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IDENTIFICATION OF BACTERIAL STRAINS ABLE TO PRODUCE CLNA ISOMERS FOR A POSSIBLE APPLICATION IN THE ELABORATION OF NEW FUNCTIONAL FOOD PRODUCTS

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IDENTIFICATION OF BACTERIAL STRAINS ABLE TO PRODUCE CLNA ISOMERS FOR A POSSIBLE APPLICATION IN THE ELABORATION OF NEW FUNCTIONAL FOOD PRODUCTS

Identificação de estripes bacterianas capazes de produzir isómeros de CLNA para uma possível aplicação na elaboração de novos produtos alimentares funcionais

Thesis presented to *Escola Superior de Biotecnologia* of the *Universidade Católica Portuguesa* to fulfill the requirements of Master of Science degree in Biotechnology and Innovation

by

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All I have accomplished with this work is dedicated to you, my dear grandfather Manuel da Rosa Fontes. It was you that ignited the light at the end of the tunnel, it was you that did not let me adrift, it was you that made it possible to get here. Thanks to you I could find so many opportunities, I could live new experiences, I could grow as a person, I could conquer my personal realizations. Thank you for all the support, kindness and concern, I am so grateful for all you have done for me. Even though you do not still among us, I hope I have not disappointed you, I have done my best to keep making you proud of me.

Abstract

Functional food products consist of a modified foodstuff that can provide additional health and well-being benefits beyond their basic nutrition. *In vitro* and *in vivo* studies have reported that conjugated linolenic acid (CLNA) exerts anti-inflammatory, anti-cancer, anti-obesity and antioxidant activities. This group of conjugated isomers is naturally present in vegetable oils and ruminant meat and milk fat, however, lactobacilli, bifidobacteria and propionibacteria strains have revealed the capacity to produce CLNA isomers. Thus, the aim of this work was to identify CLNA producing strains, among a total of 12 *Lactobacillus* and 3 *Bifidobacterium* strains, that could be applied in the future formulation of new functional food products.

CLA and CLNA microbial producing ability have been related to the presence of the linoleate isomerase (LAI) gene. Therefore, after the optimization of bacterial growth conditions, it was performed a molecular detection assay of LAI gene in *Lactobacillus* strains. *Lactobacillus brevis* D24, *Lactobacillus plantarum* D36 and *Lactobacillus plantarum* 299v revealed a positive result for the presence of LAI gene.

In order to test strains for their CLNA producing ability, selected strains were first tested for their capacity to produce CLA isomers given previous evidence, experimental knowledge and chemical similarity with CLNA group of isomers. Low concentrations of CLA were detected, even for the positive LAI gene strains, although LA reduction rate was considerably high. Two strategies were then applied in order to optimize CLA production: addition of LA to the pre-inocula and inocula and addition of LA after 7 h of incubation. Despite the efforts, CLA production continued to be low in comparison to the percentages of LA reduction. LA was detected in strains' pellet fatty acid composition, suggesting that the substrate could be being incorporated in the membrane. The amount of LA absorbed did not correspond to the level of substrate that was reduced, therefore, it was concluded that LA must have been converted into other unexpected compounds through an alternative transformation pathway not yet described.

Among the strains tested, *B. breve* NCIMB 702258 was the only strain to demonstrate CLA-producing capacity. Further assays showed that this strain was also able to convert α-LNA into CLNA isomers, even in a food matrix (1.6% reduced-fat milk). Therefore, *B. breve* NCIMB 702258 has the potential to be applied in the elaboration of CLA and CLNA enriched products, however, the organoleptic characteristics will need to be optimized.

Resumo

Os alimentos funcionais consistem em géneros alimentícios modificados capazes de providenciar benefícios adicionais para a saúde e bem-estar além da sua nutrição básica. Estudos *in vitro* e *in vivo* revelaram que o ácido linolénico conjugado (CLNA) exerce actividades anti-inflamatória, anti-cancerígena, anti-obesidade e antioxidante. Este grupo de isómeros conjugados está naturalmente presente em óleos vegetais e na gordura da carne e leite de ruminantes, contudo, estirpes dos géneros lactobacilli, bifidobacteria e propionibacteria têm demonstrado capacidade para produzirem isómeros de CLNA. Assim, o objectivo deste trabalho foi de se identificar estirpes produtoras de CLNA, entre um total de 12 estirpes *Lactobacillus* e 3 *Bifidobacterium*, que possam vir a ser aplicadas na formulação futura de novos produtos funcionais.

A capacidade de produção de CLA e CLNA tem sido relacionada com a presença do gene da linoleato isomerase (LAI). Por isso, após a optimização das condições de crescimento, foi realizado um ensaio de detecção molecular do gene LAI em estirpes do género *Lactobacillus*. *Lactobacillus brevis* D24, *Lactobacillus plantarum* D36 e *Lactobacillus plantarum* 299v deram resultado positivo relativamente à presença do gene LAI.

De forma a se testar a capacidade de produção de CLNA das estirpes, estirpes seleccionadas foram primeiro testadas quanto à sua capacidade de formação de isómeros de CLA dadas anteriores evidências, o conhecimento experimental e a similaridade química com o grupo de isómeros de CLNA. Baixas concentrações de CLA foram detectadas, mesmo para as estirpes com o gene LAI presente, embora as taxas de redução do LA tenham sido consideravelmente altas. Duas estratégias foram então depois tomadas de forma a se optimizar a produção de CLA: adição de LA aos pre-inóculos e inóculos e adição de LA após 7 h de incubação. Apesar das tentativas, a produção de CLA continuou a ser baixa comparando com a percentagem de redução do LA. Na composição de ácidos gordos do pellet das estirpes, foi detectada a presença de LA, o que sugeriu que o substrato pudesse estar sendo incorporado na membrana. A quantidade de LA absorvida não correspondia ao nível de substrato que foi reduzido, por isso foi concluído que o LA teria que estar a ser convertido em outros compostos não esperados através de uma via de transformação alternativa ainda não descrita.

Entre as estirpes testadas, *B. breve* NCIMB 702258 foi a única que demonstrou a capacidade de produção de CLA. Ensaios posteriores revelaram que esta estirpe também era capaz de converter α-LNA em isómeros de CLNA, mesmo numa matriz alimentar (leite meio gordo). Assim sendo, *B. breve* NCIMB 702258 tem o potencial para ser aplicada na elaboração de produtos enriquecidos em CLA e CLNA, contudo, as características organolépticas terão que ser optimizadas.

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First I want to thank to Escola Superior de Biotecnologia - Universidade Católica Portuguesa for having me during this past 2 years where I could achieve with success one of my life goals and experience many situations that have made me grow and discover capabilities that I thought I did not have.

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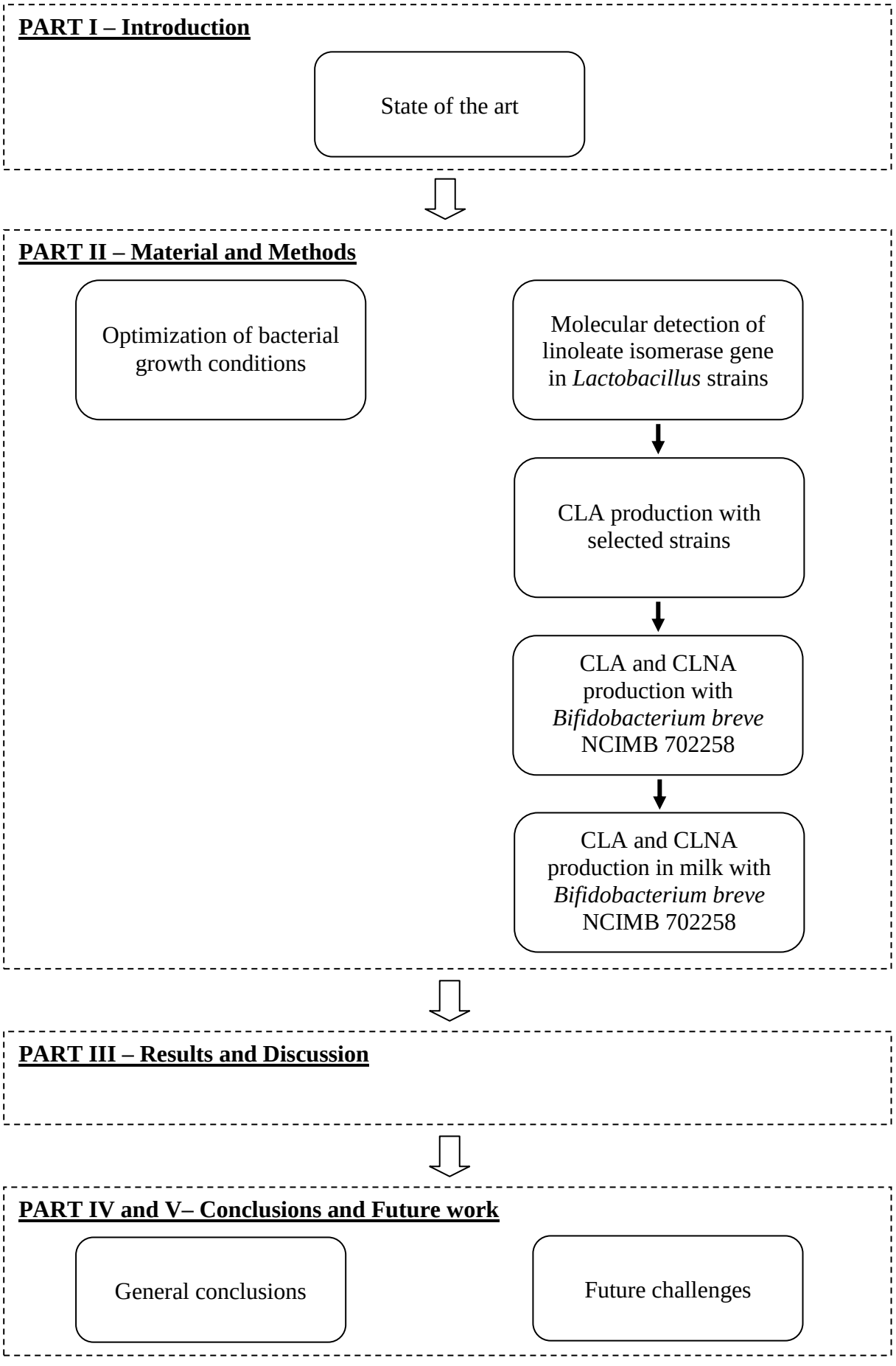
Finally, but not least, my biggest thankful goes to my parents, brothers and grandparents because you made it possible to get this further, the distance that separates us makes it harder but knowing that you are proud of my achievements in life gives me strength to keep going. I hope to never disappoint you and keep making you prouder of me every day.

Thesis scheme

This dissertation is divided in five parts. In the first part it is presented a state of the art regarding CLNA isomers bioactivity, natural sources, enhancement strategies in food, microbiological production, molecular screening tools and stability in enriched products. In the second part it is explained how this work was carried out in order to accomplish the goals intended. The third part shows the results obtained during this research effort along with the discussion of those results. The main conclusions achieved with this work are presented in the fourth part while the fifth refers the research work that could be performed in the future in order to consolidate the new information accomplished with this study.

A representative scheme of this thesis is following presented.


