 3% in average of *Bifidobacterium* species (Vaughan et al., 2002).

The presence of bifidobacteria in the gut has been associated with healthy promoting effects (Heller, 2001; Ventura et al., 2004) leading to the extensive use of many *Bifidobacterium* strains as functional components of probiotic foods and dietary supplements (Zomer et al., 2009; Lee & O'Sullivan, 2010). Some studies have suggested that the occurrence of bifidobacteria in the human large intestine is related to prevention of diarrhea (Saavedra et al., 1994), establishment of a healthy microbiota in premature infants (Wang et al., 2007), colon regularity (Marteau et al., 2002), immune stimulatory effects (O'Mahony et al., 2005) and cancer prevention (Rafter et al., 2007).

The optimum growth pH of bifidobacteria is between 6.5 and 7.0, with low growth occurring at below pH 4.5, or above pH 8.0 (Maus & Ingham, 2003). These thermophilic bacteria have the optimum growth temperature ranging from 37 to 41℃, with the maximum at 45℃ and the minimum between 25 and 28℃ (Gavini et al., 1991; Cronin et al., 2011). Bifidobacteria are anaerobic microorganisms. However, the sensitivity to oxygen changes accordingly to the species and the different strains of each species (Biavati et al., 2000).

Bifidobacteria are saccharolytic microorganisms that possess a large arsenal of enzymes capable to catabolize a wide range of carbohydrates such as glucose, fructose, galactose and fructose, as well as xylo-oligosaccharides (XOS), galacto-oligosaccharides (GOS), fructo-oligosaccharides (FOS), pectin and mucin. The products resulted from the carbohydrate metabolism are lactic, acetic and formic acids and ethanol, as well as small amounts of carbon dioxide and succinic acid (Gomes & Malcata, 1999; Ruas-Madiedo et al., 2008).

The great ability of bifidobacteria in using many carbohydrates was acquired from genetic adaptations derived from their survival in environmental conditions richer in complex carbohydrates (Cronin et al., 2011). *Bifidobacterium* strains prefer metabolize di - or oligosaccharides (Vernazza et al., 2006) rather than metabolize simple sugars like glucose, as carried out by other Gram-positive bacteria. This preference is related to their enzyme fructose-6-phosphate phosphoketolase (F6PPK) which is present in few bacteria (Lee & O'Sullivan, 2010).

Many organisms studied for their probiotic potential are members of the genus *Bifidobacterium*. Among bifidobacteria species, *Bifidobacterium animalis* subsp. *lactis* is considered less sensitive to stressful conditions such as bile salts (Sánchez et al., 2006), acid (Maus & Ingham, 2003) and oxygen (Shah et al., 2000). For this reason, this specie is largely applied in fermented or acidified dairy products (Briczinski et al., 2009).

As the health effects in the host are strain-dependent, few characteristics and health benefits associated to some *Bifidobacterium* strains used in this work are shown in Table 3.

Table 3 Main characteristics of some *Bifidobacterium* strains employed on the production of probiotic fermented milks

Characteristics	*Bifidobacterium animalis* subsp. *lactis* BB12	*Bifidobacterium animalis* subsp. *lactis* BL04	*Bifidobacterium animalis subsp. lactis* B94	*Bifidobacterium animalis subsp. lactis* HN019	*Bifidobacterium longum subsp. infantis* ATCC 15697
Temperature	37 °C – 43 °C[a]	37 °C – 43 °C[a]	37 °C – 43 °C[a]	37 °C – 43 °C[a]	36 °C – 38 °C[a]
Acid tolerance	++++[b, c]	++++[d]	+++[e]	++++[f]	+[g]
Bile salts tolerance	++++[c]	++++[h]	++++[i]	++++[f]	+[j, k]
Oxygen tolerance	+++[l, m, n]	++[l, n]	++[l, n]	++[l, n]	+[l]
Health effects	Reduction viable cells of *H. pylori*[o], and *Clostridium spp.*[p] during infection	Induction of IgG; ability to stimulate specific immune response; Restoration of microbiota after antibiotic treatment[c]	Anti-inflammatory effects and reduction of diarrhea in colite models[q] and diminution of *H. pylori* colonization[r]	Reduction of infection by *E. coli*, *Clostridium spp.* and *Bacteroides spp.*[s]	Adhesion to human intestinal epithelial cells; inhibition of enteropathogen-cell interactions[t]

Attributes based on data about *Bifidobacterium* species and /or strains: [a] Gavini et al. (1991); [b] Maus & Ingham (2003); [c] Vernazza et al. (2006);[d] Korzenik et al. (2005); [e] Crittenden et al. 2001; [f] Gopal et al. (2001); [g] Pereira & Gibson, 2002; [h] Barrangou et al. (2009); [i] Crittenden et al. 2005; [j] Noriega et al. (2004); [k] Hansen et al. (2002); [l] Li et al. (2010); [m] Simpson et al.(2005); [n] Lee & O'Sullivan (2010); [o] Wang et al. (2004); [p] Mohan et al. (2006); [q] Peran et al. (2007); [r] Zhang et al. (2008); [s] Gopal et al. (2003); [t] Ouwehand et al. (2003).

++++ Excellent; +++ Very good; ++ Good; + Fair.

2. Cultivation process

Fermented processes of dairy products have received great attention over the decades, ranging from chemical changes of the product components through to the final product properties (Walstra et al., 2006). Manufacturing processes are slightly different depending on the country, but it always comprises acidification that brings milk to gelification due to destabilization of the protein system (Sodini et al., 2004).

During fermentation, many reactions occur, including hydrolysis of milk proteins, drop in pH value, increase in viscosity, and generation of bacterial metabolites, which contribute to the flavor and health-promoting properties of fermented milks (Tamime & Robinson, 2001). Nevertheless, some of these reactions affect cultivability of probiotic bacteria that might be incorporated to fermented milk products (Donkor et al., 2006).

During fermented milk process using probiotic cultures, the main difficult for achieving and maintaining the required levels concerns their poor survival in milk due to the increased acidity and the presence of oxygen (Dave & Shah, 1997) and nutrient depletion in the product (Donkor et al., 2007).

In general, there are 56 species of *Lactobacillus* and 29 species of *Bifidobacterium*, which are used worldwide in dairy products (Shah, 2001). From bifidobacteria species, *B. animalis* subsp. *lactis* had been widely applied in fermented dairy products due to its lower sensitivity against acidification and higher oxygen tolerance, which is remarkable within the bifidobacteria genus (Masco et al., 2004). Therefore, the use of bifidobacteria in fermented milk requires some growth factors, known as bifidogenic factors, such as N-acetyl-D-glucosamine found in human milk (Liepke et al., 2002) and lactulose, in processed milk products (Oliveira et al., 2011).

The current practice to manufacture probiotic fermented milks recommends the addition of yogurt starter cultures to reduce incubation time

and improve texture properties (Oliveira et al., 2001). The level required of probiotic counts is variable, but assuming daily consumption of 100 g of dairy products, these products might contain at least 10^7CFU/g of probiotic bacteria at the end of cold storage (Vinderola & Reinheimer, 2000).

In order to increase bacterial concentration and improve fermented milk process conditions, some strategies are applied. Fermented milks can be affected by supplementation of the raw material (Oliveira et al., 2001; Akalin et al., 2007; Oliveira et al.; 2009), by acting on culture composition (Béal et al., 1999; Oliveira et al., 2001), by modifying the incubation temperature and final pH (Mortazavian et al., 2006; Shafiee et al., 2010) and by adapting cooling conditions (Tamime, 2002).

Supplementation of milk bases with casein hydrolysates improved acidification activity and reduced in 55% the fermentation time (Oliveira et al., 2001). The increment of skim milk powder, whey protein concentrate and sodium caseinate also reduced fermentation time and improved texture properties of yogurt and fermented milks (Damin et al., 2009). Moreover, the supplementation of milks with fructooligosaccharide or whey protein concentrate improved stability and viability of *B. animalis* during cold storage (Akalin et al., 2007). Additionally, the use of some diet fibers such as oligofructose and polydextrose influenced the acidification kinetic of probiotic strains, stimulated bifidobacteria counts and improved the relative levels of conjugated linoleic acid (CLA) after milk fermentation (Oliveira et al., 2009).

As mentioned before, the use of mixed cultures affects fermented milk processing. The mixed cultures strongly stimulated the growth of *Streptococcus thermophilus* 404 whereas *Lactobacillus bulgaricus* 398 metabolism was not significantly improved. The final total population was 1.4 to 4.9 higher than in pure cultures (Béal et al., 1994). Likewise, the use of ropy strain of *S. thermophilus* R increased the time to reach the maximum acidification rate as well as higher texture properties as compared to acidifying strain

S. thermophilus A (Béal et al., 1999). In addition to these results, a significant effect of culture composition on the rate of acidification was observed among pure and co-cultures of *L. acidophilus* La-5, *L. rhamnosus* Lc-35 and *S. thermophilus* St-7. The use of mixed cultures increased four times the acidification activity as compared to pure cultures in fermented milks (Oliveira et al., 2001).

The effect of fermentation temperature and final pH was shown to affect the production of exopolysaccharides (EPS), as a combination of final pH equal to 5.5 and incubation temperature of 40°C were suitable for EPS production by *Streptococcus thermophilus* 1275 (Zisu & Shah, 2003). Furthermore, pre-heat treatment 95°C for 15 minutes of milk bases and incubation temperature at 37°C increased the viability of probiotic microorganisms in ABY-type probiotic yogurt (*Lactobacillus acidophilus*, *Bifidobacterium lactis* and yogurt bacteria) at the end of fermentation (Mortazavian et al., 2006). The use of adequate heat treatments contributes to the reduction of the dissolved oxygen and redox potential in the milk that enhances the growth of *Bifidobacterium* strains (Dave & Shah, 1997).

At last, the combined effects of milk non-fat dry matter content, incubation temperature and final fermentation pH acted on biochemical and microbiological characteristics of probiotic fermented milk produced with *L. acidophilus* La-5, *Bifidobacterium lactis* BB12 and yogurt cultures. The highest cultivability of both probiotic microorganisms were obtained in fermented milk with 12% of dry matter, incubation temperature of 37°C and final pH 4.5 (Shafiee et al., 2010).

As a final point, the method of cooling controls the enzymatic activity of starter cultures in order to retain an abundant count of these organisms in fermented milks (Tamime, 2002). This method can be applied in one or two phase cooling by reducing the temperature of the product until 4-5°C or dropping fermentation temperature from 45°C to 20-25°C prior to addition of

fruit or packaging before the temperature of 10°C (Tamime et al, 2001). However, some cooling conditions lead to a supplementary decrease of pH of fermented milks, as observed by Dave & Shah (1997), when products were cooled during overnight until the temperature of 4°C being reached.

2.1. Chemical and physiological changes resulting from the fermentation process

During fermented milk manufacture, bacterial fermentation process result in the production of organic acids as a consequence of lactose and proteins hydrolysis, leading to the drops in pH and protein milk coagulation. Mechanisms involved in casein dissociation and aggregation during acid-induced gelation of milk are pH, ion concentration and temperature-dependent (Tamime, 2002). Moreover, acidification of milk by lactic acid bacteria results in typical sensorial properties (texture and flavor) which depend on the employed starter culture and in the final pH at the end of fermentation (Oliveira, 2009).

The final fermentation pH can vary from 4.6 to 4.0 and together with the accumulation of organic acids can be considered relevant factors affecting the metabolism, the growth and the viability of probiotic bacteria (Donkor et al., 2006; Oliveira, 2009). The production of organic acids during bacterial growth in milk depends on the nutrient requirements and the different pathways - homofermentative (lactic acid production) or heterofermentative (mainly lactic and acetic acid production), involved in the metabolism of lactic acid bacteria (Sauer et al., 2008) and bifidobacteria (Cronin et al., 2011). As an example, fermentation process using bifidobacteria results in the production of L (+)-lactic acid isomer that is easier to metabolize by infants in comparison with the D (-)- lactic acid isomer produced by other bacteria such as *L. acidophilus* and *L. bulgaricus* (Swidan, 2009).

Besides, biogenic metabolites released during the fermentation process include peptides and bioactive fatty acids (Ebringer et al., 2008). Among these

metabolites, some peptides exert hypocholesterolemic, antithrombotic, antimicrobial, antihypertensive, immunomodulatory and pharmacological opiate-like effects as observed with casomorphines, lactorphines and serorphines (Le Blanc et al., 2002; Silva & Malcata, 2005; Oliveira, 2009).

The production of bioactive fatty acids, like CLA by some bacteria, has been documented since 1960 (Bisig et al., 2007). Propionibacteria (Jiang et al., 1998), yogurt starter cultures (Coakley et al., 2006), *Lactococcus lactis* (Kim & Liu, 2002), *Lactobacillus rhamnosus* (Oliveira et al., 2009) and *L. acidophilus* (Espirito Santo et al., 2012), as well as bifidobacteria (Coackley et al., 2008; Florence et al. 2009) were considered organisms with the potential to be used the increase of CLA in dairy products. This fatty acid is a mixture of positional and geometric isomers of octadecadienoic acids (C18:2) with conjugated double bonds that have received interest since its beneficial effects were observed. These benefits include anticarcinogenic and antiatherogenic properties, as well as effects on body composition and metabolism of fat (Pariza et al. 1999; Turpeinen et al., 2002).