PART I – Introduction
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PART II – Material and Methods
Optimization of bacterial growth conditions
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CLA production with selected strains
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List of abbreviations

α-CDA	Alpha-Calendic acid
β-CDA	Beta-Calendic acid
α-ESA	Alpha-Eleostearic acid
β-ESA	Beta-Eleostearic acid
α-LNA	Alpha-linolenic acid
γ-LNA	Gama-linolenic acid
ACF	Aberrant crypt foci
AOM	Azoxymethane
ARA	Arachidonic acid
ATL	Adipose triglyceride lipase
BHI	Brain Heart Infusion
CFU	Colony forming units
CLA	Conjugated linoleic acid
CLNA	Conjugated linolenic acid
CPA	Catalpic acid
CVD	Cardiovascular diseases
ERK 1/2	Extracellular signal-regulated kinases 1 and 2
FA	Fatty acid
FAME	Fatty acid methyl ester
GSH	Glutathione
HFA	Hydroxy fatty acid
HSL	Hormone-sensitive lipase
HTST	High temperature short time
IBD	Inflammatory bowel disease
IL	Interleukin
JA	Jacaric acid
LA	Linoleic acid
LAI	Linoleate isomerase
MRCA	Myosin cross-reactive antigen
MRS	Man-Rogosa-Sharpe
MUFA	Monounsaturated fatty acid

NEC	Necrotising enterocolitis
NOS	Nitric oxide synthase
OA	Oleic acid
PAF	Platelet activating factor
PARP	Poly ADP ribose polymerase
PKA	cAMP-activated protein kinase
PKC	Protein kinase C
PPARγ	Peroxisome proliferator-activated receptor gamma
PUA	Punicic acid
PUFA	Polyunsaturated fatty acid
qPCR	Real time polymerase chain reaction
RLA	Rumelenic acid
ROS	Reactive oxygen species
SCD	Stearoyl coenzyme A desaturase
SFA	Saturated fatty acid
TAG	Triacylglycerol
TNF-α	Tumor necrosis factor alpha
TVA	Trans-vaccenic acid

1. Introduction

Cardiovascular diseases (CVD), like ischemic heart disease and stroke, are the leading worldwide death causes, being responsible for 17 million deaths in 2008 (Alwan, 2011). Overweight and obesity are the major risk factors for cardiovascular diseases, and it was estimated that between 1980 and 2008 the prevalence of overweight and obese adults (≥20 years old) increased from 24.6% to 34.4% and from 6.4% to 12.0%, respectively (Stevens *et al*., 2012). Although lipids are involved in the development of these conditions, recent studies have also stated their positive health effects on humans. Thus, dietary sources of omega 3 have shown to be capable of reducing the incidence of CVD, through decreasing the number of sudden deaths as well as the effect of risk factors such as obesity, hypertension and cholesterol (Harris, 2008). Furthermore, the bioactivity of conjugated linoleic acid (CLA) has been well characterized, namely for its anticarcinogenic, antiobese, antidiabetic and antihypertensive properties effects (Koba and Yanagita, 2014). However, CLA is not the only group of bioactive conjugated fatty acids and recently conjugated linolenic acid (CLNA) have also shown bioactive potential as anti-carcinogenic, anti-inflammatory, anti-obese and antioxidant compounds (Yuan *et al*., 2014) being a promising new bioactive ingredient. Thus, this chapter aims to summarize the evidences about the beneficial health effects of CLNA and the possibilities of their utilization in new functional foodstuffs thus establishing the rationale for the research project developed herein.

1.1. CLNA bioactivity

1.1.1. Anti-cancer activity

Some studies have reported the cytotoxic effect of CLNA isomers on different human tumor cell lines, including MDA-MB-231, MCF-7 (breast) (Moon *et al*., 2010), HT-29 (colon) (Degen *et al*., 2011), MDA-MB-231 (estrogen insensitive breast cancer cells), MDA-ERα7 (estrogen sensitive breast cancer cells cloned from MDA-MB-231 cells) (Grossmann *et al*., 2010), T24 (bladder) (Sun *et al*., 2012), HeLa (cervix) (Eom *et al*., 2010), LNCaP, PC-3 (prostate) (Gasmi and Sanderson, 2013) and DLD-1 (colorectal) (Shinohara *et al*., 2012b). The works found that the anti-proliferative activity was due to an increment in the number of cells in G0/G1 phase (cells are arrested in the initial phases of cell cycle and do not continue

to proliferate) and the increase of apoptosis rate are the main anti-cancer activity evidences verified for CLNA isomers.

These activities seem to be influenced by the configuration of the double bonds. Degen *et al*. (2011), when evaluating growth inhibition effects of pure CLNA isomers against HT-29 colon cancer cells, found that in the presence of an all-*trans* isomer, as C18:3 t9,t11,t13 resulted in greater inhibition than with C18:3 c9,t11,t13. Furthermore, Shinohara *et al*. (2012b) assaying the cytotoxic effects of several pure CLNA isomers from vegetable sources on DLD-1 colorectal cancer cells, observed that jacaric acid (JA) (C18:3 c8,t10,c12) exerted a stronger effect in terms of decreasing cell survival and inducing apoptosis when compared with α-eleostearic acid (α-ESA) (C18:3 c9,t11,t13), punicic acid (PUA) (C18:3 c9,t11,c13), catalpic acid (CPA) (C18:3 t9,t11,c13) and the *trans*-isomers β-eleostearic acid (β-ESA) (C18:3 t9,t11,t13) and α-calendic acid (α-CDA) (C18:3 t8,t10,t12), all tested at the same concentration (10 μM). Further studies partially confirmed these previous results since when pure JA, PUA, α-CDA and β-CDA cytotoxicity was investigated against LNCaP and PC-3 prostate cancer cells results pointed out to PUA and JA as the most effective (Gasmi and Sanderson, 2013). At the time PUA and JA 3-D conformations were analyzed and overlapped, shape and feature similarity values were highly correlated, indicating that the *cis*, *trans*, *cis* configuration of their double bonds is the reason of their bioactivity.

It has also been suggested that the mechanism of CLNA anti-cancer activity may be related to lipid peroxidation, since the addition of α-tocotrienol, an antioxidant, lead to the loss of PUA cytotoxic properties against MDA-MB-231 and MDA-ERα7 breast cancer cells (Grossmann *et al*., 2010). These authors also associated the CLNA´s cytotoxicity to the induction of protein kinase C (PKC) that leads to the inhibition of cell proliferation and activation of apoptosis. Other studies have reported increase of caspase-3 expression, decrease of apoptosis suppression factor Bcl-2 expression, formation of reactive oxygen species (ROS), activation of caspase-9 cascade, DNA fragmentation, poly ADP-ribose polymerase (PARP) cleavage, increase of tumor suppressor gene p53 expression, activation of peroxisome proliferator-activated receptor gamma (PPARγ) and inhibition of extracellular signal-regulated kinases 1 and 2 (ERK 1/2) (Sun *et al*., 2012; Moon *et al*., 2010).

CLNA anti-carcinogenic activity has also been assayed *in vivo*, mainly with natural plant sources. Tung oil, which is rich in α-ESA, when administered at doses of 50 and 100 mg/kg body to mice transplanted with DLD-1 colorectal cancer cells, suppressed vessel formation: in doses of 100 mg/kg body weight tumor vessel length was significantly lower than with 50

mg/kg body weight (Tsuzuki and Kawakami, 2008). Bitter gourd seed oil (rich in α-ESA) was also able to inhibit aberrant crypt foci (ACF) formation, induced by azoxymethane (AOM), in rats fed with at 0.01, 0.1 and 1% (Kohno *et al*., 2002). Furthermore, feeding mice treated with nitrobenzene, a genotoxic inductor, with 100, 200 and 400 mg/kg body weight of pomegranate seed oil (PUA rich source), caused a significant reduction in the percentage of aberrant cells and sperm shape and chromosome aberrations (Aly *et al*., 2014). When pomegranate seed oil was administered to rats, this led to a significant suppression of adenocarcinomas incidence and multiplicity in colon. Most of the studies described above have associated CLNA cytotoxicity *in vivo* to enhanced PPARγ expression. CLNA anti-cancer properties could also be due to the metabolic conversion of CLNA to CLA, since lipid analysis in liver and colon of rats fed with pomegranate seed oil did not detect CLNA isomers, but CLA content was elevated in a dose-dependent manner (Kohno *et al*., 2004). When compared to CLA, α-ESA suppressed tumor vessel formation at doses 10 times lower (Tsuzuki and Kawakami, 2008) supporting the fact that CLNA isomers could exert a more powerful bioactivity than CLA.

However, despite these promising results no studies focusing on CLNA anti-cancer properties have been carried out on human subjects so far.

1.1.2. Anti-inflammatory activity

Cells (*e.g.* neutrophils, macrophages and monocytes) and biochemical compounds, like tumor necrosis factor (TNF-α), interleukins (IL), platelet activating factor (PAF), leukotrienes and ROS are involved in inflammatory diseases. Therefore, an increment of these mediators is an indicator of ongoing inflammation.

Anti-inflammatory activity of CLNA *in vitro* has only been reported for PUA on human breast cancer cells (MDA-MB-231 and MCF-7) in the presence of pomegranate seed oil for 24 h at 37 ºC (Costantini *et al*., 2014). The levels of 9 pro-inflammatory cytokines, including ILs and TNF-α, were significantly decreased at amounts ranging from 0.24 to 0.6 µL.

Necrotising enterocolitis (NEC) is characterized pathologically by inflammatory and coagulative necrosis that occurs throughout the intestinal tract of, especially, newborns. Pomegranate seed oil administration at 1.5% to NEC rats caused significant reduction on ileal damage while expression levels of IL-6, IL-8, IL-12, IL-23 and TNF-α mRNA were significantly lower (Coursodon-Boyiddle *et al*., 2012).

On the other hand, inflammatory bowel disease (IBD) is a chronic inflammatory disease that can manifest itself as ulcerative colitis (affects only the large intestine) or Crohn's disease (affects the whole digestive tract). α-ESA was shown to be able to ameliorate IBD phenotypes when incorporated in induced-IBD mice diet, through equally PPARγ-dependent and independent mechanisms (Lewis *et al*., 2011). Both α-ESA and PUA, were also capable to significantly reduce IL-6, Il-1β and TNF-α expressions in rats treated with sodium arsenite and streptozotocin (Saha *et al*., 2012b; Saha and Ghosh, 2012). According to Saha and Ghosh (2011), high concentrations of arachidonic acid (ARA: C20:4 ,c8,c11,c13,c16) together with low levels of γ-linolenic acid (γ-LNA: C18:3 c6,c9,c12) in tissues are indicators of inflammation. Vegetable oils containing α-ESA or PUA were capable of normalizing ARA and γ-LNA amounts in rats when an inflammatory process altered their concentrations.

Both *in vitro* and *in vivo* studies, suggest that CLNA can interfere with inflammation mediators. At the current moment the anti-inflammatory effects of CLNA have not been tested on humans.