Another fatty acid that gained attention the last decades is α-linolenic acid or (ALA, C18:3 *cis*-9, 12, 15) an essential omega-3 polyunsaturated fatty acid (Barceló-Coblijn & Murphy, 2009). As this fatty acid cannot be synthesized by mammals, consequently, it has to be present in the diet. The potential health effects related to the ingestion of ALA are: the decrease in triacylglycerol (TAG) levels (Balk et al., 2006), antiarrhythmic properties and anti-inflammatory properties (Barceló-Coblijn & Murphy, 2009), as well as some neuroprotective effects (Barceló-Coblijn et al., 2005).

Additionally, the ability of *Bifidobacterium* species to produce conjugated α-linolenic acid, (CLNA, C18:3 *cis*-9, *trans*-11, *cis*-15) through linoleic acid isomerase activity in conjugated linoleic and α-linolenic acid has been recently tested (Gorissen et al., 2010; Hennessy et al., 2012). As CLNA displays potent anti-inflammatory properties and ability to improve biomarkers of cardio-

vascular health (Hennessy et al., 2011) it represents a novel interest of research concerning bioactive fatty acid produced during fermentation.

Finally, during the fermentation process, bacteria suffers some physiological changes resulted from the growth temperature, pH, oxygen tension, growth phase and medium composition (Guerzoni et al., 2001). It is well known that bacterial membrane fatty acid composition is altered when growth temperature is different from the optimum state (Russel, 2002). Normally, when the temperature of growth is lowered, the proportion of palmitic acid and unsaturated fatty acids (UFA) increases (Fernandez-Murga et al., 2000). Similarly, the acid pH resulted from the fermentation process increases the relative levels of UFA (Cotter & Hill, 2003) such as oleic (Béal et al., 2001) and α-linolenic acid (Montanari et al., 2010).

2.2. Chemical changes during storage at 4 ℃ of fermented milks

Several factors have been recognized to affect the stability of bacteria during storage of fermented products. Among them, the choice of strain, the possible interactions between species, the post-acidification generated by organic acids production (Dave & Shah, 1997), the oxygen content, nutrients limitations and storage temperature and duration are related to the decrease in survival of microorganisms(Donkor et al., 2007).

Considering the stability of fermented product until consumption, rate of cooling, the composition of the medium, the temperature and duration of storage along with post-acidification can be regarded as crucial factors for probiotic survival in fermented dairy products during the storage period (Shah, 2001; Beales, 2004; Boylston et al., 2004).

Studies conducted by Dave & Shah (1997) and Biavati et al., (2000), revealed the weakly growth and survival of some *Bifidobacterium* species in milk. The maintenance of bifidobacteria viability in fermented milks represents a challenge to dairy industries due to their great sensibility to low pH and

temperatures during storage (Shin et al., 2000). In addition, the decrease in cell numbers can also be a consequence of high sensitivity to oxygen (Maus & Ingham, 2003).

The duration of refrigerated storage also impacts on the viability of bifidobacteria, and lactic acid bacteria in yogurts. Shin et al. (2002) noted that viable counts remained higher than 10^6 CFU/g after one and two weeks past of the expiration day, however, a significant decrease in bacterial cultivability was established three weeks after the expiry date. Similarly, Jayamanne et al. (2006) observed no significant loss in *Bifidobacterium animalis* subsp. *lactis* counts after two weeks of storage at 4℃, nevertheless, on the expiry date, bacterial concentration declined less than 1 log cycle.

The optimum survival temperature for *B. lactis* ranges from 4 to 8℃ (Russel et al., 2011). Temperatures below 4℃ are detrimental to bifidobacteria cells (Kailasapathy & Rybka, 1997). Comparing the temperatures of 4℃ and 10℃, a higher increase in titratable acidity and a decline in pH for fermented products stored at 10℃, indicating residual activity of bacteria at this temperature (Dave & Shah, 1997). Likewise, Mortazavian et al. (2007) tested the effect of different refrigerated temperatures: 2, 5 and 8℃ on the viability of probiotics in ABY (*Lactobacillus acidophilus*, *B. lactis* BB12 and yogurt cultures) "yogurt-like" product. This study showed that storage at 2℃ resulted in the highest viability of *L. acidophilus* while 8℃ was considered the best temperature to keep the viability of *B. lactis* BB12.

In addition to these two factors that affect probiotic viability during shelf-life bacterial metabolic activity results in the production of organic acids (lactic and acetic acids) and consequently reduction in pH values. This phenomenon is known as post-acidification which diminishes cell counts of probiotics (Shah & Ravula, 2000). In general, post-acidification during cold storage is mainly ascribed by the activity of *L. bulgaricus* in fermented milks (Oliveira et al., 2001).

According to Ranadheera et al. (2012), a continued decline in pH values from the first day of storage until the expiry date was observed in stirred yogurts.

Finally, the maintenance of bacterial cell membranes functions is essential for probiotic survival during storage in acidified products (Saarela et al., 2009). From Castro et al. (1995), survival of *L. bulgaricus* lyophilized in skim milk powder decreased during storage at low temperatures. This decline in cell counts concurred with the reduction in the ratio of unsaturated to saturated fatty acids (UFA/SFA) in its cellular membranes during the storage period.

3. Environmental factors that influence viability of *Bifidobacterium* in fermented milk products

In the latest years, bifidobacteria strains have gained scientific attention due to their health claims and utilization in dietary supplements and numerous food products (Briczinski et al., 2009). However, the health benefits displayed for these bacteria depend on their colonization on the gut. For these reasons, bifidobacteria constitute great interest in the knowledge concerning molecular and physiological changes resulting from the stress suffered from the product preparation until consumption and adhesion in the human gut (De Dea Lindner et al., 2007).

Numerous stress factors influence the viability of bifidobacteria in fermented milks. These include the low temperature during storage, the acidic pH, the dissolved oxygen, the availability of nutrients and the presence of growth promoters (Donkor et al., 2007; Briczinski et al., 2009; Oliveira, 2009). After fermented milk consumption, *Bifidobacterium* strains have to overcome the stress conditions encountered in human GIT, such as the presence of oxygen and amylase in the mouth, the acidic conditions (pH ~ 2.0) in the presence of pepsin and gastric lipase in the stomach, the pancreatin and bile salts in the small intestine (duodenum and jejunum), and the nutrient starvation

and competition with indigenous microbiota in the colon in order to promote a transient colonization in the host (Ruiz et al., 2011).

According to these authors, such stress conditions can be defeated or attenuated by applying some bacterial stress treatments. These include shock (short exposure time to stress factor), transient adaptation (growth for a given time in the presence of sub-lethal concentration of the stress factor) and stable adaptation (long-time incubation with sub-lethal concentration of the stress factor) (Ruiz et al., 2011).

Nevertheless, the stress adaptation mechanisms in *Bifidobacterium* strains are still poorly characterized and understood. Some responses at molecular or physiological levels are described in the next sections.

3.1. Effect of refrigerated storage

Storage temperature is an important parameter regulating the activities of microorganisms in fermented milk products. At low temperature, the metabolism probiotics is drastically reduced (Heller, 2001) and the affinity for substrate uptake is decreased (Beney & Gervais, 2001). Cold storage is as a critical time during the shelf-life of fermented milks as *Bifidobacterium* strains are subjected to low temperature stresses which are not encountered in their normal intestinal environment (Lee & O'Sullivan, 2010). Nevertheless, some of them are more resistant to cold stress such as *B. animalis* subsp. *lactis*, while others display a small tolerance to low temperatures like *Bifidobacterium bifidum* and *B. longum* strains (Ruiz et al., 2011).

The low temperature in refrigerated products can influence the physiological responses of microorganisms by affecting their enzymatic activity, their cell composition or their nutritional requirements. Moreover, the ion transport and diffusion, the surface tension and the cell density can be influenced by the temperature during storage (Beales, 2004).

A supplementary tolerance to reduced temperatures can be achieved after pre-treatment of microorganisms to sub-lethal temperatures, resulting in greater survival (Wang et al. 2005b). The incubation of *Streptococcus thermophilus* at 20°C before freezing resulted in better survival as compared to no pretreated cells (Wouters et al., 1999). Other study conducted by Maus & Ingham (2003) tested various combinations of reduced temperature in order to adapt *Bifidobacterium longum* and *B. lactis* cells to subsequent cold-tolerance. The results showed that *B. longum* had less cold-tolerance than *B. lactis* and the stress generated by the low temperature is specie-dependent (Maus & Ingham, 2003).

For some microorganisms, a “cold shock response” has been observed in reaction to sudden changes to lower temperatures. Some strategies to lead with cold stress is an alteration of membrane fatty acid composition after a decrease in temperature (Beales, 2004) or the synthesis of DNA and RNA-binding proteins that counteract the stabilizing effect of cold temperatures on nucleic acid secondary structures (De Angelis & Gobbetti, 2004).

The exposure to low temperature results in the development of a close array of acyl chains of fatty acids in the cytoplasmic membrane, resulting in a gelling effect on the lipid bilayer (Russell, 2002). The synthesis of cold-shock proteins (CSPs), cold-shock acclimatation proteins (CAPs) and cold-induced proteins (CIPs) is also promoted in response to cold stress (Phadtare et al., 2004). CSPs can be transiently overexpressed in low temperatures while CAPs and CIPs are labeled according to the size of the protein and the method by which the microorganism was transfered to low temperatures (Panoff et al., 2000).

When cells are in normal physiological state, the membrane must be fluid to allow the transport of ions, the uptake of nutrients, as well as to perform cellular respiration (Berry & Foegeding, 1997). At low temperatures, membrane components change from the liquid crystalline to gel-like state, avoiding the

correct functioning of proteins (Beales, 2004). In order to maintain the membrane fluidity which is crucial to the survival of bacteria at low environmental temperature during storage, the membrane fatty acid composition must be modified. The term membrane fluidity concerns the effects of chain conformation, lateral, microviscosity and the resistance of the membrane to sheer forces (Denich et al., 2003).

Some alterations in membrane fatty acid composition can occur after a fall in temperature. Commonly these changes are obtained by increasing the unsaturation degree or shortening chain length of membrane fatty acids, by changing iso and anteiso positions of branched fatty acids and by reducing the proportion of cyclic fatty acids (Russell et al., 1995). All these modifications in cells membranes increase the membrane fluidity (Russel, 2002).

3.2. Effect of pH lower than pH 5.0

The reduction in survival of bifidobacteria has been mainly attributed to the decrease in the pH and the accumulation of organic acids during fermentation (Shah, 2000). The pH at the end of the fermentation process appears to be a significant factor affecting the bacterial concentration of *Bifidobacterium* strains (Shah & Ravula, 2000).

In general, bifidobacteria have low tolerance to acidic conditions as compared with *Lactobacillus* strains. However, *B. animalis* subsp. *lactis* is an exception amongst *Bifidobacterium* strains (Kheadr et al., 2007). As reported by Abe et al. (2009), human bifidobacteria are more sensitive to environmental factors such as acidic pH than animal bifidobacteria, and for this reason, it is more difficult to guarantee their survival in fermented dairy products. Moreover, it was observed that *B. animalis* subsp. *lactis* was also capable of survival during *in vitro* gastric fluids with pH lower than 3.5. The exposure to sub-lethal acid stress during fermented milk production improved bifidobacteria survival to the adverse conditions found in GIT (Maus & Ingham, 2003).

The effect of different final fermentation pH (4.45, 4.50, 4.55 or 4.60) during yogurt manufacture on the viability of probiotic bacteria during chilled storage was tested by Donkor et al. (2006). Surprisingly, the different termination pH has no effect on the viability of probiotic organisms. The survival of probiotic bacteria was considered strain-dependent, and the reduction in pH values during cold storage coincided with the increase in organic acids level, resulting in low counts of *B. lactis* B94 (Donkor et al., 2006).

One of the mechanisms implicated into acid tolerance of bifidobacteria is related to the F_1F_0 ATPase, encoded by the ATP operon (Ventura et al., 2004), which is essential for their growth under acidic conditions. The activity of the F_1F_0 ATPase is enhanced at low external pH (Ventura et al., 2007). Nevertheless, the acid tolerance is not managed in the same way for all the bifidobacteria. For an example, the F_1F_0 ATPase was not up regulated in acid conditions for *B. dentium* Bd1 that may control its acid tolerance by amino acid degradation (Cronin et al., 2011). Other mechanisms involved into the control of the environmental pH are the glutamate decarboxylases (GAD) and the transporters which are able to increase intracellular pH after acid exposure (Cotter & Hill, 2003).

Moreover, the function of the cell membrane is demonstrated by changes in membrane fatty acid profiles in reaction to acidic conditions. The reduction of growth conditions of LAB to pH 5.0 increased levels of monounsaturated (MUFA) and long chain fatty acids (LCFA) as compared to the fatty acid composition at pH 7.0 (Cotter & Hill, 2003).