1.1.3. Antioxidant activity

Some works have also reported antioxidant properties for CLNA. Karela seed oil (rich in α-ESA) was added at 0.05 and 0.1% to human blood samples of diabetic and non-diabetic subjects reducing lipid peroxidation at both doses in samples from diabetic subjects (Dhar *et al*., 2007). Other assays comparing bitter (α-ESA) and snake (PUA) gourd seed oils revealed that the antioxidant activity was greater for bitter gourd seed oil, possibly due to α-ESA's better oxidative stability (Saha *et al*., 2012c), since *trans* double bonds are more stable than *cis*.

Some *in vivo* studies have been carried out to test the activity of CLNA isomers against chemically-induced oxidative stress. As in the *in vitro* research works mentioned above, α-ESA and PUA showed antioxidant properties in rats treated with sodium arsenite to induce oxidative stress being α-ESA the most effective at the lowest dose since it was able to increase the activity of antioxidant enzymes (SOD, CAT and GPx) and glutathione (GSH) levels while it decreased nitric oxide synthase (NOS) activity and lipid and protein oxidation in plasma and kidney (Saha and Ghosh, 2013). Some of these effects were also observed with α-ESA against the oxidative stress caused by methyl mercury and induced diabetes in rats (Paul *et al*., 2014).

On the other hand, the effect of PUA was tested on 15 healthy young humans, consuming an equivalent of 3 g/day of PUA from *Trichossanthes kirilowii* seeds during 28 days (Yuan *et al*., 2009); results concluded that PUA exhibited a pro-oxidant activity. These findings seem to be controversial with the *in vivo* studies described above, however, animal studies revealed that PUA has both antioxidant and pro-oxidant activity depending on the dose used: feeding rats with an equivalent of 0.6 g/kg of PUA lead to antioxidant effects, whereas a higher dose of 1.2 g/kg caused pro-oxidant activity (Mukherjee *et al*., 2002).

1.1.4. Anti-obesity activity

CLA isomers have been described to exert positives effects on body weight and due to the similarities among these fatty acids, some works have been focused on the study of the possible anti-obesity properties of CLNA. When 3T3-L1 adipose cells were exposed to a mixture of two CLNA isomers: RLA and C18:3 c9,t13,c15 at 10 and 100 µM triacyglycerol content decreased at both concentrations (Miranda *et al*., 2011). However, a dose of 10 µM increased the expression of hormone-sensitive lipase (HSL) while 100 µM affected adipose triglyceride lipase (ATL).

Moreover, oral administration of α-ESA and PUA, caused decrease of triacylglycerol (TAG), cholesterol and LDL-C levels and increase of HDL-C level in plasma, erythrocytes, liver and brain of obese and hypercholesterolemic rats (Saha *et al*., 2012a; Sengupta *et al*., 2015). Although no significant weight loss was observed, body weight gain and fat mass were reduced. Interestingly, the activity of HMG-CoA reductase, responsible for cholesterol biosynthesis, was found to decrease. Authors concluded that α-ESA was more effective than PUA and associated such effects to the *trans* configuration of this fatty acid.

It has been elsewhere suggested that effects of CLNA on lipolysis are due to the activation of cAMP-activated protein kinase (PKA) pathway and apoptosis in white adipose tissue (Chen *et al*., 2012). Nevertheless, the administration of JA (5 mg/day) for 1 week to normal rats increased the accumulation of palmitic acid (C16:0) and stearic acid (C18:0) while decreasing palmitoleic acid (C16:1 c9) and oleic acid (C18:1 c9) in liver and white adipose tissues (Shinohara *et al*., 2012a). The results were associated to the inhibition of stearoyl coenzyme A desaturase (SCD) activity, an endoplasmic reticulum enzyme that catalyzes the biosynthesis of monounsaturated fatty acids (MUFAs) from saturated fatty acids (SFAs), since the expression level of SCD-1 mRNA was significantly decreased.

Recently a study was carried out with humans. The effect of pomegranate seed oil, was investigated by monitoring lipid profile of 23 volunteer hyperlipidaemic subjects (<20 yr) enrolled in a parallel, randomized, double-blind and placebo-controlled study (Mirmiran *et al*., 2010). The test group consumed one capsule containing 400 mg of seed oil twice a day during 4 weeks. The results revealed a significantly decrease in TAG and TAG:HDL-C ratio levels although cholesterol and LDL-C levels remained unchanged.

According to the already reported research works, there are strong evidences supporting the bioactivity of CLNA isomers (mainly α-ESA and PUA) and their potential to be used as a functional ingredient. However, little research has been performed on humans. Thus, to fully understand the mechanisms and possibilities of these compounds more investigations focused on this topic need to be accomplished.

1.2. CLNA in foods: main sources and contents

It has been reported that CLNA isomers occur naturally in milk fat and meat of ruminants, however, it is mostly found in vegetable oils (Mapiye *et al*., 2013) (Table 1.1). Despite all the possible isomers, only seven compounds are found in plant seed oils: jacaric acid (JA) (C18:3 c8,t10,c12), α-eleostearic acid (α-ESA) (C18:3 c9,t11,t13), β-eleostearic acid (β-ESA) (C18:3 t9,t11,t13), punicic acid (PUA) (C18:3 c9,t11,c13), α-calendic acid (α-CDA) (C18:3 t8,t10,c12), β-calendic acid (β-CDA) (C18:3 t8,t10,t12) and catalpic acid (CPA) (C18:3 t9,t11,c13) (Tanaka *et al*., 2011). The main source of JA is the seed oil of the argentine native tree *Jacaranda mimosifolia*, with 36 g/100 g of oil (Kraus *et al*., 2005). The isomer α-ESA is the main compound of tung oil (*Aleurites fordii*) (>70 g/100 g of oil), a native tree from Southeast Asia and the Pacific Islands (Burrows and Tyrl, 2013), bitter melon (*Momordica charantia*) (>50 g/100 g of oil) (Dhar *et al*., 1999) and *Parinarium* spp. (>60 g/100 g of oil) (Scrimgeour and Harwood, 2007). This isomer can be found in white mahlab (*Prunus mahaleb*) (~40 g/100 g of oil), a small tree native to southern Europe that grows wild in the Mediterranean region across to Turkey (Sbihi *et al*., 2014).

Tung and bitter melon seeds also contain β-ESA but in lower concentrations (3.5 and 2.6 mol/100 mol of oil, respectively) than those of α-ESA (Tsuzuki *et al*., 2004). PUA is mainly found in pomegranate (*Punica granatum*) (>70 g/100 g of oil) (Spilmont *et al*., 2013) and balsam apple (*Momordica balsamina*) (~50 g/100 g of oil) (Gaydou *et al*., 1987), while snake gourd of *Trichosanthes anguina* and *Trichosanthes kirilowii* species have approximately 40

g/100 g of oil and above 30 g/100 g of oil, respectively (Yang *et al*., 2012). *Trichosanthes anguina* is a native vine from tropical Asia and it is cultivated in China, while *Trichosanthes kirilowii* is distributed through several Chinese provinces and also occurs in Korea and Japan (Hu, 2005). In pot marigold (*Calendula officinalis*) it is possible to find α-CDA (>50 g/100 g of oil) and a small amount of β-CDA (<1 g/100 g of oil) (Dulf *et al*., 2013). CPA is mainly present in *Catalpa ovata* (>40 g/100 g of oil), a species originating in China (Suzuki *et al*., 2006). Among the seeds that contain CLNA isomers, only those from pomegranate, snake gourd *T. kirilowii* and white mahlab are edible. Pomegranate is native form Persia and cultivated in Mediterranean countries, Afghanistan, India, China, Japan, Russia and some parts of the United States. Its edible parts are comprised of 80% juice and 20% seeds, also used to produce fresh juice, syrups, canned beverages, jelly, jam and for flavouring drinks (Fadavi *et al*., 2006). *Trichosanthes kirilowii* seeds have long been used as a traditional Chinese medicine to reduce infections and are a popular snack food in China (Yuan *et al*., 2014). White mahlab powder is used in Greece, Cyprus, Turkey and neighboring Arab countries from Syria to Saudi Arabia for flavouring breads and pastries while in Sudan it is also used as a medicine for diarrhea in children (Ieri *et al*., 2012).

The isomers C18:3 c9,t11,c15 (Rumelenic acid, RLA) and C18:3 c9,t11,t15 have been described as the main CLNA isomers in milk fat and meat of ruminants. In bovine milk, RLA has been detected at concentrations between 0.03 and 0.39 g/100 g of fat and C18:3 c9,t11,t15 between 0.02 and 0.06 g/100 g of fat (Lerch *et al*., 2012; Plourde *et al*., 2007b). Relatively to ruminant meat, RLA and C18:3 c9,t11,t15 isomers, were found in steer (0.08 and 0.02 mg/g, respectively), cow (0.06 and 0.02 mg/g, respectively) and goat (0.28 and 0.03 g/100 g of meat fat, respectively) (Ebrahimi *et al*., 2014; Mapiye *et al*., 2013; Nassu *et al*., 2011). In comparison to the vegetable oils, the concentration of CLNA in these foodstuffs is very low, which suggests that the intake of CLNA, in order to highlight its potential health benefits, would not be viable through this natural source, therefore, strategies to enhance its intake will have to be studied.

Table 1.1 Naturally occurring CLNA isomers, main sources and respective amounts.

Isomer	Chemical structure	Source	Amount	Reference
Jacaric acid (JA)	C18:3 c8,t10,c12	*Jacaranda mimosifolia* seed oil	36 g/100 g of oil	Tulloch, 1982
α-eleostearic acid (α-ESA)	C18:3 c9,t11,t13	*Aleurites fordii* seed oil	>70 g/100 g of oil	Tsuzuki *et al*., 2006
		Momordica charantia seed oil	>50 g/100 g of oil	Dhar *et al*., 1999
		Parinarium spp. seed oil	>60 g/100 g of oil	Scrimgeour and Harwood, 2007
		Prunus mahaleb seed oil	~40 g/100 g of oil	Sbihi *et al*., 2014
β-eleostearic acid (β-ESA)	C18:3 t9,t11,t13	*Aleurites fordii* seed oil	3.5 mol/100 mol of oil	Tsuzuki *et al*., 2004
		Momordica charantia seed oil	2.6 mol/100 mol of oil	Tsuzuki *et al*., 2004
Punicic acid (PUA)	C18:3 c9,t11,c13	*Punica granatum* seed oil	>70 g/100 g of oil	Spilmont *et al*., 2013
		Momordica balsamina seed oil	~50 g/100 g of oil	Gaydou *et al*., 1987
		Trichosanthes anguina seed oil	~40 g/100 g of oil	Mukherjee *et al*., 2002
		Trichosanthes kirilowii seed oil	>30 g/100 g of oil	Yang *et al*., 2012
α-calendic acid (α-CDA)	C18:3 t8,t10,c12	*Calendula officinalis* seed oil	>50 g/100 g of oil	Dulf *et al*., 2013
β-calendic acid (β-CDA)	C18:3 t8,t10,t12	*Calendula officinallis* seed oil	<1 g/100 g of oil	Dulf *et al*., 2013
Catalpic acid (CPA)	C18:3 t9,t11,c13	*Catalpa ovate* seed oil	>40 g/100 g of oil	Suzuki *et al*., 2006
Rumelenic acid	C18:3 c9,t11,c15	Bovine milk	0.03-0.39 g/100 g of fat	Lerch *et al*., 2012; Plourde *et al*., 2007a
		Bovine meat	0.06-0.08 mg/g of muscle	Mapiye *et al*., 2013; Nassu *et al*., 2011
		Goat meat	0.28 g/100 g of meat fat	Ebrahimi *et al*., 2014
	C18:3 c9,t11,t15	Bovine milk	0.02-0.06 mg/100 g of fat	Lerch *et al*., 2012
		Bovine meat	0.02 mg/g of muscle	Mapiye *et al*., 2013; Nassu *et al*., 2011
		Goat meat	0.03 g/100 g of meat fat	Ebrahimi *et al*., 2014

1.3. Strategies for CLNA enhancement in food

It is accepted that the effective dose of CLA to obtain the beneficial effects in humans is 3 g/day (Ip *et al*., 1994) and for CLNA 2-3 g/day (Shinohara *et al*., 2012b). However, both values were assumed based on animal models. Specifically, the CLNA dose was calculated from the amount of jacaranda seed oil administered to mice (0.001 g/day), exerting anti-cancer properties against transplanted human colorectal adenocarcinoma cells. Since it is a value obtained from animal studies, it cannot be completely assumed as the correct effective dose for humans as our metabolism and absorption of nutrients is different from that in animals.

Furthermore, obtaining this amount from milk and meat of ruminants, would be difficult according to the concentration of CLNA in these foodstuffs. On the other hand, the vegetable oils may be an alternative but currently pomegranate seed oil is the only edible oil that is commercialized. When this vegetable oil was administered to hyperlipidaemic subjects, no side effects were reported, however, the dose administered (0.80 g/day of oil that is equivalent to 0.56 g/day of PUA) is much lower than the abovementioned effective dose (Mirmiran *et al*., 2010). Moreover, pomegranate seed oil has been successfully incorporated in margarine (0.5 g/100 g PUA) and goat milk was naturally enriched with PUA (1.19 g/100 g of fat) using 12% of pomegranate seed pulp added to the feeding of the animals (Franczyk-Żarów *et al*., 2014; Modaresi *et al*., 2011).

Some other studies have assayed a different approach: the production of enriched supplements like nanoparticles and nanoemulsions containing vegetable oils rich in CLNA isomers (0.05 g/kg body weight/day in nanoparticles and 2 g in nanoemulsions) (Paul *et al*., 2014; Sengupta *et al*., 2015). These matrices were used to enhance CLNA bioavailability and stability and according to the results were efficient in attenuating the effects of hypercholesterolemia and diabetes in an animal model.

The manipulation of ruminants' diet had led to some interesting results in the enhancement of CLA concentrations in milk and ruminants' meat and it has also been assayed for CLNA. The studies focused on the addition of extruded seeds or oils rich in LNA to animal diet but, in general, the results obtained were very limited (Ebrahimi *et al*., 2014; Mapiye *et al*., 2013).

1.4. Microbiological production of CLNA: From rumen to cultures

Ruminant products, such as meat, milk and other dairy foods, represent the main source of CLA for humans. CLA isomers are formed during LA (C18:2 c9,c12) and α-LNA (C18:3 c9,c12,c15) biohydrogenation process that occurs in rumen, a multi-step pathway carried out by different microorganisms on unsaturated fatty acids (Figure 1.1). Once in the rumen, LA and α-LNA from diet are hydrolyzed through microbial lipases from *Butyrivibrio fibrisolvens* (hydrolyses phospholipids) and *Anaerovibrio lipolytica* (hydrolyses di- and triacylglycerols), for further reactions of isomerization and hydrogenation (Buccioni *et al*., 2012). The biohydrogenation of LA involves two main steps: isomerization of LA to C18:2 c9,t11 (CLA) and then hydrogenation of the *cis* double bond of the conjugated diene to yield C18:1 t11 (trans-vaccenic acid, TVA), that is further hydrogenated to stearic acid (C18:0) while C18:2 9,11 isomers, C18:2 8,10 and C18:2 t10,c12 isomers are also produced through this pathway (Chilliard *et al*., 2007). LNA biohydrogenation involves similar reactions in rumen, differing in the intermediate products, yielding CLA and CLNA: after hydrolysis, this fatty acid is first isomerized at *cis*-12 position producing RLA. Subsequently, this compound is reduced to C18:2 t11,c15 and further converted to three different products: C18:1 t11 (TVA); C18:1 c15 and C18:1 t15. Only TVA is reduced up to stearic acid (C18:0) (Van Nieuwenhove *et al*., 2012). Previous works suggested that CLA isomers can be formed from α-LNA as well, namely, C18:2 c9,c11, C18:2 t8,t10, C18:2 c10,c12 and C18:2 11,13 (Jouany *et al*., 2007; Lee and Jenkins, 2011; Loor *et al*., 2005). All intermediates of biohydrogenation are absorbed in the gut and transported through the blood stream to different body tissues, thus CLA and CLNA can appear in milk and meat fat (Gorissen *et al*., 2015). CLA is also produced at the mammary gland of lactating cows, through the conversion of TVA coming from rumen by the Δ9-desaturase. This pathway represents the primary source of CLA in milk (64%) (Griinari *et al*., 2000). On the other hand, CLNA does not have another synthesis pathway yet evidenced, so all ruminant milk content apparently comes exclusively from α-LNA biohydrogenation and ruminants diet.

The main ruminal bacterium that is involved in the biohydrogenation process is *Butyrivibrio fibrisolvens*, where the linoleate isomerase (LAI) bound to the bacterial membrane is responsible for its transformation (Bauman *et al*., 1999). However, it has been reported that ruminal bacteria are not the only ones capable of producing CLA. Species isolated from dairy products and human intestine showed a similar capacity, namely, strains

of lactobacilli, bifidobacteria and propionibacteria. Therefore, it has been proposed that other microorganisms can produce CLNA including those able to produce CLA. This hypothesis has been confirmed in some studies using strains of lactobacilli, propionibacteria and bifidobacteria (Annexes I and II).

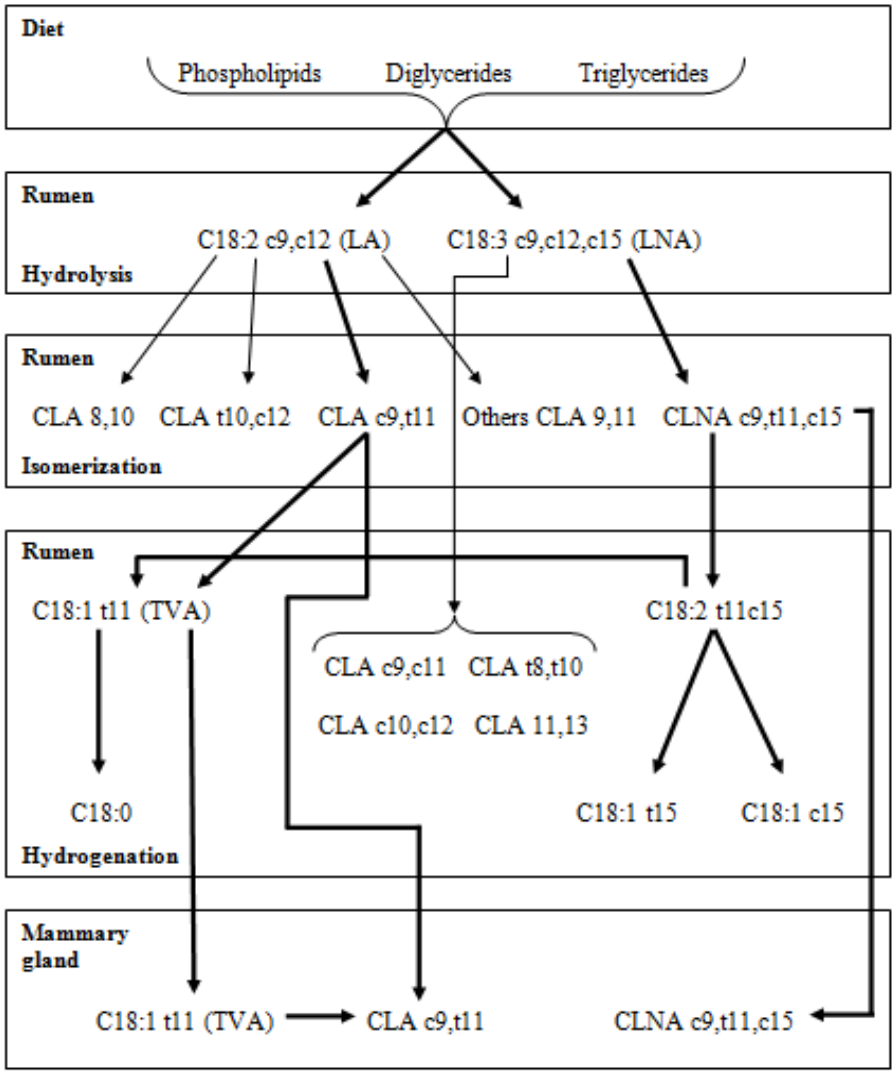


Figure 1.1 Scheme of LA and LNA biohydrogenation. Bold arrows indicate the principal pathways and simple arrows indicate secondary pathways (Buccioni *et al*., 2012; Chilliard *et al*., 2007; Gorissen *et al*., 2015; Jouany *et al*., 2007; Lee and Jenkins, 2011; Loor *et al*., 2005; Van Nieuwenhove *et al*., 2007).

1.4.1. CLNA production by lactobacilli

Lactobacillus plantarum AKU 1009a was able to produce CLNA isomers in Man-Rogosa-Sharpe (MRS) medium supplemented with LNA (Kishino *et al*., 2003). At optimal conditions

(37 ºC, 72 h, 63.00 mg/mL of pure LNA and anaerobic conditions) CLNA was formed at a conversion rate of 40.0% under two isomer forms: RLA and C18:3 t9,t11,c15 which corresponded to 67.0% and 33.0% of total CLNA, respectively. In a further study by these authors (Kishino *et al*., 2009) the same strain (MRS, 37 ºC, 72 h, 4.00 mg/mL of pure substrate and anaerobic conditions) evidenced a 47.0% conversion rate of α-LNA after 48 h, yielding RLA and C18:3 t9,t11,c15 corresponding to 17.0% and 83.0% of total CLNA, respectively. This study also revealed the formation of conjugated isomers of γ-LNA (C18:3 c6,c9,c12) at a 46.0% rate after 24 h. Those isomers were C18:3 c6,c9,t11 (19.0% of total CLNA) and C18:3 c6,t9,t11 (81.0% of total CLNA).

Besides *L. plantarum*, other lactobacilli strains have also been tested (Gorissen *et al*., 2011): *L. curvatus* LMG 13553, *L. plantarum* ATCC 8014, *L. plantarum*, IMDO 130201, *L. plantarum* LMG 6907, *L. plantarum* LMG 13556, *L. plantarum* LMG 17682, *L. sakei* 23K, *L. sakei* CG1, *L. sakei* CTC 494 and *L. sakei* LMG 13558. These strains assayed with 0.50 mg/mL of pure LA or α-LNA for 72 h at 37 ºC (except for *L. sakei* and *L. curvatus* strains that were cultured at 30 ºC), converted α-LNA to different CLNA isomers (RLA and C18:3 t9,t11t,c15). Authors concluded that α-LNA conversion was strain dependent, since there were significant statistical differences. On the other hand, LA was converted by only three of these ten strains, pointing out to higher efficiency in converting α-LNA than LA. The highest CLNA conversion percentage was observed for *L. sakei* LMG 13558 (60.1%). Using this particular bacterial strain in a bioreactor with MRS medium and 0.50 mg/mL of pure LA or α-LNA for 48 h, the authors tested the effects of temperature (20, 25, 30 and 37 ºC) and pH (5.5 and 6.2) during fermentation. Temperature and pH influenced LA and α-LNA conversion, as well as bacterial growth. These authors also reported the presence of linoleate isomerase genes in the genome of strains able to produce CLA and CLNA.