In conclusion, the study of Veerkamp (1971) showed that the age of the culture in combination with the decrease of pH, appeared to affect the fatty acid composition of *B. bifidum* subsp. *pennsylvanicus*. The main fatty acids modification was done by the increase of average chain length of the fatty acids, resulting in a decrease of the permeability of the cells during growth by a decreased fluidity of the lipid barrier of membrane.

3.3. Effect of oxygen

Since *Bifidobacterium* strains are strictly anaerobic, the dissolved oxygen levels during product manufacture and refrigerated storage have also been mentioned as significant factors affecting the survival of *Lactobacillus* and *Bifidobacterium* in dairy products (Talwalkar & Kailasapathy, 2004). In contrast to aerobic bacteria, the oxygen is not entirely reduced to water, because of the reduced oxygen-scavenging system of these probiotic bacteria (Miller et al., 2003).

It was observed that when bifidobacteria strains are cultivated in co-culture of *Streptococcus thermophilus*, high-oxygen tolerant specie, the effect of dissolved oxygen in the product is strongly reduced. *S. thermophilus* can act as an oxygen scavenger, by consuming the oxygen in the fermented product, reducing the exposition of bifidobacteria to this lethal stress (Talwalkar & Kailasapathy, 2004).

The packaging system also exerts a high impact on the dissolved oxygen content of fermented milks (Miller et al., 2002). It was noted that, during the shelf-life of the product, the oxygen level increased from 20ppm to 50ppm in polystyrene packaging. In contrast, the use of a packaging material with oxygen barrier layer lowered the initial oxygen content from 20ppm to 8ppm over 42 days (Miller et al., 2003).

Bifidobacteria are classified as catalase negative microorganisms, and this enzyme is essential for the decomposition of oxygenic metabolites. Consequently, an oxidative damage is induced by the formation of reactive oxygen species (ROS), such as hydrogen peroxide (H_2O_2) or superoxide ion, leading to bifidobacteria cell death (Sánchez et al., 2012).

As observed by Shimamura et al. (1992), the oxygen tolerance is specie-dependent. Three strains *Bifidobacterium breve*, *B. infantis*, and *B. longum* of four bifidobacteria analyzed species were able to growth under partial aeration conditions, whereas the growth of the fourth, *B. adolescentis* was significantly

reduced by low concentrations of oxygen. Additionally, *Bifidobacterium animalis* subsp. *lactis* showed the most aerotolerance of this genus (Masco et al., 2005; Li et al., 2010). In contrast, *B. bifidum* JCM 1255T and *B. longum* subsp. *longum* JCM 1217T can support just 5% of dissolved oxygen in the medium and *B. boum* JCM 1211T and *B. thermophilum* JCM 1207T are capable to grown under 20% oxygen (Kawasaki et al., 2006).

The oxygen tolerance of some strains was related to NADH oxidase, NADH peroxidase and superoxide dismutase (SOD). *B. animalis* subsp. *lactis* possesses NADH enzymes and some catalase activity helping to eliminate hydrogen peroxide from the environment (Lee & O'Sullivan, 2010). It has also been reported that cells exposure to oxygen promote the fermentation of some carbohydrates, even though, bacteria could not increase in number by cell division due to oxygen toxicity (Amor et al., 2002).

In order to investigate what type of stress response has to oxygen stress, Ahn et al. (2001) tested five oxygen-tolerant bifidobacteria during cell growth in the presence of oxygen. Their results showed that the lag phase increased, and some dissolved oxygen was removed in the early stage of growth. These authors also observed that cells become longer, and the membrane surface contained many nodes, derived from incomplete cell division. Moreover, the chain length of cellular fatty acid was shortened and, cyclopropane fatty acid (9, 10-methyleneoctadecanoic acid; C19:0cyc9,10) was increased (Ahn et al., 2001). Similarly, Oberg et al. (2012) tested the oxygen tolerance of two *B. animalis* subsp. *lactis* strains DSM10140 and BL04. Higher resistance to H_2O_2 was observed for BL04 strain in comparison to DSM10140 strain. Coincidently, this strain was characterized by significant higher percentage of C19:0cyc (15.5%) than 6.7% in DSM10140.

Additionally, a recent study at proteomic level revealed that oxidative stress-protective proteins and DNA oxidative damage-protective proteins are

involved in the defense of *Bifidobacterium longum* BBMN68 against oxygen stress conditions (Xiao et al., 2011).

3.4. Effect of combined stress

During fermented milk production and storage at 4°C, *Bifidobacterium* strains are exposed to combined stress. Firstly, the acidic conditions generated by the organic acids production and decrease in pH values occur during the whole process (Østlie et al., 2005). Secondly, the exposure to oxygen is inevitable during fermented milk processing in the food industry, and the oxygen levels tend to increase during refrigerated storage (Fortin et al., 2011). Thirdly, these two technological factors along with the low temperature during chilled storage are considered detrimental to survival of bifidobacteria in fermented products (Donkor et al., 2007).

Some studies were performed in order to improve knowledge concerning the effects of these environmental stress during fermentation and cold storage of fermented milk on the viability of yogurt and bifidobacteria (Damin et al., 2006; Donkor et al., 2006; Abe et al., 2009; Jayamanne et al; 2009).

The main effects of combined stress into *B. lactis* BL04 viability were noted in the first week as the greater decrease in pH values occurred at this period of storage (Damin et al., 2006). Studies realized by Donkor et al. (2006) showed that cell counts of *B. lactis* B94 decreased by one log cycle at the end of storage as a consequence of the increased proteolysis that improved survival of *L. delbrueckii* subsp. *bulgaricus* Lb1466 during storage, resulting in reduction of pH and production of higher levels of organic acids, occasioning the low cell counts for *B. lactis* B94.

Moreover, bifidobacteria survival in yogurt after 35 days of cold storage was higher than 1.0 10^7 CFU/g (Abe et al., 2009). Similarly, Jayamanne et al. (2009) observed that *B. animalis* had better survival in the fermented milk

during chilling in comparison with *B. longum.* Indeed, these authors showed that cold storage temperature, pH and the oxidoreduction potential (Eh) could be manipulated to enhance survival during storage. Nevertheless, the Eh reduction appears to be the best way to reduce the loss of viability for probiotic strains.

At last, there are no available results concerning the degradation of physiological responses of bifidobacteria when submitted to the combined stress during storage at 4°C of fermented milks.

3.5. Effect of gastrointestinal stress

The incorporation of bifidobacteria in dairy products implies the survival in the product, as well as their resistance through the human GIT with high viable population in order to display their health benefits (Oozeer et al., 2006). These beneficial effects of bifidobacteria can be envisaged when these microorganisms are able to colonize the GIT (Kailasapathy & Chin, 2000).

The survival of bifidobacteria during gastrointestinal transit depends on many variables, such as the choice of strain, the fermented milk process conditions and the choice of food matrix for cell delivery to the gut (Corcoran et al., 2008).

During the passage through the gastrointestinal region, bifidobacteria are exposed to sequential challenges of low pH in the stomach, followed by bile secretions and pancreatic enzyme in the small intestine. Bifidobacteria survival also implicates into their ability to withstand to these stressful conditions (Corcoran et al., 2008; Kleerebezem & Vaughan, 2009).

The adaptive ability to resist acidic stress or bile salt differ a lot within the members of the *Bifidobacterium* genus (Sánchez et al., 2007), suggesting that the mechanisms to deal with acid pH and to bile salts stresses may vary in expression or composition among the different *Bifidobacterium* species (Cronin et al., 2011).

The pH in gastric environment is in constantly in flux varying from pH 2.0 to pH 5.0, depending on the nature of the consumed product (Marteau et al., 1997). The common response to acidic conditions is the acid tolerance response (ATR), which is induced in bifidobacteria cells after exposure to sub-lethal acid conditions during food processing. This mechanism allows improving the bacterial survival to the severe pH conditions (pH 2.0) in gastric environment (Maus & Ingham, 2003). Furthermore, the ATR also depends on the pH profile of the F_1F_0 ATPase enzymes and the cytoplasmic membrane composition, as well as the manufacturing conditions in which bifidobacteria were produced (Madureira et al., 2011).

The acidic conditions in gastric environment cause structural cellular membrane damage, intracellular accumulation of protons and reduce the intracellular pH (pHi) (Corcoran et al., 2008). The acid resistance against these damages is strain-specific. For example, *B. animalis* subsp. *lactis* submitted to pH 3.0 during 60 minutes demonstrated superior acid tolerance with no decrease in viability, while the concentration of *B. adolescentis* strains decreased under the same conditions (Mättö et al., 2004).

The main strategies of response of bifidobacteria to acid environmental challenges are related to the branched-chain amino acid production, the glutamine synthetase (Ruiz et al., 2011), the ATP synthesis and proton extrusion (Mills et al., 2011).

After exposure to low pH in stomach, bifidobacteria encounter the stress barriers in the intestine. These stress barriers in the small intestine are the high bile salts concentration, the presence of pancreatin and the nutrient competition in the large intestine (Ruiz et al., 2011).

Bile salts promote an extremely complex response in bifidobacteria, involving numerous cellular mechanisms. Study of the bile-adapted strain *B. animalis* subsp. *lactis* 4549dOx and the wild type strain, IPLA4549, has shown changes in enzymatic profiles, protein expression patterns, cell surface

properties and production of extracellular exopolysaccharides in response to bile stress (Ruas-Madiedo et al., 2009). In addition, changes in the levels of proteins with essential functions, such as some ribosomal proteins were affected during exposure to bile salts concentration (Ruiz et al., 2009).

According to Ruiz et al. (2007), the influence of bile stress in *B. animalis* 4549dOx resulted in a decrease in membrane fluidity, as well as severe deformation of the cell surface, affecting bifidobacteria survival.

Finally, in the large intestine, the ingested bifidobacteria interact with the endogenous microbiota. When bifidobacteria population is lowered in the small intestine, the numerical abundance of these microorganisms decreases to eventually become a minor subpopulation in the large intestine, lowering the transiently colonization in the gut and also reducing the possibilities of probiotic bifidobacteria to exert their health effects on the host (Kleerebezem & Vaughan, 2009).

CHAPTER 2 – Fatty acid profile, *trans*-octadecenoic, α-linolenic and conjugated linoleic and acid contents differed in certified organic and conventional probiotic fermented milks

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Fatty acid profile, *trans*-octadecenoic, α-linolenic and conjugated linoleic acid contents differing in certified organic and conventional probiotic fermented milks

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α-Linolenic acid

ABSTRACT

Development of dairy organic probiotic fermented products is of great interest as they associate ecological practices and benefits of probiotic bacteria. As organic management practices of cow milk production allow modification of the fatty acid composition of milk (as compared to conventional milk), we studied the influence of the type of milk on some characteristics of fermented milks, such as acidification kinetics, bacterial counts and fatty acid content. Conventional and organic probiotic fermented milks were produced using *Bifidobacterium animalis* subsp. *lactis* HN019 in co-culture with *Streptococcus thermophilus* TA040 and *Lactobacillus delbrueckii* subsp. *bulgaricus* LB340. The use of organic milk led to a higher acidification rate and cultivability of *Lactobacillus bulgaricus*. Fatty acids profile of organic fermented milks showed higher amounts of *trans*-octadecenoic acid (C18:1, 1.6 times) and polyunsaturated fatty acids, including *cis*-9 *trans*-11, C18:2 conjugated linoleic (CLA-1.4 times), and α-linolenic acids (ALA-1.6 times), as compared to conventional fermented milks. These higher levels were the result of both initial percentage in the milk and increase during acidification, with no further modification during storage. Finally, use of bifidobacteria slightly increased CLA relative content in the conventional fermented milks, after 7 days of storage at 4 °C, whereas no difference was seen in organic fermented milks.

1. Introduction

Organic methods of food production have gained increased public interest over the last couple decades, mainly in the western world. Organic and conventional dairy productions differ in feeding regimens, use of antibiotics and chemotherapeutic treatments, and handling of the animals (Collomb et al., 2008). Organic milk is produced in an agro-system under more constrained conditions in which the use of synthetic livestock additives or other artificial inputs, as well as genetically modified organisms are forbidden. This production relies on ecological practices that prohibit the use of antibiotics, hormones and any synthetic chemical fertilizers (Toledo et al., 2002).

Milk is an excellent source of lactose, dairy proteins as caseins and whey proteins, calcium and it contains other minerals and trace elements. According to Ellis et al. (2006) there is little or no difference between organic and conventional milk samples by considering their carbohydrate, proteins and minerals contents. Conversely, significantly higher amounts of polyunsaturated fatty acids (PUFA), conjugated linoleic (CLA) and n-3 fatty acids are found in organic milk (Collomb et al., 2008). This is also confirmed by Butler, Stergiadis, Seal, Eyre, & Leifert (2011), who indicated that fatty acid profile and antioxidant content of milk are influenced by management (organic or conventional), season and brands. The distribution of these fatty acids in milk is important as it confers different characteristics to the milk (Ekinci et al., 2008).