1.4.2. CLNA production by propionibacteria

To the best of our knowledge, Verhulst *et al*. (1987) were the first to evidence CLNA production using *Propionibacterium* strains. The strains were cultured under anaerobic conditions for 48 h at 37 ºC in Brain Heart Infusion (BHI) medium with 0.02 mg/mL of different pure substrates, including α-LNA and γ-LNA (C18:3 c6,c9,c12), in order to test their capability in isomerizing polyunsaturated long chain fatty acids. *Propionibacterium freudenreichii* subsp. *freudenreicbii* NCIB 8896 and NCIB 5959, *P. freudenreichii* subsp.

shermanii NCIB 10585, NCIB 5964 and NCIB 8099, *P. acidipropionici* NCIB 8070 and NCIB 5958 and *P. tecbnicum* NCIB 5965 converted, on total, over 30.0% of the α-LNA to RLA. *Propionibacterium acnes* was not able to convert α-LNA, but did form C18:3 c6,t10,c12 (>50.0%) from γ-LNA.

In a recent study (Hennessy *et al*., 2012) dairy starter propionibacteria were cultured under anaerobic condition at 30 ºC for 72 h in cys-MRS medium with 0.45 mg/mL of pure LA, α-LNA, γ-LNA or stearidonic acid (C18:4 c6,c9,c12,c15), for evaluation of further conversion into the conjugated forms. For α-LNA, *P. freudenreichii* subsp. *shermanii* 9093 showed the highest conversion rate, producing two different isomers: RLA (50.3%) and C18:3 t9,t11,c15 (3.2%). In comparison with the abovementioned study, this strain showed higher conversion capacity than the other eight propionibacteria. Nevertheless it may be noted that *P. freudenreichii* subsp. *shermanii* 9093 was cultured during a longer period of time, at lower temperatures and in a different medium. This strain was also grown in the presence of a twenty-fold higher α-LNA concentration (0.45 mg/mL vs. 0.02 mg/mL).

Despite the good production of CLNA by propionibacteria strains, this group is the less studied among the CLNA-producing bacteria. Thus, more studies are needed to support the α-LNA isomerizing ability of these microorganisms.

1.4.3. CLNA production by bifidobacteria

According to the current literature, this genus is the most promising and has attracted many of the investigations. Coakley *et al*. (2009), investigated α-LNA isomerization ability of 6 CLA-producing bifidobacteria, derived from human intestinal sources: *Bifidobacterium lactis* Bb12 and *Bifidobacterium breve* NCIMB 702258, NCTC 11815, NCIMB 8815, NCIMB 8807 and DPC 6035. The strains were grown in cys-MRS medium containing 0.24 and 0.41 mg/mL of pure α-LNA at 37 ºC for 42 h under anaerobic conditions. The *B. breve* strains were capable of converting α-LNA to RLA and C18:3 t9,t11,c15 at conversion rates between 67.6% and 80.7% (assaying 0.24 mg/mL) and 49.4% and 79.1% (with 0.41 mg/mL). Recently, *Bifidobacterium breve* strains, isolated from human breast milk, isomerized between 94.0 and 97.0% of 500 μg/mL α-LNA to RLA in milk-based medium (24 h at 37 ºC) under anaerobic conditions (Villar-Tajadura *et al*., 2014).

According to Jiang *et al*. (1998), the conversion of LA to CLA may be a detoxification mechanism for the bacteria. Previous results suggest that α-LNA is more toxic than LA, since

B. bifidum, *B. pseudolongum* subsp. *pseudolongum* and *B. breve* strains, cultured at 37 ºC for 72 h in cys-MRS medium with 0.50 mg/mL of α-LNA under anaerobic conditions, showed a higher conversion rate of α-LNA (55.6%-78.4%) than LA (19.5%-53.5%) and strains' growth was inhibited in the presence of α-LNA (Gorissen *et al*., 2010).

Gorissen *et al*. (2012b) conducted a research work with the aim of producing a fermented milk with enhanced CLA and CLNA concentrations. Three bacterial strains, namely *B. bifidum*, *B. breve* and *B. pseudolongum* subsp. *pseudolongum*, were each inoculated in milk containing sunflower and rapeseed oils (originating milk with 0.50 mg/mL of LA and 0.75 mg/mL of α-LNA) and incubated at 37 ºC for 24 h under anaerobic conditions, with or without co-inoculation of a commercial yoghurt starter culture (*Lactobacillus delbrueckii* subsp. *bulgaricus* Danisco LYO120 and *Streptococcus thermophilus* YC180). The starter culture was added to increase the amount of free LA and α-LNA due to their lipolytic activity. The results demonstrated that the growth of bifidobacteria strains was not inhibited when the starter culture was added, however, no significant differences were found for rumenic acid content in comparison to control (0.08 mg/g fermented milk) and CLNA was not even detected with or without starter culture added. The results suggest that the amount of free LA and α-LNA was too low to initiate isomerization, possibly because of lack of lipolytic activity.

Furthermore, human-derived bifidobacteria strains previously described as CLA and CLNA producers, *B. breve* DPC 6330 and *B. breve* NCIMB 702258 (Hennessy *et al*., 2012), have been incorporated in a mice diet to evaluate the *in vivo* production of conjugated fatty acids and their effect on lipid metabolism (Barrett *et al*., 2012; Wall *et al*., 2009). A significant modification of the lipid profiles in liver, adipose tissue, brain, serum and intestines was observed, being palmitoleic acid (C16:1 c9), eicosapentaenoic acid (C20:5 ω3) and docosahexaenoic acid (C22:6 ω6) the principal FAs altered. CLA levels were also significantly higher in comparison to the control samples. However, CLA could have been metabolized from CLNA, since this has been observed *in vivo* (Tsuzuki *et al*., 2006). These results are very promising but further research must be accomplished since mechanisms behind lipid profile modification are still unknown.

In comparison to other groups of microorganisms, bifidobacteria appear to be the best CLNA producers showing high potential for future development of new CLNA-enriched foods.

1.5. Linoleate isomerase as a screening tool

Commercial high CLA oils are currently produced mainly through alkaline isomerization of LA. However this strategy has not been utilized for the elaboration of high CLNA oils to bring more easily available sources. Furthemore, chemical catalysts are not isomer selective, resulting in a mixture of different positional isomers that cannot be selected (Reaney *et al*., 1999). An alternative to this process is the bioconversion of LA and α-LNA into pure CLA and CLNA single isomers using specific enzymes. As previously mentioned, specific microorganisms are capable of producing CLA and CLNA as an intermediate in the biological process of biohydrogenation. Isomerization represents the initial step of this pathway, occurring in FA containing a c9,c12 double bond structure. Linoleate isomerase (LAI) (EC 5.2.1.5) is the enzyme responsible for the conversion of LA and α-LNA into their conjugated forms, by forming conjugated double bonds from the c9,c12 double bond system (Bauman *et al*., 1999). The presence of a double bond at the c15 position in α-LNA has little or no effect on the system and therefore biohydrogenation of LNA follows the same pathway as that of LA in rumen microorganisms (Kepler and Tove, 1967).

LAI from the rumen bacterium *B. fibrisolvens* was first described in 1967 by Kepler and Tove (1967). Thereafter, LAI enzymes from *Clostridium sporogenes* (Peng *et al*., 2007) and *Propionibacterium acnes* (Liavonchanka *et al*., 2006) have been characterized. Several attempts to solubilize the *B. fibrinogens* LAI were ineffective, suggesting an association of this enzyme to the cell membrane (Kepler and Tove, 1967). Similarly, the *C. sporogenes* LAI is membrane associated and both isomerases are only active on substrates containing c9,c12 double bonds in C18 fatty acids (Peng *et al*., 2007). However, *C. sporogenes* LAI shows higher V_{max} and K_m with LNA than with LA, suggesting that the extra double bond increases the reaction rate while decreasing its affinity for the substrate. On the other hand, although the *P. acnes* LAI has similar substrate specificity to that of the *B. fibrisolvens* and *C. sporogenes*, it is an intracellular soluble cytoplasmic protein capable of converting LA to CLA t10,c12 single isomer (Rosberg-Cody *et al*., 2007). Due to its high solubility and high catalytic activity, *P. acnes* LAI is a promising candidate for the biosynthesis of conjugated fatty acids (He *et al*., 2015).

To the present date, different LAI protein sequences have been annotated in GenBank including sequences of the strains *P. acnes*, *L. acidophilus*, *L. plantarum*, *L. reuteri*, *Lactococcus lactis* subsp. *lactis*, *B. dentium*, *B. breve*, *Rhodococcus erythropolis*, *L.*

delbrueckii subsp. *bulgaricus*, *P. freudenreichii* subsp. *shermanii*, *B. fibrisolvens* and *C. sporogenes*. According to the amino acid identity percentage of the annotated sequences, LAIs can be divided into four groups: 1) LAI from *P. acnes* that did not show any identity to other LAIs; 2) LAI from *L. reuteri*, *L. acidophilus* and *Lact. lactis* spp. *lactis*; 3) LAIs of *L. plantarum* and *R. erythropolis* and 4) LAIs of *B. dentium* and *B. breve* (Farmani *et al*., 2010). The *P. acnes* isomerase shares no significant sequence homology to other enzymes except a flavin-binding domain in the N-terminal region (Deng *et al*., 2007). In addition, with the exception of *P. acnes*, LAIs from the species mentioned have significant homology with myosin cross-reactive antigen (MRCA) proteins. MCRA-like LAIs are mostly 9,11-isomerases and are mainly associated with the cell membrane (Farmani *et al*., 2010).

Molecular techniques have been recently used to screen and study the putative LAI of different species within the genera *Lactobacillus* and bifidobacteria (Macouzet *et al*., 2010; Gorissen *et al*., 2011). Among *Lactobacillus* strains studied the LAI gene sequence is highly identical and the putative LAI gene sequence was only identified in the genome of strains able to produce CLA and CLNA isomers. Therefore, genotypic screening appears to be a reliable method to detect the presence of CLA and CLNA producing strains.

In order to increase the CLA and CLNA production levels, the use of recombinant technologies has been tested during the last decade. The LAI gene has been successfully introduced in other microorganisms using either a novel yeast cell surface display system (He *et al*., 2015) or heterologous technologies (Hornung *et al*., 2005) in *Saccharomyces cerevisiae* and expression as an intracellular enzyme in *Escherichia coli* (Rosberg-Cody *et al*., 2007; Luo *et al*., 2013) and *Bacillus* spp. (Saengkerdsub, 2013). In addition, LAI gene has been introduced in plants such as in tobacco seeds (Hornung *et al*., 2005) and rice (Kohno-Murase *et al*., 2006). Most of these studies focused on the recombinant production of LAIs from *P. acnes* since it is more difficult to develop a recombinant biocatalyst based on membrane-bound enzymes and problems regarding their solubility may rise during recombinant production.