Among the unsaturated fatty acids, the relative concentration of three main long chain fatty acids (LCFA) differed according to the kind of milk. Conjugated linoleic acid, an isomer of linoleic acid (C18:2), has gained considerable attention due to its potentially beneficial biological effects (Gnädig et al., 2003), including anticarcinogenic, antiatherogenic, antidiabetic and immune stimulation. The *trans* fatty acids content in milk represents about 2% of total fatty acids, which can be increased to 4–10% of total fatty acids by

enhancing dietary unsaturated oils content in the cow's diet. *Trans*-vaccenic acid, known as (*E*)-11-octadecenoic acid (TVA, C18:1 *trans*-11), is the main *trans* fatty acid isomer found in the fat of ruminants and in dairy products such as milk and yogurts (Santora et al., 2000). It participates in CLA production, through enzymatic action of Δ-9-desaturase in mammary glands (Gnädig et al., 2003), and contributes to supply the human body's in CLA (Butler et al., 2011). It is also an intermediate fatty acid of CLA biohydrogenation pathway (Bergamo et al., 2003). Finally, α-linolenic acid (ALA), the major omega-3 fatty acid in milk, has been related to the ability to exert anti-arrhythmic effect in the heart, to have a positive impact on neurological function by limiting central nervous system injury and to protect against coronary heart disease (Barceló-Coblijn & Murphy, 2009). It is also the dietary precursor for three long-chain omega-3 polyunsaturated fatty acids (LC-PUFA) synthesis: eicosapentaenoic acid (EPA), docosapentaenoic acid (DPA), and docosahexaenoic acid (DHA) (Brenna et al., 2009).

Production of fermented milks using bifidobacteria is a big challenge in dairy industry because milk, on the whole, is not a suitable matrix for the growth of lactic and probiotic bacteria since they lack essential proteolytic activity (Oliveira et al., 2001). Interest of bifidobacteria for human health is related to their survival through gut intestinal tract and to their role for stimulating the immune system and for preventing microbial gastroenteritis (Hols et al., 2005; Foligne et al., 2007). In addition, CLA production by bifidobacteria was shown to be a possible mechanism for their health enhancing properties (Oh et al., 2003).

Until now, few studies have explored the effect of organic milk on the growth of bifidobacteria and yogurt starters. From our knowledge, only the work of Florence et al. (2009) described the acidification profile, fatty acids contents, and chemical composition of organic and conventional milks fermented by bifidobacteria in co-culture with *Streptococcus thermophilus.* These authors detected higher protein and iron concentrations in organic fermented milks,

although no difference was observed in the initial milk. In addition, they found higher relative concentrations of TVA and CLA in organic fermented milks. From this information, a better knowledge about acidification kinetics and milk composition of organic and conventional fermented milk products is needed. In this context, this study aimed at characterizing the behavior of bifidobacteria and yogurt starters during organic and conventional milk fermentation. Their impact on milk composition, in terms of overall fatty acid composition, and *trans*-octadecenoic, conjugated linoleic and α-linolenic acid relative contents, were determined and compared, during fermentation and cold storage of the fermented milks.

2. Material and methods

2.1. Milks

Commercial organic (Naturallis, São Paulo, Brazil) and conventional (Batavo, São Paulo, Brazil) UHT whole milks were purchased from local supermarket. They were heat treated at 85°C for 15 minutes in a water-bath (Lauda, Type A100, DR. R. Wobser GmbH & Co. KG, Germany), under constant stirring. They were cooled down to 10°C and stored overnight at 4°C before manufacture of fermented milks.

Skimmed milk powder (Molico, Nestlé, São Paulo, Brazil) was reconstituted at 10% (w/w) and heat treated at 121°C for 15 minutes. It was used for inoculum preparation.

2.2. Preparation of cultures

Three commercial freeze-dried strains of probiotic and yogurt cultures were employed: *Streptococcus thermophilus* TA040 (Danisco, Dangé-Saint-Romain, France), *Lactobacillus delbrueckii* subsp*. bulgaricus* LB340 (Danisco,

Madison, WI) and *Bifidobacterium animalis* subsp. *lactis* HN019 (Danisco, Madison, USA).

Each lyophilized strain was weighted and rehydrated in 50 mL of sterilized skimmed milk at 42°C for 15 min before use, as recommended by the manufacturer. One mL of each rehydrated culture was inoculated in 500 mL of organic and conventional milk allowing initial counts of 6.0 $\log_{10}$ colony forming units (CFU)/mL.

2.3. Experimental procedure

Organic and conventional UHT heat treated milks were tempered at 42°C, divided into two batches, and inoculated with two combinations of starter cultures. Yogurt was achieved by inoculating both *S. thermophilus* TA040 (50%) and *L. bulgaricus* LB340 (50%) and probiotic fermented milk was prepared by inoculating these two strains (33% each) and *Bifidobacterium lactis* HN019 (33%). Inoculated milk samples were incubated at 42°C in a thermostatically controlled water bath until pH reached 4.5. The pH and the acidification rate (dpH/dt, in upH/min) of each microbial blend were monitored by using the Cinac system (Ysebaert, Frépillon, France). The time to reach pH 4.5 ($t_{pH4.5}$, in hours) was used to differentiate the mixed cultures. After achievement of pH 4.5, the fermentations were stopped by rapid cooling in an ice bath until 10°C. The samples were dispensed into 50 mL polypropylene cups, thermally sealed using Selopar equipment (BrasHolanda, Pinhais, Brazil) and stored at 4°C until required for analysis. The samples were prepared in duplicate, and the experiment was replicated twice on different days.

Before fermentation, at final fermentation time and after 7 days of storage at 4°C, the cultivability (CFU/mL) of yogurt and probiotic bacteria, the fatty acids profile of milk and fermented milks, including *trans*-octadecenoic acid, CLA and ALA relative contents were determined.

2.4. Chemical composition of milks

Fat, proteins, total solids content and density were determined with an ultrasonic milk analyzer Ekomilk (Eon Trading, Stara Zagora Bulgaria). Titratable acidity was analyzed as recommended by Association of Official Analytical Chemists (AOAC, 1995) and lactose concentration was determined according to the Lane–Eynon method based on the reduction of copper (AOAC, 1995). A digital potentiometer (Mod.8603, Mettler-Toledo, Scherzenbach, Switzerland) was used for pH measurements. All analyses were duplicated.

2.5. Cultivability measurements

The CFU counts (log_{10} CFU/mL) were determined in triplicate. *S. thermophilus* and *L. bulgaricus* were respectively plated onto M17 lactose agar and MRS agar (Oxoid, Basingstoke, UK), previously acidified to pH 5.4 with acetic acid. *B. lactis* was enumerated in RCA (Oxoid, Basingstoke, UK) added with 2 µg/mL of dicloxacillin (pH 7.1) and 0.3 g/L aniline blue (InLab, São Paulo, Brazil). They were incubated at 37°C for 48 h under anaerobic conditions (AnaeroGen, Oxoid, Basingstoke, UK). CFU were counted after anaerobic incubation at 37°C for 72 h of at least four replicates.

2.6. Fatty acids extraction and analysis

The lipids were extracted from organic and conventional UHT milks, yogurts and probiotic fermented milks, according to International Organization for Standarzation ISO 14156-IDF 172:2001 (ISO, 2001), which is a dedicated method for extraction or separation of lipids and liposoluble compounds from milk and milk products. Fatty acids methyl esters (FAME) of milk lipids were prepared by transesterification according to ISO 15884-IDF 182:2002 (ISO, 2002), that consists in a base-catalyzed methanolysis of the glycerides,

followed by a neutralization with crystalline sodium hydrogen sulfate to avoid saponification of esters.

Analyses of FAME were carried out in a gas chromatograph, model 3400CX (Varian, Walnut Creek, Ca., USA) equipped with a split-injection port, a flame-ionization detector and a software package for system control and data acquisition (model Star Chromatography Workstation version 5.5). Injections were performed in a 30 m long fused silica capillary column with 0.25 mm internal diameter, coated with 0.25 μm Chrompack CP-Wax 52CB (ChromTech, Apple Valley MN., USA). Helium was used as carrier gas at a flow rate of 1.5 mL/min and a split ratio of 1:50. The injector temperature was set at 250°C and the detector at 280°C. The oven temperature was initially set at 75°C for 3 min, then programmed to increase to 150°C at a rate of 37.5°C/min, and then to 215°C at a rate of 3°C/min (Luna et al., 2004). Samples (1 μL) were injected manually after a dwell-time of *ca* 2s. Qualitative fatty acid composition of the samples was determined by comparing the retention times of the peaks with those of standards 05632 and 189-19 (Sigma, Chemical Co., St Louis, MO, USA). The relative content of each FAME was calculated from the area of each peak, and expressed as a percentage, according to the official method Ce 1-62 of American Oil Chemists' Society (AOCS, 1997). Results were grouped and expressed as percentages of short chain fatty acids (SCFA - C4:0 and C6:0), medium chain fatty acids (MCFA - C8:0 to C15:0), long chain fatty acids (LCFA - C16:0 to C18:3), saturated fatty acids (SFA), monounsaturated fatty acids (MUFA), and polyunsaturated fatty acids (PUFA), according to Ackman (2007). All samples were analyzed in quadruplicate.

2.7. Statistical analysis

General Linear Models (GLM), multifactor analyses of variance (ANOVA) and multiple comparison tests were done using Statistica 8.0 (Statsoft, Tulsa,

USA) in order to determine statistical significance of differences among samples. Mean values were compared using the Newman Keuls test at $P < 0.05$.

3. Results and discussion

3.1. Composition of organic and conventional milks

The chemical composition, which is expressed as percentage (%), was similar by considering conventional and organic milks. The contents in fat (3.0 ± 0.05%), total solids (11.7 ± 0.09%) and lactic acid (0.15 ± 0.01%) were similar in both milks as measured before fermentation (day 0). Conversely, protein (2.4 ± 0.0%) and lactose (4.7 ± 0.1%) concentrations were significantly lower in organic milk than in conventional milk (2.8 ± 0.1% and 4.9 ± 0.1%, respectively). The chemical composition of organic and conventional cow milks found in the present study was comparable to those reported by (Sola-Larrañaga & Navarro-Blasco, 2009). On the contrary, Toledo et al. (2002) reported similar levels of lactose but higher fat and protein concentrations. Differences in milk composition can be attributed to management system, season, and sampling periods in which the milk was purchased (Butler et al., 2011).

Table 1 summarizes the percentage of total identified fatty acid composition of the four kinds of fermented milks, before (0) and after fermentation (1) and after (7) days of storage at 4°C.

Table 1 Evolution of identified fatty acids methyl esters composition (%) in organic and conventional milks fermented by yogurt cultures or probiotic yogurt cultures

Kind of milk	Sample	Time (days)	SCFA	MCFA	LCFA	SFA	MUFA	PUFA
Organic	Y	0	5.51±0.18^{abc}	21.69±0.36^{a}	71.76±0.25^{abc}	68.86±0.20^{ab}	27.89±0.26^{ab}	3.36±0.04^{bc}
	Y	1	5.08±0.16^{a}	22.08±0.19^{ab}	72.82±0.312^{bc}	68.25±0.18^{a}	28.13±0.18^{b}	3.61±0.02^{cd}
	Y	7	5.29±0.14^{ab}	21.87±0.16^{ab}	72.84±0.30^{bc}	68.11±0.24^{a}	28.24±0.21^{b}	3.65±0.04^{d}
	PY	0	5.61±0.13^{abc}	22.69±0.15^{ab}	71.72±0.25^{abc}	68.89±0.15^{ab}	27.73±0.13^{ab}	3.39±0.01^{bc}
	PY	1	5.10±0.08^{a}	21.71±0.33^{a}	73.20±0.36^{c}	68.44±0.32^{a}	27.97±0.19^{ab}	3.60±0.05^{bcd}
	PY	7	5.48±0.22^{abc}	22.07±0.26^{a}	72.46±0.46^{abc}	68.48±0.13^{a}	27.92±0.13^{ab}	3.60±0.02^{bcd}
Conventional	Y	0	6.31±0.38^{bc}	23.97±0.84^{b}	70.00±0.97^{ab}	71.08±0.87^{c}	26.60±0.40^{a}	2.62±0.10^{a}
	Y	1	5.00±0.09^{a}	21.64±0.16^{a}	73.35±0.21^{c}	69.01±0.18^{ab}	28.19±0.16^{b}	2.58±0.08^{a}
	Y	7	5.56±0.25^{abc}	23.47±0.61^{ab}	70.97±0.85^{abc}	70.42±0.37^{bc}	26.96±0.32^{ab}	2.61±0.04^{a}
	PY	0	6.30±0.41^{bc}	23.99±0.89^{b}	70.08±1.01^{ab}	71.12±0.85^{c}	26.67±0.49^{a}	2.58±0.08^{a}
	PY	1	5.44±0.21^{abc}	22.79±0.60^{ab}	71.39±0.93^{abc}	69.67±0.50^{abc}	27.27±0.49^{ab}	2.69±0.14^{a}
	PY	7	4.89±0.01^{a}	21.66±0.04^{a}	73.44±0.04^{c}	69.18±0.01^{ab}	28.11±0.03^{b}	2.71±0.02^{a}

Abbreviations: Y = yogurt culture; PY = probiotic yogurt culture; Short Chain fatty acid (SCFA, C4:0 to C6:0); Medium Chain fatty acid (MCFA, C8:0 to C15:0); Long Chain fatty acid (LCFA, C16:0 to C18:3); SFA, Saturated fatty acid; MUFA, Monounsaturated fatty acid; PUFA, Polyunsaturated fatty acid; 0 = days 0 (before fermentation), 1 (1 day after fermentation) and 7 = (7 days storage at 4°C). Mean values (N = 4), ± standard deviation with different letters in the same column are significantly different ($P \leq 0.05$).