1.6. Stability of CLNA in enriched products

A bioactive compound must be stable after elaboration and during storage period of the product. Otherwise it may result in ingestion of hazardous compounds (e.g peroxides from lipid oxidation) and/or an insufficient intake as to obtain the effective dose. There is an utter

lack of data describing the stability of high CLNA products during storage and after elaboration.

However, interesting information can be obtained from studies focused on CLA. Its stability in enriched food matrices has been investigated in terms of influence of elaboration (i.e thermal processing) and storage conditions (time and temperature). Thus, CLA content was stable in naturally enriched milk after UHT processing (142 ºC; 2 s), since total CLA content (4.67 g/100 g of FA) was similar to that in raw milk (4.68 g/100 g of FA) (Jones *et al*., 2005). Furthermore, after elaboration of butter and cheese, CLA contents were 4.34 g/100 g of fat and 4.80 g/100 g of fat, respectively and not significantly different from that in the UHT milk. However, Campbell *et al*. (2003) observed a significant loss of C18:2 c9,t11 in fortified milk (2% CLA) after HTST pasteurization (77.2 ºC; 16 s).

The first clear insight about the effects of processing were reported after assaying cooking and frying of milk fat (200-300 ºC, 15 min) as elaidic acid (C18:1 t9) increased proportionally to temperature as a result of isomerization of oleic acid (OA, C18:1 c9) while C18:1 c9,t11 decreased (Precht *et al*., 1999). In further experiments it was reported that linoleic acid can be oxidized at high temperatures to produce CLA isomers (namely C18:2 c9, t11 and C18:2 t10, c12) or isomerize to the *trans, trans* moieties through a intramolecular sigmatropic rearrangement (Destaillats and Angers, 2005) that can also affect CLA isomers (Destaillats *et al*., 2005). According to these findings it could be hypothesized that these effects occur only with high temperature processing. However, it was demonstrated that *trans* fatty acids and CLA isomers in milk increased as a result of pasteurization and sterilization processes in agreement with the abovementioned reactions (Herzallah *et al*., 2005; Rodríguez-Alcalá *et al*., 2014).

It is known that the deodorization of linseed oil led, as expected, to the formation of *trans, trans* isomers (Wolff, 1993) while oxidation conditions during derivatization of pomegranate oil transformed PUA into *trans, trans, trans* compounds (Chen *et al*., 2007). Moreover, the stability of CLNA to oxidation was compared to that of CLA, LA and α-LNA and it was quite lower than in these latter fatty acids (Yang *et al*., 2009).

Finally, other investigations focused on the study of the stability of CLA during storage. It was found elsewhere that throughout 14 d at 4°C in the dark or with light exposure, the fatty acid concentration including CLA (18:2 c9,t11) did not change in naturally enriched pasteurized milk (Lynch *et al*., 2005) while CLA butter aged (8 wk, 6 ºC) in a similar way to regular samples (Mallia *et al*., 2008). On the other hand, skimmed-milk added with high CLA oils showed loss of C18:2 c9, t11 while in fresh cheese were observed losses of C18:2 c11,

t13, C18:2 c9, c11 and C18:2 c10, c12 as result of microbiological growth after 3 and 10 wk of refrigerated storage (Campbell *et al*., 2003; Rodríguez-Alcalá and Fontecha, 2007). Recent results seem to confirm these findings as in yoghurts elaborated with cow´s milk, the concentration of C18:1 c9, t11 decreased after 14 d (5 ºC) (Serafeimidou *et al*., 2013) while in Feta cheese it decreased during aging (Laskaridis *et al*., 2013).

Furthermore, studies concerning CLA stability in enriched products after temperature treatments have shown that this FA is affected by temperature, originating changes in both total content and isomers distribution. Due to the recent interest in CLNA as well as the lack of rich edible and commercial sources, little is known about the thermal and storage stability of these compounds. These issues must be considered in future research works; available data suggests that temperature will increase *trans* moieties while during storage, if microbiological growth takes place, some CLNA isomers may decrease. However, chemical differences with CLA as well as presence of other compounds in the assayed high CLNA sources (*e.g.* antioxidants in oils), may lead to unexpected results.

1.7. Objectives

Due to the potential CLNA bioactivity and the possibility to become a new functional ingredient, the main aim of this work was to identify CLNA-producing strains, since some groups of dairy bacteria have demonstrated the ability to produce those isomers. In order to achieve such identification the following specific objectives were pursued: (i) molecular detection of linoleate isomerase gene in *Lactobacillus* strains, (ii) selection of the strains capable of converting LA into CLA isomers in optimized growth conditions, since CLA-producing strains have been demonstrated to also isomerize LNA into CLNA isomers and (iii) assessment of the capacity of the producing strains to form CLA and CLNA in a dairy food matrix.

2. Material and Methods

2.1. Analytical reagents

Hexane, methanol and dimethylformamide were HPLC grade (VWR Chemicals, West Chester, PA), GLC-Nestlé36, methyl undecanoate (99.9%), heptadecnoic acid (99.9%) and tritridecanoin (99.9%) were obtained from Nu-Chek Prep, inc. (Elysian, Minnesota, USA) while butterfat CRM-164 (EU Commission; Brussels, Belgium) was from Fedelco Inc. (Madrid, Spain), sulphuric acid was from Panreac AppliChem (Darmstadt, Germany) and Supelco 37 FAME, sodium hydroxide, linolenic acid (LA; C18:2 c9,c12), α-linolenic acid (α-LNA; C18:3 c9,c12,c15) and γ-linolenic acid (γ-LNA; C18:3 c6,c9,c12) were from Sigma-Aldrich (St. Louis, MO, USA). Stock solutions of LA and α-LNA were prepared at 15 mg/mL and at 10 mg/mL for γ-LNA with 2% (w/v) Tween 80 (Sigma) and filter-sterilized through a 0.45 µm-pore size membrane (Millipore, Carrigtwohill, Ireland).

2.2. Bacterial strains

A pool of 15 strains was used in this study, including 12 strains of *Lactobacillus* and 3 strains of *Bifidobacterium* (Table 2.1). All strains had been stored at -80 ºC in glycerol (Fisher Chemical, Loughborough, UK), a cryoprotectant, at 30% (w/w). They were activated at 2% (v/v) in MRS broth (10 mL) (Biokar Diagnostics, Beauvais, France) at 37 ºC overnight. About 1 mL of each culture was then transferred to 9 mL of new medium containing 0.1% (w/v) of Tween 80 (pre-inoculation) and incubated at 37 ºC for 16 h. Two percent (v/v) of these cultures were inoculated in fresh MRS broth (10 mL) for the following experiments. For the *Bifidobacterium* strains the culture media were supplemented with 0.05% (w/v) L-cysteine-HCl (Sigma) and these strains were grown under anaerobic conditions in an anaerobic workstation (Whitley DG 250; Don Withley Scientific, Yorkshire, UK.) that was continuously sparged with a mixture of 80% nitrogen, 10% hydrogen and 10% carbon dioxide. The *Lactobacillus* strains were grown under aerobic conditions.

Table 2.1 Bacterial strains used in this study and corresponding sources.

Strain	Source
Bifidobacterium animalis subsp. *lactis* Bb12®	Christian Hansen (HØrsholm, Denmark)
Bifidobacterium breve NCIMB 702258	NCIMB (Aberdeen, Scotland)
Bifidobacterium lactis LAFTI® B94	DSM (Moorebank, NSW, Australia)
Lactobacillus acidophilus Ki	CSK (Ede, Netherlands)
Lactobacillus acidophilus LAFTI® L10	DSM (Moorebank, NSW, Australia)
Lactobacillus acidophilus LA-5®	Christian Hansen (HØrsholm, Denmark)
Lactobacillus brevis D24	Persimmon isolate (ESB-UCP, Portugal)
Lactobacillus casei 01	Christian Hansen (HØrsholm, Denmark)
Lactobacillus casei 431®	Chrristian Hansen (HØrsholm, Denmark)
Lactobacillus delbruecki subsp. *bulgaricus* 20081	DSMZ (Braunschweig, Germany)
Lactobacillus paracasei LAFTI® L26	DSM (Moorebank, NSW, Australia)
Lactobacillus plantarum 299v	PROBI AB (Lund, Sweden)
Lactobacillus plantarum D36	Persimmon isolate (ESB-UCP, Portugal)
Lactobacillus rhamnosus 20021	DSMZ (Braunschweig, Germany)
Lactobacillus rhamnosus R11	Lallemand (Montréal, QC, Canada)

2.3. Optimization of bacterial growth conditions

Lactobacillus plantarum 299v and *Bifidobacterium animalis* subsp. *lactis* Bb12 were selected to optimize the growth conditions of *Lactobacillus* and *Bifidobacterium* strains, respectively. These strains were first activated and pre-inoculated according to the growth conditions mentioned previously (section 2.2), where *B. animalis* subsp. *lactis* Bb12 was always incubated under anaerobic conditions and, in order to compare the influence of the atmosphere on *Lactobacillus* growth, *L. plantarum* 299v was incubated under aerobic and anaerobic conditions throughout the whole experiment. About 2% (v/v) of the activated cultures were transferred to new MRS medium (10 mL) in the absence and presence of 0.5 mg/mL of LA and incubated at 37 ºC during 24 and 48 h for *L. plantarum* 299v and during 24, 48 and 72 h for *B. animalis* subsp. *lactis* Bb12. All experiments were carried out in duplicate. Viable cell numbers were obtained via sequential decimal dilutions in peptone water 0.1% (w/v) (Sigma) and plating on MRS agar (Biokar Diagnostics, Beauvais, France) or on cys-MRS agar by the drop method (Miles and Misra, 1938). The plates were incubated

at 37 ºC for 24 h at the respective atmosphere conditions and viable cell counts were expressed as log (Colony Forming Units (CFU)/mL). Upon incubation, each culture was centrifuged at 3 500 rpm, 18 ºC, 5:30 min and the supernatant was collected to be analyzed for CLA quantification.

2.4. Molecular detection of linoleate isomerase gene in *Lactobacillus* strains

A molecular screening of the LAI gene was performed in all *Lactobacillus* strains (Table 2.1), in order to determine which strains carried the potential as CLA-producers. *Bifidobacterium* strains were not included, since no specific LAI gene primers have been described for these bacteria so far. The strains were first activated at 2% (v/v) in MRS broth (10 mL) at 37 ºC overnight and then 1 mL of each culture was transferred to fresh medium (9 mL) and incubated at 37 ºC for 24 h. The bacterial growth was performed under aerobic conditions. After recovery, each culture was centrifuged at 7 000 rpm for 15 min at room temperature and the pellet was used for the DNA extraction. Bacterial DNA was extracted using the FastDNA® Spin Kit (QBIOgene, Carlsbad, CA, USA) according to the manufacturer's instructions. DNA of each sample was then quantified and the purity measured using a nanophotometer (Implen, Isaza, Portugal). DNA concentrations were standardized to 1 ng/µL.

Real time polymerase chain reaction (qPCR) technique was used to detect the presence of the LAI gene in all 12 mentioned strains in a CFX96 Touch Deep Well real-time PCR detection system (Biorad). qPCR amplification reactions were optimized using the forward primer 5'-CGGACNTACGTYGAYTTAATGG and the reverse primer 5'-TGGTGMACMACRATCGACAT, according to Gorissen *et al*. (2011). The amplifications were performed in a total volume of 20 µL containing 10 µL of iTaq Universal SYBR Green Supermix (Biorad), 0.5 µL of forward primer, 0.5 µL of reverse primer, 4 µL of H_2O and 5 µL of DNA template. Before amplification, the reaction mixtures were incubated at 95 ºC for 3 min. The PCR conditions were 40 cycles of amplification consisting of denaturation at 95 ºC for 10 s, annealing at 56 ºC for 25 s and extension at 72 ºC for 25 s. The specificity of all the reactions was confirmed through analysis of the melting profile, obtained by dissociation of the DNA after amplification with 0.5 ºC increments between 65 and 95 ºC at 2-5 s/step.

2.5. CLA production with selected strains

Lactobacillus brevis D24 and *Lactobacillus plantarum* D36 were selected to test their ability in producing CLA. In order to also include *Bifidobacterium* strains, *Bifidobacterium lactis* B94 and *Bifidobacterium breve* NCIMB 702258 were also chosen for this experiment. The strains were first activated and pre-inoculated according to the procedures mentioned previously (section 2.2) and then inoculated at 2% (v/v) in new MRS broth (10 mL) containing 0.5 mg/mL of LA. All inocula were incubated at 37 ºC for 24 and 48 h. The culture medium for *Bifidobacterium* strains was supplemented with 0.05% (w/v) L-cysteine-HCl and they were grown under anaerobic conditions. *Lactobacillus* strains were incubated under aerobic conditions throughout the whole experiment.

In order to optimize CLA production, two additional strategies were undertaken using *B. breve* NCIMB 702258, *B. lactis* B94 and *L. brevis* D24. *L. plantarum* 299v and *B. animalis* subsp. *lactis* Bb12 were also included in this experiment since their behavior in the presence of linoleic acid was well-known from the previous optimization of bacterial growth conditions (section 2.3). In the first strategy, it was hypothesized that an early adaptation to precursor substrate could enhance metabolic conversion, hence an amount of 0.05 mg/mL of LA was also added during pre-inocula preparation. In the second strategy, it was hypothesized that early exposure to the precursor LA could inhibit bacterial growth hence no LA was added to the pre-icocula, instead, 0.5 mg/mL of LA was added to the culture medium after 7 h of incubation of the inocula. Bacterial cultures without substrate were also grown under the conditions of both strategies.

All experiments were carried out in duplicate. Bacterial growth was analyzed through plating of sequential decimal dilutions followed by log (CFU/mL) calculation. The samples collected were centrifuged at 3 500 rpm, 18 ºC for 5:30 min and both the supernatant and the pellet were used for CLA quantification.

2.6. CLA and CLNA production with *Bifidobacterium breve* NCIMB 702258

Bifidobacterium breve NCIMB 702258 was selected to study its CLNA-producing capacity. This strain was first activated and pre-inoculated according to the growth conditions mentioned previously (section 2.2). Then it was inoculated at 2% (v/v) in new cys-MRS broth (10 mL) without any substrate or containing 0.5 mg/mL of α-LNA, or γ-LNA, or LA, α-LNA

and γ-LNA, where, in this combination, the substrates were added separately at a final concentration of 0.5 mg/mL each. The inoculated media were incubated at 37 ºC for 24 h under anaerobic conditions. All experiments were carried out in duplicate. Bacterial growth was analyzed through plating of sequential decimal dilutions followed by viable cell numbers determination. The samples collected were centrifuged at 3 500 rpm, 18 ºC for 5:30 min and both the supernatant and the pellet were used for CLA and CLNA quantification.

2.7. CLA and CLNA production in milk with *Bifidobacterium breve* NCIMB 702258

Bifidobacterium breve NCIMB 702258 was chosen to analyze CLA and CLNA microbial production in a food matrix, more precisely, a commercial 1.6% reduced-fat milk. After *B. breve* NCIMB 702258 activation and pre-inoculation in cys-MRS medium, this strain was inoculated at 2% (v/v) in milk (10 mL) containing 0.05% (w/v) of L-cysteine-HCl and no precursor substrate or 0.5 mg/mL of LA, or α-LNA, or both, added separately at a final concentration of 0.5 mg/mL each. The inoculated milks were incubated at 37 ºC for 24 h under anaerobic conditions. All experiments were carried out in duplicate. Bacterial growth was analyzed through plating of sequential decimal dilutions followed by viable cell numbers calculation. The samples collected were used for CLA and CLNA quantification.

2.8. Fatty acids analysis

For the analysis of the total fatty acid (FA) composition, growth medium (500 µL), pellets or milk samples (500 mg) were prepared according to Castro-Gómez *et al*. (2014). For quantification purposes samples were added with 50 µL of tritridecanoin (1.4 mg/mL in hexane) and heptadecanoic acid (1.4 mg/mL in hexane) prior to derivatization and extracts were added with 100 µL of methyl undecanoate (1.4 mg/mL in hexane). Fatty acid methyl esters (FAME) were analysed in a gas chromatrograph HP6890A (Hewlett-Packard, Avondale, PA, USA), equipped with a flame-ionization detector (GLC-FID) and a BPX70 capillary column (50m x 0.32 mm x 0.25 µm; SGE Europe Ltd, Courtaboeuf, France). Analysis conditions were as follows: injector (split 10:1; injection volume 1 µL) and detector temperatures were 250 ºC and 270 ºC, respectively; carrier gas was Hydrogen (11 psi) and the oven temperature program started at 60 ºC (held 2 min), was raised at 10 ºC/min to 135 ºC (held 2 min), then at 10 ºC/min to 165 ºC (held 2 min) and finally at 10 ºC/min to 230 ºC (held 7 min). Supelco 37 and CRM-164 were used for identification of fatty acids. GLC-Nestlé36

was assayed for calculation of response factors and detection and quantification limits (LOD: 0.079 µg FA/mL; LOQ: 0.264 µg FA/ mL).

2.9. Statistical analysis

Results are reported as mean values with their standard deviation. Data were analyzed using t-Student test when comparing two groups ($p<0.05$) and one-way ANOVA for three or more groups. In this latter case, Levene's test was applied to verify the homogeneity of the variances. Tamhane's T2 (equal variances not assumed) or Bonferroni (equal variances assumed) post hoc tests were used to determine differences within groups ($p<0.05$). Analyses were performed using the IBM SPSS Statistics 21 (SPSS Inc., an IBM Company).

3. Results and Discussion

3.1. Optimization of bacterial growth conditions

To guarantee that the strains used in this work would grow well, *Lactobacillus plantarum* 299v and *Bifidobacterium animalis* subsp. *lactis* Bb12 were selected to optimize the growth conditions of *Lactobacillus* and *Bifidobacterium* strains, respectively. Since these strains were the first available in stock, at the time, they were selected for this experiment. The parameters analyzed were the incubation time (0, 24 and 48 h) and the atmosphere conditions (aerobic and anaerobic) in the presence and absence of LA.

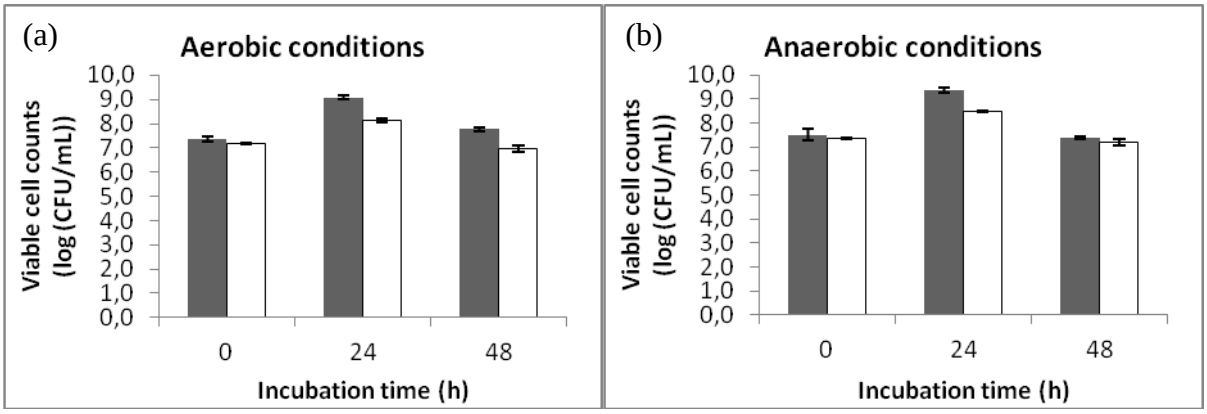


Figure 3.1 Bacterial growth of *L. plantarum* 299v at 0, 24 and 48 h of incubation in the absence (■) and presence (□) of LA (0.5 mg/mL) under aerobic (a) and anaerobic (b) conditions. Results are expressed in log (CFU/mL).

Concerning *L. plantarum* 299v (Figure 3.1a, b), no significant differences ($p>0.05$) were observed between 0, 24 and 48 h of incubation at both atmospheres, although this strain growth tended to be higher after 24 h under anaerobic conditions (8.48 and 9.38 log (CFU/mL) with and without LA, respectively) (Figure 3.1b). Smetanková *et al*. (2012) observed that after incubating *L. plantarum* 115 and *L. plantarum* 2L2 in MRS broth at 37 ºC for 48h under aerobic and anaerobic conditions, these strains have grown faster under anaerobic conditions. Similar results were obtained by Fu and Mathews (1999) that incubated *L. plantarum* ATCC 21028 in a synthetic lactose medium at 37 ºC and saw a faster growth under anaerobic conditions during the exponential growth phase between the 15th and 25th hour of fermentation. On the other hand, Murphy and Condon (1984) in their study with *L. plantarum* P5 have not seen considerable differences on growth patterns between aerobic and

anaerobic conditions when this strain was incubated at 30 ºC in MRS medium supplemented with glucose, although the anaerobic cultures departed from exponential growth at a higher cell density.

Some studies (Dilika *et al.*, 2000; Zheng *et al.*, 2005) reported that LA has anti-bacterial activity, however, the growth inhibition observed in this experiment was minimum. Li *et al.* (2011) did not detect a significant alteration in cell counts of *L. acidophilus* F0221 when treated with LA at concentrations lower than 1 mg/mL, but in the presence of 0.040 mg/mL of LA *L. casei* Shirota and *L. bulgaricus* growth was significantly reduced in Kankaanpää *et al.* (2001) study. At an even lower LA concentration (0.025 mg/mL), bacterial growth of *L. acidophilus* ATCC 4356, *L. bulgaricus* and *L. fermentum* was reduced in 20% to 50% and *L. reuteri* ATCC 23272 was inhibited between 50-70%, while the growth of two *L. casei* strains and *L. helveticus* ATCC 15009 was not significantly affected (Jiang *et al.*, 1998). *Lactobacillus* behavior in the presence of LA seems to be a strain-specific trait, since at 0.5 mg/mL of LA the number of viable cells of *L. plantarum* 299v was not greatly affected, while at lower concentrations other lactobacilli strains have been significantly inhibited.