The fatty acid composition of conventional and organic milks differed according to the kind of milk used for the fermentation. Their distribution according to chain length allowed separating short chain (SCFA), medium chain (MCFA) and long chain fatty acids (LCFA). The saturation degree allowed classifying the fatty acids into saturated (SFA), monounsaturated (MUFA) and polyunsaturated (PUFA) fatty acids.

The main fatty acids encountered in milk corresponded firstly to saturated fatty acids such as myristic acid (C14:0, 12.1-12.7%), palmitic acid (C16:0, 28.9-31.9%) and stearic acid (C18:0, 9.6-12.2 %). Monounsaturated fatty acids were also found. Among them, oleic acid (C18:1 *cis*-9, 21.3-21.8%), palmitoleic acid (C16:1 *cis*-9, 1.5-1.9%) and *trans*-octadecenoic acid (*trans*-C18:1, 2.1-3.3%) were the more abundant. Thirdly, polyunsaturated fatty acids were detected. The PUFA fraction was mostly composed by linoleic acid (LA, C18:2 *cis*-9, 12, 1.6-1.9%), conjugated linoleic acid (CLA, C18:2 *cis*-9, *trans*-11, 0.7-1.0%) and α-linolenic acid (ALA, C18:3, *cis*-9, 12, 15, 0.3-0.5%). PUFA and MUFA concentrations were, in this study, lower (2.5-3.5% and 27-28%, respectively) than those found by Rodríguez-Alcalá, Harte, & Fontecha (2009) in cow milk (5.7% for PUFA and 32.9% for MUFA). As a consequence, higher relative contents in SFA were found in the present study, 68-71% as compared to 60% obtained by Rodríguez-Alcalá et al. (2009).

From Table 1, the fatty acid composition results expressed as % of total fatty acids differed according to chain length in organic and conventional milks, as measured before fermentation. The relative content of short chain fatty acids (SCFA; C4:0 and C6:0) was lower in organic milk (5.6% instead of 6.4%) than in conventional milk. The medium chain fatty acid (MCFA; C8:0-C15:0) percentage was slightly lower in organic milk (difference of 0.6%). These data are in agreement with those reported by Collomb et al. (2008) that equally did

not find significant difference according to the long chain length (LCFA; C16: - C18:3) in organic and conventional milks.

The proportion in saturated fatty acids (SFA) was slightly higher in conventional milk (+2%). Conversely, for Ellis et al. (2006) and Collomb et al. (2008), organic and conventional milks did not significantly differ with respect to SFA. By regarding MUFA, their proportion was always lower in conventional fermented milks (-2%). Nevertheless, these results are contradictory with that obtained by Ellis et al. (2006) who found higher amounts of MUFA in conventional milks. More specifically, *trans*-C18:1 relative content was 1.6 times higher in organic products (Fig. 1A), in agreement with data reported by (Bergamo et al., 2003). After all, percentage of PUFA fraction was 1.3 times higher in organic products, when compared to conventional milks, as previously reported by (Ellis et al., 2006). Among these PUFA, the linoleic acid (LA - C18:2) was higher in organic milk, with 1.9 ± 0.02% instead of 1.6 ± 0.01% for conventional products. The initial relative contents of CLA (1.0 ± 0.01%) and ALA (0.5 ± 0.00%) were 1.4 and 1.6 times higher in organic milk (Fig. 1B and 1C). Even if Ellis et al. (2006) did not confirm that as a general rule, similar findings were reported by Bergamo et al. (2003) and Collomb et al. (2008).

Finally, the main difference observed in fatty acid composition of conventional and organic milks was related to the higher unsaturated fatty acid content in organic milk. It could be ascribed to the feeding regiment of the cows, as demonstrated by Bergamo et al. (2003), Collomb et al. (2008) and Butler et al. (2011).

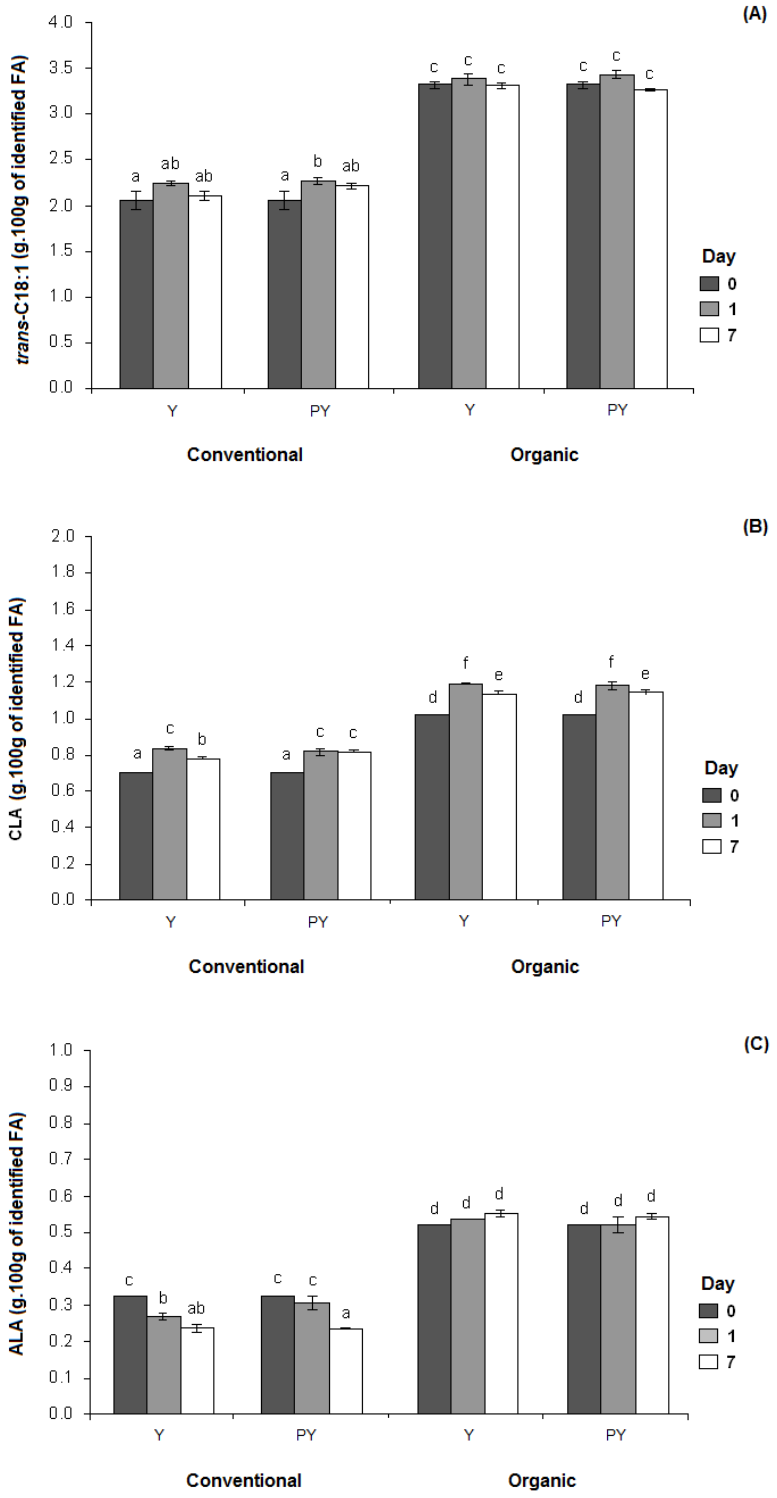

Fig. 1 Evolution of *trans*-octadecenoic acid (*trans*-C18:1, A), conjugated linoleic acid (CLA, B) and α-linolenic acid (ALA, C) relative contents in organic and conventional milks during fermentation by *Streptococcus thermophilus* TA040 and *Lactobacillus bulgaricus* LB340 (Y), and *Streptococcus thermophilus* TA040, *Lactobacillus bulgaricus* LB340 and *Bifidobacterium animalis* subsp. *lactis* HN019 (PY). 0: day 0 (before fermentation); 1: day 1 (1 day after fermentation); 7: day 7 (7 days storage at 4°C); Means (n = 4) with different letters are significantly different; $P \leq 0.05$.

3.2. Fermentation profile

The acidification profiles of yogurt made with *S. thermophilus* TA040 and *L. delbrueckii* subsp. *bulgaricus* LB340, and probiotic fermented milk containing the same yogurt culture plus *B. animalis* subsp. *lactis* HN019, in organic and conventional UHT milks, are shown on Fig. 2.

A similar acidification profile was observed for yogurt culture in both milks (Fig. 2A). Even if initial pH slightly differed (pH 6.54 ± 0.01 conventional milk, instead of pH 6.65 ± 0.01 in organic milk), the higher rate of acidification in organic milk (15.3 10^{-3} upH/min) than in conventional milk (11.7 10^{-3} upH/min) (Fig. 2B) allowed the final pH to be reached at the same time (tpH4.5 = 6.2 ± 0.3 h in both fermented milks). From Fig. 2B, two maximum acidification rates were observed whatever the kind of milk. This is explained by Pernoud et al. (2004), who demonstrated that *S. thermophilus* is an urease positive species, thus allowing urea conversion into ammonia and carbon dioxide. This transitory ammonia synthesis neutralized lactic acid, thus explaining the temporary pH stabilization, which resulted in these two peaks. This phenomenon has a direct impact on acidification profiles, due to natural variation of the urea level in milk (Hols et al., 2005). The previous phenomenon engendered by urease activity was not observed in the acidification profile of organic milk fermented with probiotic plus yogurt culture (Fig. 2C) that displayed a typical sigmoid behavior. This could be explained by the lower urea level in organic milk than in conventional milk, as previously reported by Toledo et al. (2002).

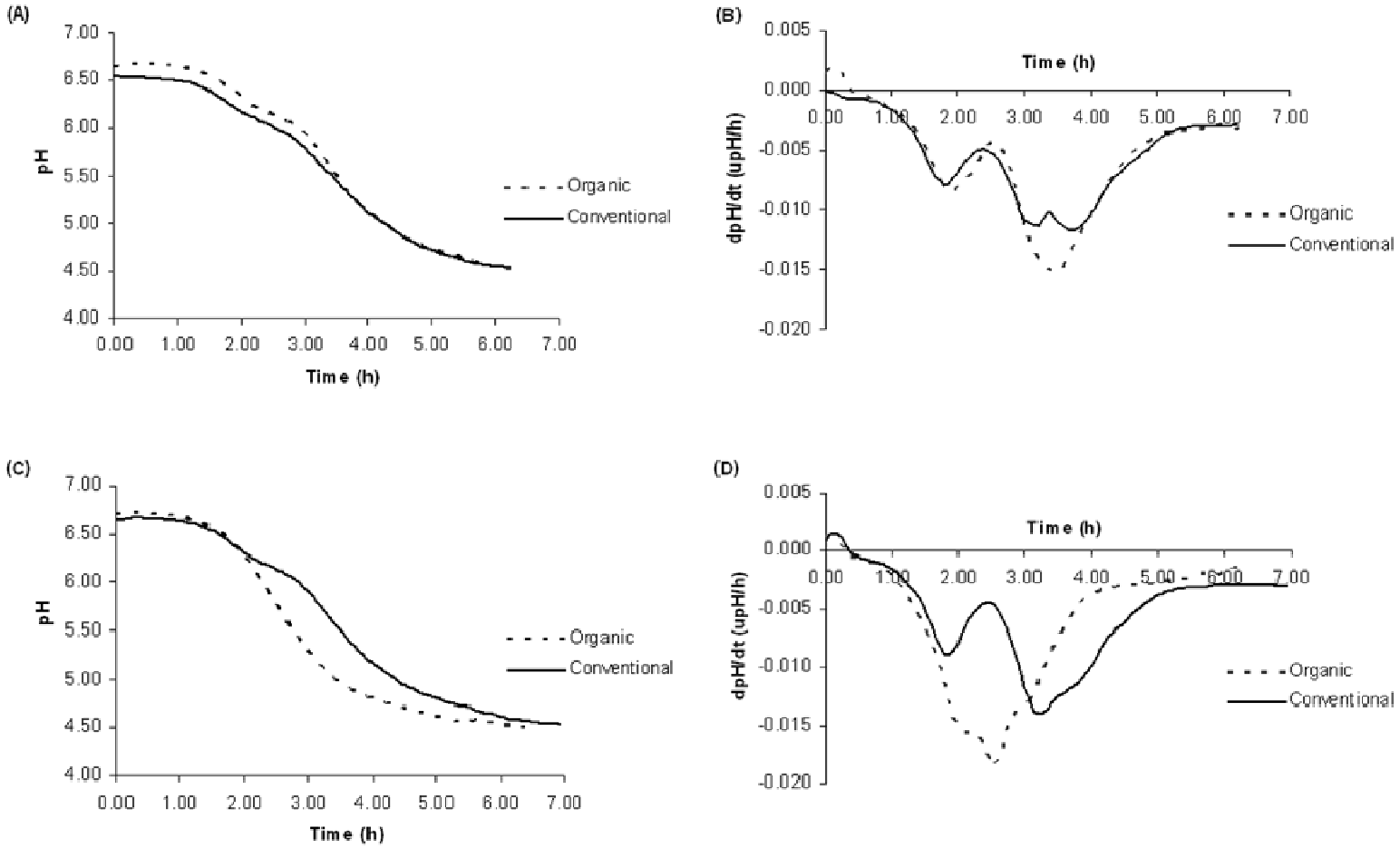

Fig. 2 Acidification kinetics in organic and conventional milks incubated at 42°C until pH 4.5, with (A and B) *Streptococcus thermophilus* TA040 and *Lactobacillus bulgaricus* LB340, and (C and D) *Streptococcus thermophilus* TA040, *Lactobacillus bulgaricus* LB340 and *Bifidobacterium animalis* subsp. *lactis* HN019. - - - - Organic milk; —— Conventional milk.

By considering the mixed culture including *B. lactis* HN019, the use of organic milk increased acidification rates as compared to conventional milk (Fig. 2B and 2D). This difference allowed the acidification of organic milk to be significantly more rapid (18.6 10^{-3} upH/min instead of 14.2 10^{-3} upH/min, ($P < 0.05$) with bifidobacteria, lactobacilli and streptococci than with only yogurt bacteria. The time to reach pH 4.5 was equal to 6.2 ± 0.2 h in organic milk instead of 6.9 ± 0.1 h in conventional milk, which was significantly different ($P < 0.05$). This result is in agreement with those of Florence et al. (2009) that reported shorter fermentation time using binary cultures of *B. animalis* subsp. *lactis* and *S. thermophilus* in organic milks. It can be supposed that the strain *B. lactis* HN019 required specific nutriments that were found in organic milk, but not in conventional milk.

3.3. Bacterial concentrations during fermented milks production and storage

Bacterial growth differed by considering both type of milk and mixed culture composition. Indeed, microbial interactions can result either in stimulation, delay, inhibition, or the absence of effects, depending on bacterial species and strains (Vinderola et al., 2002; Roy, 2005).

Growth of *S. thermophilus* TA040 occurred during the first two hours of fermentation, resulting from its rapid lactose assimilation, in agreement with earlier works of Béal & Corrieu (1994). Final concentrations of *S. thermophilus* achieved at the end of the fermentation ranged from 8.9 to 9.1 $\log_{10}$ CFU/mL, with no significant differences ($P > 0.05$) between the two different kinds of milk and types of cultures employed.

Growth of *L. bulgaricus* LB340 started after four hours of fermentation, in agreement with previous studies (Oliveira et al., 2009). Final concentrations were significantly higher ($P < 0.05$) in organic milk fermented by yogurt culture (8.1± 0.03 $\log_{10}$ CFU/mL) as compared to the other conditions (7.8 ± 0.03 $\log_{10}$

CFU/mL). A positive effect of organic milk was thus demonstrated on *L. bulgaricus* growth, which can be related to the higher poly-unsaturated fatty acid content (1.3 times higher) in this kind of milk as compared to conventional milks. Higher viability of lactic acid bacteria was previously achieved when the ratio between unsaturated and saturated fatty acids was increased (Castro et al., 1995; Béal et al., 2001). This could be explained by the stereochemistry of the double bounds of unsaturated fatty acids, which control membrane fluidity during exposure to the adverse environmental conditions found in fermented milks, such as low pH or low temperature. Moreover, the more rapid acidification observed in organic milk could be another factor of *L. bulgaricus* improvement.

No significant difference ($P > 0.05$) was noted for *B. lactis* HN019 growth in organic and conventional milk. Bacterial counts at the end of fermentation were equal to 7.9 ± 0.03 $\log_{10}$ CFU/mL and 8.1 ± 0.06 $\log_{10}$ CFU/mL for organic and conventional milk, respectively.

Final concentrations of *L. bulgaricus* and *S. thermophilus* at the end of the cultures were not significantly influenced by the presence of the probiotic culture *B. lactis* HN019 ($P > 0.05$). This result differs from those obtained by Vinderola et al. (2002) on the one hand and Donkor et al. (2006) on the other hand, who demonstrated that *L. bulgaricus* and *S. thermophilus* were either inhibited or stimulated by *Bifidobacterium* strains, respectively. This contradictory information indicates that the interactions between yogurt bacteria and *Bifidobacterium* are strongly strain dependent.

Growth of *B. lactis* HN019 in milk remained weak as final concentrations were around 8.1 ± 0.06 $\log_{10}$ CFU/mL. This result agreed with those reported by (Vinderola et al., 2002), who showed that addition of probiotic cultures to yogurt starters generally results in slower growth of the probiotic strains than if they were added alone in milk. This was explained firstly by the accumulation of

lactic and acetic acids that affect the viability of bifidobacteria, and secondly by the low proteolytic activity of these bacteria (Roy, 2005).

Finally our results demonstrated that fermentation was mainly ascribed to *S. thermophilus*, which reached a final concentration 1 log higher than *L. bulgaricus* and *B. lactis*. Only a slight effect of the type of milk was noticed on the growth of *L. bulgaricus,* when associated with *S. thermophilus*, organic milk leading to a better growth of this species. The faster grow of starter cultures allowed rapid acidification which resulted in reduced availability of nutrients; thus, probiotic cultures do not have time to grow extensively (Roy, 2005).

By considering the bacterial concentrations measured after 7 days of storage at 4°C, the kind of milk did not affect the survival of the three bacterial species that was stable during cold storage. Concentrations were equal to 8.8 ± 0.2 $\log_{10}$ CFU/mL for *S. thermophilus* TA040, 7.6 ± 0.2 $\log_{10}$ CFU/mL for *L. bulgaricus* LB340 and 7.9 ± 0.1 $\log_{10}$ CFU/mL for *B. lactis* HN019*,* in both milks. Moreover, no significant difference ($P > 0.05$) was observed with the counts measured just after fermentation. This result differs from that obtained by Donkor et al. (2006), who indicated that the viability of *L. bulgaricus* Lb1466 was enhanced in the presence of probiotic organisms during cold storage. It was thus strain dependant.

3.4. Fatty acid profiles of milk during fermentation and storage

The fatty acid profiles varied during milk fermentation, as a result of the kind of milk and the type of starter culture. In contrast, no modification was observed during storage at 4°C for 7 days.

The relative content of SCFA was slightly reduced during fermentation ($P < 0.05$), in both conventional and organic fermented products, independently of co-culture employed. During cold storage for 7 days, the SCFA of the fermented milks did not change anymore, whatever the type of milk. These data

differ from those achieved by Ekinci et al. (2008), who observed higher amounts in short chain fatty acids in products fermented with other bacterial species. In conventional milks, independently of the co-culture used, the MCFA concentration decreased during fermentation, whereas no significant difference was observed during 7 days storage at 4°C. In organic milk, the MCFA relative contents did not change during fermentation and after 7 days of cold storage. In addition, no significant difference ($P \geq 0.05$) was pointed out between organic and conventional milks. Nevertheless, relative concentrations of C14:1 and C15:0 were slightly higher ($P < 0.05$) in fermented conventional milks, which agrees with the study of Butler et al. (2011) who found higher concentration of MCFA in conventional milk. Finally, a significant increase in LCFA concentration was observed during fermentation (between 1 and 2%), but not during storage at 4°C, for both organic and conventional fermented milks. The relative contents of LCFA did not show significant difference ($P > 0.05$) between the two kinds of milks, in agreement with the findings of (Collomb et al., 2008; Ellis et al., 2006). Among these LCFA, higher relative contents of C16:0; C16:1 and C17:0 were found in conventional products, whereas relative amounts of C18:0 and C18:2 were higher in organic fermented milks.

In addition to these results that concerned the chain length of milk fatty acids, important changes were observed on the fatty acid saturation degree during fermentation ($P < 0.05$). In conventional milk, the proportion of saturated fatty acids (SFA) strongly decreased during fermentation (1-2%), whereas it diminished only slightly in organic milk (~0.4%). As a result of SFA level decrease during fermentation, the relative concentration of MUFA increased in conventional milk (1%) but not in organic milk (Table 1).

The levels of MUFA measured after fermentation were practically alike for both milks in our study. The percentage of PUFA increased during fermentation in organic milk (~0.2%) but remained stable in conventional milk.

These results are in agreement with those obtained by Florence et al. (2009) with the cultures of *S. thermophilus* and four strains of *B. lactis*. They could be explained either by the different balance with MUFA or SFA, or by the synthesis of some polyunsaturated fatty acids by the bacteria (Oh et al., 2003).

The relative percentages of SFA, MUFA and PUFA at day 7 remained close to those measured at day 1 (Table 1). At 4°C, the metabolic activity of the bacteria was reduced as a consequence of the low temperature, and no more change occurred in the fatty acid content as a result of their metabolic activity. This result is in agreement with those reported by Rodriguez-Alcala & Fontecha (2007) with CLA fortified dairy products. They showed that the relative contents in SFA, MUFA and PUFA remained stable during storage. In contrast, Van de Guchte et al. (2006) observed that the total n-3 PUFA concentration slightly decreased during storage of conventional fermented milks. This difference can be ascribed to the different strains used.

Moreover, no significant effect of the type of starter culture was noticed on the chain length of milk fatty acids. The relative proportions of each group of fatty acids varied in the same way, whatever the probiotic culture was added to the yogurt culture or not. The same conclusion was achieved by comparing the fatty acid composition after 7 days of storage at 4°C, which was not affected by the starter and remained stable. Finally, fermentation allowed increasing MUFA relative concentration in conventional milk, whereas organic fermented milks were characterized by an increase in PUFA relative contents. This indicates that the fatty acid composition of the fermented milk was the result of initial saturation degree as well as modification during fermentation. This result confirmed those obtained by Van de Guchte et al. (2006) with conventional fermented milks enriched or not with PUFA or whey proteins.

From these results, differences were observed according to fatty acid chain length and saturation degree by comparing organic and conventional fermented milks. We ascribed these differences to both initial milk composition

and modification by fermentation. The initial fatty acid profile of milk was primarily determined by the balance of fatty acids in feeding regiment and the extent of rumen hydrogenation and mammary desaturase activity that differed in both systems of dairy production (Butler et al., 2011). Moreover, fatty acid composition of fermented milks was affected by growth and corresponding enzymatic activities of bacterial cells, which differed according to the milk, as a result of initial fatty acid profile (Kim & Liu, 2002; Ekinci et al., 2008).

In contrast, no differences were noted during cold storage of fermented milks. This fact may be due to the slower metabolic activity of bacteria at low temperature (Béal et al., 2001).

3.5. Evolution of trans-octadecenoic acid (trans-C18:1), conjugated linoleic acid (CLA) and α-linoleic acid (ALA) relative contents during fermentation and cold storage

During fermentation, *trans*-C18:1 relative concentration (Fig. 1A) showed a 20% increase in conventional fermented milks, with no significant difference among the starter cultures, whereas an enhancement of 8% was observed in organic milk. As the initial relative concentration in *trans*-C18:1 was 1.6 times higher in organic milk, the final *trans*-C18:1 percentage in the fermented milks was the result of both initial milk composition and modification during fermentation. It is interesting to maintain a high relative content of *trans*-C18:1 as it participates in CLA production in human's body (Gnädig et al., 2003; Butler et al., 2011) and acts as intermediate fatty acid in biohydrogenation pathway (Bergamo et al., 2003). During storage of the fermented products, the *trans*-C18:1 concentration remained stable, whatever the kind of milk and starters used. Finally, after 7 days storage at 4°C, it was higher in organic fermented milks (3.3 ± 0.03%) than in conventional milks (2.2 ± 0.03%).

During fermentation, CLA relative content significantly increased ($P < 0.05$), at different levels in organic (17%) and conventional (12%) milks (Fig. 1B). This was explained by Ekinci et al. (2008), who indicated that enzymatic reactions occurred on biohydrogenation pathway, thus increasing CLA level during the production of fermented products. Similar results were reported by Oliveira et al. (2009) in fermented milks, whereas no change was observed in probiotic fermented products made with conventional milk, as reported by Van de Guchte et al. (2006). As these authors used different strains, this behaviour was thus strain dependent. The difference between conventional and organic fermented milks found in our study was considered as significant ($P < 0.05$). The CLA relative concentration was higher in organic fermented milks (1.2±0.01%) as compared to conventional fermented milks (0.8 ± 0.01%) (Fig. 1B), in accord with previous results (Oliveira et al., 2009). This higher CLA relative content in organic fermented products was the result of both initial CLA percentage in milk and changes during fermentation. In addition to these results, CLA relative concentration did not significantly vary in fermented milks according to the co-cultures. This result indicates that *B. lactis* HN019 had no effect on CLA relative content, and that the variations observed during fermentation could be ascribed to *S. thermophilus* or *L. bulgaricus*, as suggested by Lin (2003). Finally, the CLA percentage slightly decreased during cold storage of three of the fermented milks ($P < 0.05$), that may be related to the activation of reduction steps in biohydrogenation pathway (Kim & Liu, 2002). However, by considering the conventional fermented milk with yogurt starters and bifidobacteria, a significant increase of relative CLA content was observed.

Fig. 1C shows that during fermentation ALA level did not vary significantly in organic milk (0.5 ± 0.02%), for the two kinds of culture. In contrast, a significant decrease ($P < 0.05$) was noted during fermentation and storage of conventional milk products (from 0.38 ± 0.02% to 0.30 ± 0.02%).

These results are not in agreement with those of Van de Guchte et al. (2006), who showed that the content of ALA was not affected during storage of conventional fermented milks at 4°C, which can be attributed to the different strains used. No significant difference was noticed between the two kinds of starters at the end of the fermentation. Finally, the ALA content in the fermented milks mainly resulted from its initial concentration in milk and from variation during fermentation and storage. During 7 days storage at 4°C, strong difference was observed between the two kinds of fermented milks. The ALA content remained high and stable in organic milk (0.54 ± 0.02%), whereas it decreased from 0.30 ± 0.02% to 0.24 ± 0.01% in conventional milk. This decrease can be correlated to the increased levels of C18:0 and C18:1, independently of the co-culture used, as a result of modification of biohydrogenation and desaturation pathways (Destaillats et al., 2005).

4. Conclusions

Our study demonstrated that the use of organic milk allowed more rapid acidification and provided higher amounts of PUFA content in the fermented milks that was related to an improvement of *L. bulgaricus* growth. In contrast, the growth of *S. thermophilus* and *B. lactis* HN019 was not affected by the type of milk. Bacterial concentrations remained stable after 7 days of storage at 4°C.

Acidification process also provided *trans*-C18:1 and CLA enhancement, together with ALA decrease, at different levels in conventional and organic milks. This result indicates that bacterial metabolism modified the relative fatty acid milk composition. By combining these differences with the initial fatty acid composition of organic and conventional milks, which depended on variations in dairy diet manipulation, organic fermented milks showed higher relative amounts of *trans*-C18:1 (x 1.6), CLA (x 1.4) and ALA (x 1.6), as compared to

conventional fermented milks at the end of fermentation and after storage at 4 °C. Consequently, the fatty acid content of the fermented milks was the result of two factors: initial milk composition and modification during fermentation as a result of bacterial metabolic activities. The higher relative amounts of *trans*-C18:1, CLA and ALA in organic fermented milks and lower levels of SFA may be considered as desirable from a nutritional perspective.

In the future, it will be necessary to identify the specific role of each bacterial species, in pure cultures, in order to understand the biochemical mechanisms that support the changes in fatty acid composition in the fermented milks.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.16/j.foodchem.2012.07.026.

CHAPTER 3 - Survival of three *Bifidobacterium animalis* subsp. *lactis* strains is related to *trans*-vaccenic and α-linolenic acids contents in organic milk

Abstract

This study aims at relating the survival of three strains of *Bifidobacterium animalis* subsp. *lactis* (BB12, B94 and BL04), in co-culture with *Streptococcus thermophilus* TA040 and *Lactobacillus bulgaricus* LB340, to *trans*-vaccenic (TVA) and α-linolenic (ALA) fatty acids contents in organic and conventional fermented milks. Acidification kinetics, cultivability and fatty acid composition were compared after fermentation, and after 14 and 28 days of storage at 4°C. The kind of milk did not influence the acidification kinetics, whereas cultivability was improved in organic milk after 28 days of cold storage, with slight differences among strains. In addition, the relative TVA and ALA contents were respectively 1.8 and 2.4 times higher in organic fermented milks, as compared to conventional products. From these results, it was concluded that elevated levels of TVA and ALA, as found in organic fermented milks, allowed improving survival of bifidobacteria during chilled storage.

Keywords: α-linolenic acid; Bifidobacteria; Fermented milk; Organic milk; *Trans*-vaccenic acid.

Article being published

CHAPTER 4 - Viability of *Bifidobacterium* is improved in organic fermented milks as a result of membrane fatty acids composition

Abstract

This study demonstrates that survival of three *Bifidobacterium* strains in organic and conventional fermented milks, during chilled storage for 21 days, depends on their membrane fatty acid composition, as a result of milk composition. Fermented milk products were elaborated using either conventional milk or organic milk. Acidification activity, cultivability and survival for 21 days at 4°C under acidic pH (pH 4.4) of *Bifidobacterium animalis* subsp. *lactis* BB12 and *B. animalis* subsp. *lactis* BL04 were enhanced using organic milk, whereas those of the strain *Bifidobacterium longum* subsp. *infantis* ATCC15697 were less affected. Differences observed during fermentation were linked to acidification activity and oxidoreduction potential that differed among the strains and the kinds of milk used. This behavior was related to the fatty acid composition of the milks, which was characterized by higher mono- and poly-unsaturated fatty acids contents in organic products. Among them, relative contents in *trans*-vaccenic acid, conjugated linoleic acid and α-linolenic acid were greater in organic milk. These different fatty acid compositions of milks allowed the cells of *B. lactis* BB12 and BL04 to modify their membrane fatty acids composition, by increasing their linoleic and α-linolenic acids contents. The lower survival improvement observed with the strain *B. infantis* ATCC15697 was linked to the lower ability of this strain to increase the relative contents of these specific unsaturated fatty acids in cell membranes, even in organic milk. Finally, this study showed that survival of bifidobacteria during chilled storage in fermented milk products was the result of the fatty acid composition of the cellular membranes, which depended on the considered strain and on the fatty acid composition of the milk. Moreover, it demonstrated that organic milk improved survival of *Bifidobacterium* strains during chilled storage, as a result of elevated contents in *trans*-vaccenic, conjugated linoleic and α-linolenic acids.

Keywords: α-linolenic acid; Bifidobacteria; Chilled storage; Fermented milk; Organic milk.

Article being published

CHAPTER 5 - Fermented milk manufacture conditions affect *Bifidobacterium animalis* subsp. *lactis* BB12 survival as a result of membrane fatty acid composition

Abstract

The effects of the kind of milk (organic and conventional), fermentation temperature (37°C and 42°C), final pH (4.8 and 4.4), intermediate cooling temperature (22°C, 25°C and 28°C) and duration (4, 8 and 12 hours) were investigated on acidification activity, survival and bacterial membrane fatty acids composition of *Bifidobacterium animalis* subsp. *lactis* BB12 after fermentation and storage at 4°C. Acidification time was reduced when fermentation was performed at 42°C until pH 4.4. Superior cell counts were achieved in organic fermented milks at the end of fermentation and after storage, independently of process conditions. Cultivability was also higher when the cells were fermented at 42°C until pH 4.4 and maintained at 28°C for 12 hours before being cooled to 4°C. These conditions led to modifications of membrane fatty acid composition, thus resulting in changes of membrane fluidity. However, if the unsaturated fatty acid percentage was higher in organic fermented milks, it was lower when fermentation was conducted et 42°C and pH 4.4; and after cooling at 28°C for 12h. Finally, this study suggested that other biological mechanisms were requested in responses to environmental conditions.

Keywords: Adaptation; Bifidobacteria; Fermented milk; Membrane fatty acids; Organic milk; Survival.

Article being published

CHAPTER 6 – Physiological changes of *Bifidobacterium animalis* subsp. *lactis* BB12 during *in vitro* dynamic digestion of fermented milks

Abstract

During gastrointestinal transit, bifidobacteria are supposed to survive, despite several stress, including low pH and action of pepsin in stomach, followed by effects of bile salts and pancreatin in duodenum. This study aims to assess survival and changes in membrane fatty acid composition of *Bifidobacterium animalis* subsp. *lactis* BB12, in organic and conventional fermented milks, using a dynamic gastrointestinal tract model (DIDGI). Bacterial cell concentration decreased during the digestion process of fermented milks that were prepared in pure culture, at 37°C until pH 4.4, and cooled down to 4°C. This result coincided with strong alterations in membrane fatty acid composition that were mainly ascribed to the increase in palmitic (C16:0), stearic (C18:0) and unsaturated fatty acids, to the detriment of medium chain fatty acids (C10:0 to C14:0). Better survival after *in vitro* digestion (x 1.4) was observed when *B. lactis* BB12 cells were brought in organic fermented milks instead of conventional ones. This improvement was related to the fatty acid composition of organic milk that allowed the cells to increase their contents in unsaturated and cyclic fatty acids (especially, α-linolenic and dihydrosterculic acids). Finally, survival of *B. lactis* BB12 at the end of the digestion process was enhanced when fermented milks were prepared at 42°C or cooled at 28°C for 4h before 4°C. These conditions allowed modifying the membrane composition of the bifidobacteria that was characterized by higher myristic, conjugated linoleic and α-linolenic acids, as compared to control conditions. Finally, this study demonstrated that survival of bifidobacteria during gastrointestinal transit could be improved by using suitable conditions for fermented milks manufacture that allowed modifying the membrane fatty acid composition of the cells.

Keywords: Bifidobacterium; In vitro dynamic digestion; Membrane fatty acids; Organic milk; Survival

Article being published

GENERAL CONCLUSION AND PERSPECTIVES

The popularity of probiotic food products increased during the last decades due to consumer awareness and encouraging evidence of health benefits of probiotic bacteria, such as *Bifidobacterium*. The interest of these bacteria assumes, however, a good survival (e.g. bacterial concentrations higher that 10^7 CFU/mL) in the food products and inside the gut. Generally, these probiotic bacteria are consumed in fermented milks and other dairy products, where their survival is related to the composition of the food matrix, the acidity (pH 4-5) of the product and the low storage temperature (4-6°C). Besides, the use of organic products is growing, thanks to their contribution to sustainable development. In addition, organic milk demonstrates health effects as it contains elevated levels of bioactive fatty acids, as a result of specific feeding regimens and handling of the animals.

In this context, this PhD thesis allowed improving knowledge on the physiological changes of *Bifidobacterium* strains submitted to environmental stress conditions during cold storage and digestion process of organic and conventional fermented milks, in order to enhance their resistance to these deleterious conditions. Four complementary approaches were carried out to answer this main objective.

The first part (chapters 1 and 2) was dedicated to the characterization of the growth and acidification, as well as the resistance to cold and acid stress during storage at 4°C, of four strains of *Bifidobacterium animalis* subsp. *lactis* (BB12, B94, BL04 and HN019). Cultures were conducted in mixed cultures with *Streptococcus thermophilus* TA040 and *Lactobacillus delbrueckii* subsp. *bulgaricus* LB340, and in two different kinds of milk, organic and conventional. The use of organic milk promoted higher acidification activity when *B. lactis* HN019 grown with yogurt cultures, and better cultivability and survival of *B. lactis* BB12, B94 and BL04 during cold storage at 4°C, for 7 to 28 days. This

improvement was strain-dependent and related to the superior relative contents in short and medium chain fatty acids and bioactive fatty acids (*trans*-vaccenic – TVA, conjugated linoleic – CLA and α-linolenic acids – ALA) in organic milk. These specific fatty acids were thus considered as growth factors for bifidobacteria, as they may affect their membrane fatty acid composition and then their fluidity.

The second part of this PhD thesis (Chapter 3) aimed at comparing the growth and survival of three *Bifidobacterium* strains (*B. animalis* subsp. *lactis* BB12, *B. animalis* subsp. *lactis* BL04 and *Bifidobacterium longum* subsp. *infantis* ATCC 15697) in pure cultures, in order to identify some physiological mechanisms at membrane level, to explain the different behavior of the strains in organic and conventional milks. Fermentation kinetics differed according to the strains and the kinds of milk used, as a result of different acidification and oxidoreduction potential kinetics. Survival of the bifidobacteria in acidic fermented milks during storage at 4°C was also affected, with higher survival in organic fermented milks. These products were characterized by higher relative amounts of TVA, CLA and ALA that allowed changes in membrane fatty acid composition of the cells, by increasing their relative contents in linoleic acid and ALA. In addition, the *Bifidobacterium* strains were distinguished by their unsaturated fatty acid percentages, more specifically by their ALA concentration. Higher relative levels of these specific fatty acids were found in *B. lactis* strains that were more resistant during chilled storage as compared to the *B. infantis* strain, which did not contain ALA.

In the third part of this PhD thesis (Chapter 4), the effects of different conditions applied during the fermented milks manufacture were assessed on the survival and the membrane fatty acid composition of *Bifidobacterium animalis* subsp. *lactis* BB12. These conditions aimed at inducing physiological adaptations of the bacteria, by modifying culture (fermentation temperature at 37°C and 42°C and final fermentation pH at 4.8 and 4.4) and cooling conditions

(intermediate cooling temperature at 22°C, 25°C and 28°C for 4h, 8h and 12h before cooling at 4°C), in both conventional and organic milks. Fermentation time was reduced when incubation was performed at 42°C instead of 37°C, and until pH 4.8. Cultivability was improved when fermented milks production was performed at 42°C until pH 4.4, when they were maintained at 28°C for 12 hours before being cooled to 4°C, and when organic milk was used instead of conventional milk. The survival of *B. lactis* BB12 cells was positively related to the unsaturated fatty acids percentage in their membranes, as obtained in organic milk products. However, despite their improved survival, the relative content in unsaturated fatty acids was lower in membranes of cells obtained at 42°C and pH 4.4, and after cooling at 28°C for 12 h. This observation suggested that other biological mechanisms were involved in response to these adaptation procedures, such as modification of protein synthesis.

The last part of this work (Chapter 5) intended characterizing the ability of *Bifidobacterium animalis* subsp. *lactis* BB12 cells to survive the stress encountered in the digestive tract and to adapt themselves at the membrane level. By using a dynamic gastrointestinal bioreactor (DIDGI), survival and membrane fatty acids composition of the bifidobacteria were evaluated in organic and conventional fermented milks, produced under different manufacture conditions. The survival of bifidobacteria was reduced during the digestion process, mainly in the duodenal compartment, and in a less extends in the gastric compartment. Better survival after *in vitro* digestion was observed when *B. lactis* BB12 cells were cultivated in organic fermented milks as compared to conventional ones. This behavior was related to the ability of the cells to maintain higher levels in unsaturated and cyclic fatty acids (especially, α-linolenic acid and cycC19:0) in their membranes. Finally, survival of *B. lactis* BB12 was enhanced when fermented milks were prepared at 42°C instead of 37°C, but not when cooling was done at 22°C for 4h. The improvement was linked to the ability of the cells to modify their membrane fatty acid contents,

which were characterized by elevated myristic (C14:0), C18:2 (CLA) and C18:3 (ALA) acids.

Finally, this PhD thesis allowed demonstrating that survival of different *Bifidobacterium* strains in fermented milks, during refrigerated storage and *in vitro* digestion process, was linked to membrane fatty acid composition of the cells, as a result of kind of milk, fermentation and cooling conditions. High unsaturated fatty acids and α-linolenic acid relative contents in bacterial membranes were more specifically related to survival, either during storage of fermented milks and during *in vitro* digestion process. In addition, use of organic milk, as well as specific fermentation and cooling conditions, allowed the bacteria to adjust their fatty acid contents, and consequently, to improve their survival.

From these results, various perspectives can be envisaged for future research.

As positive effects of specific fermentation and cooling conditions were demonstrated on survival during storage and *in vitro* digestion of bifidobacteria, the implementation of other adaptation procedures may help defining processes dedicated to specific applications. As a second prospect, the adaptation approaches described in this study may be broadened to a large panel of microorganisms including other species and strains of bifidobacteria as well as lactic acid bacteria.

The last part of this work was focused on the characterization and the understanding of the resistance of *Bifidobacterium lactis* during *in vitro* digestion, in fermented milk in pure culture. As most of the fermented milk products are elaborated in mixed cultures, a similar approach may be carried out by associating the bifidobacteria with yogurt cultures.

Finally, the understanding of the physiological responses of the bifidobacteria to the stress encountered during fermented milk production and

storage, during *in vitro* digestion, and to the adaptation procedures, was established on membrane level. Nevertheless, some specific responses were not completely explained from membrane fatty acid analysis. Consequently, characterization of the proteome of *Bifidobacterium animalis* subsp. *lactis* and other bifidobacteria, submitted to different stress and adaptation conditions and to *in vitro* digestion, may help improving knowledge about these biological responses.

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Resumo

FLORENCE, A.C.R. **Respostas fisiológicas de bifidobactérias submetidas aos estresses ácido, frio e gastrointestinal em leite orgânico e convencional.** 2013. 203 f. Tese (Doutorado) – Faculdade de Ciências Farmacêuticas - Universidade de São Paulo, Brasil; AgroParisTech, França.

Bifidobactérias são expostas a vários tipos de estresse, devido às condições ambientais, durante a produção de leite fermentado e o armazenamento a frio e durante a digestão, no interior do trato gastrointestinal. Para melhorar a sobrevivência dessas bactérias, este estudo visou compreender os mecanismos de degradação do estado fisiológico de diferentes estirpes de *Bifidobacterium* quando expostas aos estresses frio e ácido e ao estresse gastrointestinal *in vitro*. Este estudo também visou estabelecer as relações entre a resistência das bactérias ao estresse e os conteúdos em ácidos graxos de membrana e ácidos graxos dos leites orgânico e convencional. Os resultados mostraram que a atividade acidificante de bifidobactérias é estirpe-dependente e que ela é aumentada quando bifidobactérias são associadas às culturas do iogurte, quando o leite orgânico foi utilizado em vez do condicional e quando a temperatura de incubação foi fixada em 42°C em vez de 37°C. A cultivabilidade e a sobrevivência das estirpes de *Bifidobacterium* foram determinadas após a fermentação; após o armazenamento a 4°C, durante 7 a 28 dias; e durante um processo de digestão *in vitro,* que foi simulada num modelo dinâmico do trato gastrointestinal. Estas características foram melhoradas em leites fermentados orgânicos, em comparação com os produtos convencionais, quando a fermentação foi realizada a 42°C até pH 4,4, e quando os leites fermentados foram mantidos a 28°C durante 12 horas antes de serem resfriados a 4°C. Estes procedimentos de fabricação específicos geraram assim a adaptação fisiológica das bifidobactérias aos estresses. Durante a digestão *in vitro*, a cultivabilidade das bifidobactérias foi menos degradada quando a fermentação foi realizada em leite orgânico, em vez do leite convencional, e, em menor grau, quando os processos de adaptação foram aplicados durante a fabricação de leite fermentado. Estes resultados estão relacionados com os níveis mais elevados de ácidos graxos insaturados, incluindo os ácidos *trans*-vacênico, linoleico conjugado e α-linolênico, que caracterizam os produtos orgânicos. Estes perfis de ácidos graxos, específicos do leite orgânico, permitiram às bifidobactérias modificar a composição de seus ácidos graxos de membrana, aumentando os níveis de ácidos graxos insaturados e diminuindo os ácidos graxos saturados de cadeia média; adaptando, assim, a fluidez da membrana. Quando os procedimentos de fabricação específicos foram implementados para induzir a adaptação fisiológica das bifidobactérias, a composição em de ácidos graxos de membrana foi modificada de maneira diferente do que é observado em leite orgânico. Esta diferença indica, assim, que outros mecanismos de adaptação biológicos podem estar implicados, especialmente ao nível proteômico. Finalmente, este estudo demonstra que estas modificações, ao nível de membrana, contribuem para modular a resistência aos estresses tecnológicos e gastrointestinais de estirpes de *Bifidobacterium*.

Palavras-chave: Ácidos graxos de membrana, Bifidobactérias, Leite orgânico, Leite fermentado, Estresse frio, Estresse gastrointestinal.

Abstract

FLORENCE, A.C.R. **Physiological responses of bifidobacteria subjected to acid, cold and gastro-intestinal stress in organic and conventional milks.** 2013. 203 f. PhD Thesis (Doctorat) – São Paulo University, Brazil; AgroParisTech, France.

Bifidobacteria are exposed to various stress, as a result of environmental conditions encountered during fermented milk production, cold storage and during digestion of the products inside gastrointestinal tract. In order to improve their survival, this study aimed at understanding the degradation mechanisms of the physiological state of various *Bifidobacterium* strains when exposed to cold, acid and *in vitro* simulated gastrointestinal stress. It also intended to establish relationships between stress resistance and milk and membrane fatty acids contents, in organic and conventional milks.
The results showed that acidification activity of bifidobacteria was strain-dependent and increased when bifidobacteria were associated to yogurt cultures, when organic milk was used and when incubation temperature was set at 42°C instead of 37°C. Cultivability and survival of the *Bifidobacterium* strains were determined after fermentation, after storage at 4°C for 7 to 28 days, and during *in-vitro* digestion that was simulated in a dynamic gastrointestinal tract model. These characteristics were improved in organic fermented milks as compared to conventional products, when fermentation was performed at 42°C until pH 4.4, and when the fermented milks were kept at 28°C for 12 hours before being cooled to 4°C. These specific manufacture procedures thus generated physiological adaptation of the bifidobacteria to the stress. During *in-vitro* digestion, cultivability of bifidobacteria was less deteriorated when they were grown in organic instead of conventional milk, and to a less extent, when the adaptation procedures were applied during fermented milk manufacture. These results were related to the higher unsaturated fatty acids content, including *trans*-vaccenic, conjugated linoleic and α-linolenic acids that characterize organic products. These particular fatty acids profiles of organic milks allowed bifidobacteria to modify their membrane fatty acids composition, by increasing their unsaturated fatty acids contents and by shortening the length of medium chain saturated fatty acids, thus adapting their membrane fluidity. When specific manufacture procedures were carried out to trigger physiological adaptation of the bifidobacteria, membrane fatty acid composition changed differently from what is observed in organic milk. This difference indicates that other biological adaptation mechanisms are probably involved, especially at the proteomic level. Finally, this study demonstrated that modifications at membrane level contribute to modulate resistance against technology and gastro-intestinal stress of *Bifidobacterium* strains to better withstand technological and gastro-intestinal stress.

Keywords: Bifidobacteria, Cold stress, Fermented milk, Gastro-intestinal stress, Membrane fatty acid, Organic milk.

Résumé

FLORENCE, A.C.R. **Réponses physiologiques de bifidobactéries soumises aux stress acide, froid et gastro-intestinal en laits biologique et conventionnel.** 2013. 203 f. Thèse (Doctorat) – Faculté de Sciences Pharmaceutiques - Université de São Paulo, Brésil; AgroParisTech, France.

Les bifidobactéries sont exposées à de nombreux stress, liés aux conditions environnementales rencontrées lors de la production, du stockage au froid, et pendant la digestion des laits fermentés. Afin d'améliorer leur survie, cette étude vise la compréhension des mécanismes de dégradation de l'état physiologique de différentes souches de *Bifidobacterium* soumises aux stress froid et acide et au stress gastro-intestinal simulé *in vitro*. Elle ambitionne également d'établir des relations entre la résistance aux différents stress et la teneur en acides gras membranaires et des laits biologiques et conventionnels.
Les résultats montrent que l'activité acidifiante des bifidobactéries est souche-dépendante et qu'elle augmente lorsque les bactéries sont associées aux bactéries lactiques du yaourt, avec du lait biologique et lorsque la température d'incubation est fixée à 42°C au lieu de 37°C. La cultivabilité et la survie des souches ont été déterminées après fermentation, après stockage à 4°C pendant 7 à 28 jours, et pendant un processus de digestion simulé *in-vitro* dans un digesteur dynamique reproduisant le tractus gastro-intestinal. Ces caractéristiques sont améliorées dans les laits fermentés biologiques par rapport aux produits conventionnels, lorsque la fermentation est effectuée à 42°C jusqu'à pH 4,4, et lorsque les laits fermentés sont maintenus à 28°C pendant 12 heures avant d'être refroidi à 4°C. Ces procédures de fabrication spécifiques génèrent ainsi une adaptation physiologique des bifidobactéries aux stress. Pendant la digestion *in-vitro*, la cultivabilité des bifidobactéries se dégrade moins lorsque la fermentation se déroule en lait biologique plutôt qu'en lait conventionnel et, dans une moindre mesure, lorsque les procédures d'adaptation sont appliquées pendant la fabrication du lait fermenté. Ces résultats sont liés aux teneurs plus élevées en acides gras insaturés, en particulier en acides *trans*-vaccénique, linoléique conjugué et α-linolénique, qui caractérisent les produits biologiques. Ces profils d'acides gras particuliers aux laits biologiques permettent aux bifidobactéries de modifier leur composition en acides gras membranaires, en augmentant leur teneur en acides gras insaturés et en raccourcissant la longueur moyenne des chaînes d'acides gras saturés, adaptant ainsi leur fluidité membranaire. Lorsque les procédures de fabrication spécifiques sont mises en œuvre pour induire une adaptation physiologique des bifidobactéries, la composition en acides gras des membranes se modifie différemment de ce qui est observé en lait biologique. Cette différence indique ainsi que d'autres mécanismes biologiques d'adaptation sont probablement impliqués, en particulier au niveau protéomique. Finalement, cette étude démontre que les modifications au niveau de la membrane contribuent à moduler la résistance aux stress technologique et gastro-intestinal de souches de *Bifidobacterium*.

Mots-clés: Acides gras membranaires, Bifidobactéries, Lait biologique, Lait fermenté, Stress froid, Stress gastro-intestinal.