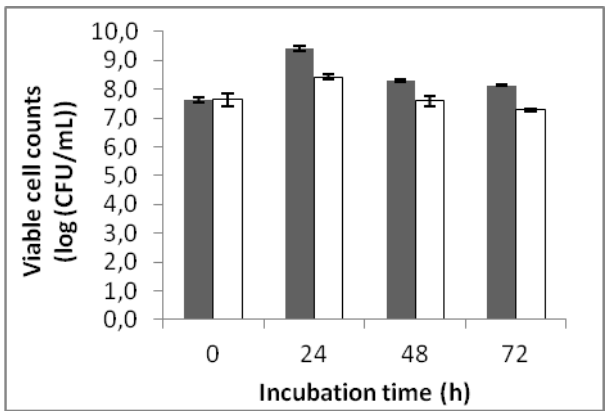


Figure 3.2 Bacterial growth of *B. animalis* subsp. *lactis* Bb12 at 0, 24, 48 and 72 h of incubation in the absence (■) and presence (□) of LA (0.5 mg/mL) under anaerobic conditions. Results are expressed in log (CFU/mL).

Bifidobacterium strains are strict anaerobes (Bernhard and Field, 2000) so *B. animalis* subsp. *lactis* Bb12 was only tested under anaerobic conditions. Viable cell numbers were determined throughout a 72 h period since some studies with *Bifidobacterium* strains have incubated these bacteria for that long (Gorissen *et al.*, 2012a; Hennessy *et al.*, 2012; Rosberg-Cody *et al.*, 2004). No differences ($p>0.05$) were detected between 0, 24, 48 and 72 h of incubation with and without LA (Figure 3.2), although the growth of this strain tended to be

higher after 24 h in media containing no LA. Similarly to *L. plantarum* 299v, the reduction of viable cell numbers of *B. animalis* subsp. *lactis* Bb12 was not considerable in the presence of LA. Van Nieuwenhove *et al*. (2007) did not detect growth inhibition of *B. bifidum* CRL 1399 with addition of up to 0.1 mg/mL of LA to the culture medium, so did not Oh *et al*. (2003) with a *B. breve* and a *B. pseudocatenulatum* strains in the presence of 0.5 mg/mL of LA. In a further study, Gorissen *et al*. (2010) also showed that the growth of five *B. animalis* subsp. *lactis* strains, including the one used in this experiment, was not inhibited with 0.5 mg/mL of LA in the culture medium, whereas cell numbers of *B. angulatum* LMG 11568, *B. longum* subsp. *infantis* LMG 11588, *B. pseudocatenulatum* LMG 10505 and *B. pseudocatenulatum* LMG 11593 were negatively affected. As for *Lactobacillus*, *Bifidobacterium* behavior in the presence of LA seems to be a strain-specific trait, since at the same concentration some strains were shown to be inhibited but others were not.

Table 3.1 LA reduction (%) and CLA production (µg/mL) by *L. plantarum* 299v and *B. animalis* subsp. *lactis* Bb12 in the culture supernatant.

Strain	Atmosphere	Incubation time (h)	LA reduction (%)*	CLA (µg/mL)*
L. plantarum 299v	Aerobic	24	56.45 ± 15.26	1.96 ± 0.42^{b}
		48	65.41 ± 8.98	2.49 ± 0.22^{a}
	Anaerobic	24	58.26 ± 14.21	2.57 ± 0.03
		48	69.28 ± 3.27	2.82 ± 0.47
B. animalis subsp. *lactis* Bb12	Anaerobic	24	51.28 ± 2.41^{c}	0.11 ± 0.01^{b}
		48	60.32 ± 2.10^{b}	0.62 ± 0.03^{a}
		72	64.54 ± 1.13^{a}	ND^{c}

* Average value ± standard deviation.

a, b, c - Different superscript letters stand for significant differences ($p<0.05$) between 24 h vs. 48 h vs. 72 h.

ND = not detected.

After the respective incubation time, the supernatant of each culture was collected to be analyzed for FA (LA and CLA) quantification. In general, LA reduction was above 50% for *L. plantarum* 299v and *B. animalis* subsp. *lactis* Bb12 (Table 3.1). No significant differences ($p>0.05$) were observed between 24 and 48 h of incubation for *L. plantarum* 299v at both atmosphere conditions, but for *B. animalis* subsp. *lactis* Bb12 the percentage of LA reduction increased over time, being significantly higher after 72 h of incubation (64.54%). Regarding CLA production, although of a very low order of magnitude, *L. plantarum* 299v under aerobic

conditions reported a statistically significant ($p<0.05$) increase in CLA production between 24 and 48 h having reached 2.49 µg/mL by 48 h. On the other hand, under anaerobic conditions, the amount of CLA was not statistically different ($p>0.05$) between incubation times, yet absolute amounts were always higher compared to those obtained under aerobic conditions. The isomers detected were C18:2 c9,t11 and C18:2 t10,c12 (data not shown). Kishino *et al*. (2002b) was able to detect higher amounts of CLA with *L. plantarum* AKU 1138, *L. plantarum* AKU 1009a, *L. plantarum* JCM 8341 and *L. plantarum* JCM 1551 when grown at 37 ºC for 72 h. The values ranged from 190 to 3 410 µg/mL corresponding to C18:2 c9,t11 and C18:2 t9,t11. These higher levels of CLA could be due to the use of an eight-fold higher LA concentration (4 mg/mL). Nevertheless, with a five-fold lower concentration of added LA (0.1 mg/mL) than that applied in our research, Liu *et al*. (2011) observed that ca. 25% of the LA was converted to C18:2 c9,t11 and C18:2 t10,c12 by *L. plantarum* Ip15 grown at 30 ºC for 48 h, while in our experiment the concentration of CLA formed by *L. plantarum* 299v was very low in comparison to the percentage of LA reduction.

In the case of *B. animalis* subsp. *lactis* Bb12, CLA production reached maximum concentration by 48 h (0.62 µg/mL) and C18:2 c9,t11 and C18:2 t10,c12 were the identified isomers (data not shown). At an equal LA concentration to the one used in this study (0.5 mg/mL) and with the same strain grown at 37 ºC for 72 h, Gorissen *et al*. (2010) were not able to observe any CLA production, whereas at a higher concentration (1 mg/mL) and with the same strain, Rodríguez-Alcalá *et al*. (2011) were able to detect the production of 7.12 µg/mL of CLA isomers, mostly C18:2 c9,t11 and C18:2 t10,c12. Therefore, the concentration of LA in the culture medium seems to affect the amount of CLA formed by *B. animalis* subsp. *lactis* Bb12, even though it is not a substantial production. Moreover, similarly to the results reported for *L. plantarum* 299v, the low concentrations of CLA produced are not duly correlated with the considerably high LA reduction percentages (over 50%). A possible hypothesis for this scenario could be the degradation of LA, although that could have exposed the strain to an oxidative environment and toxic compounds causing a considerable growth inhibition, which was not observed in this work in the presence of LA. Other possible hypotheses are that the strains could be using the substrate in a different way than the expected, for example the incorporation of LA into their cells' membrane (Jenkins and Courtney, 2003; Rosberg-Cody *et al*., 2007) or the conversion of LA to hydroxy fatty acids (HFAs) (Kim *et al*., 2003; Kishino *et al*., 2002b). Therefore, in the following experiments the pellet was also analyzed, in order to try and find a possible explanation for the results observed.

According to the bacterial growth and CLA production results, the following experiments with *Lactobacillus* were set to be performed under aerobic conditions, since the exposure to an anaerobic environment did not notoriously improve *L. plantarum* 299v performance (viable cell numbers and CLA concentration values under anaerobic conditions were not considerably higher). Regarding the incubation time, although *L. plantarum* 299v and *B. animalis* ssp *lactis* Bb12 tended to report higher viable cell numbers at 24 h growth, a higher CLA concentration was detected by 48 h incubation for both strains. Based on these observations the incubation time in the following experiments with *Lactobacillus* and *Bifidobacterium* strains was set to 24 and 48 h.

3.2. Molecular detection of linoleate isomerase gene in *Lactobacillus* strains

In order to identify high functionality potential in *Lactobacillus* strains to produce CLA isomers, a molecular screening of the LAI gene was performed in the 12 *Lactobacillus* strains. The DNA extracted from all strains revealed a purity degree above 1.75 (A260/A280) (data not shown). The real-time PCR amplification curves and melting curves for the positive strains demonstrating the presence of the linoleate isomerase gene are presented in Figures 3.3 and 3.4, respectively.

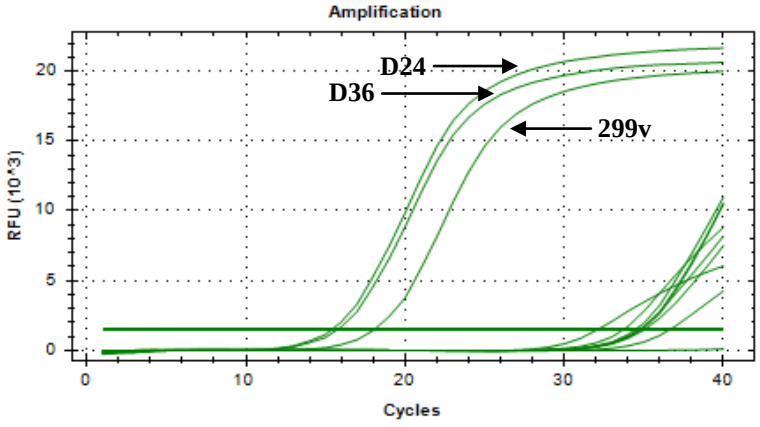


Figure 3.3 Real-time PCR amplification curves demonstrating the presence of the linoleate isomerase gene in *L. brevis* D24, *L. plantarum* D36 and *L. plantarum* 299v.

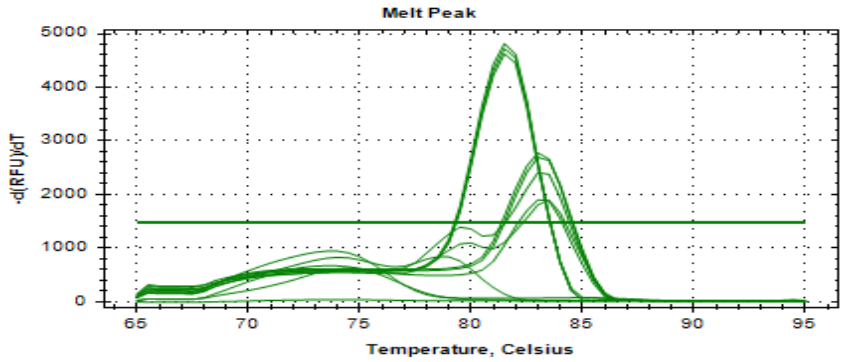


Figure 3.4 Real-time PCR melting curves of *L. brevis* D24, *L. plantarum* D36 and *L. plantarum* 299v at the highest peak and overlapped.

Among all the 12 strains tested, only three revealed the presence of LAI gene according to the real-time PCR amplification curves: *Lactobacillus brevis* D24, *Lactobacillus plantarum* D36 and *Lactobacillus plantarum* 299v (Figure 3.3). The specificity of the amplification product formed can be confirmed through the real-time PCR melting curves (Figure 3.4), where the overlap of the corresponding melting curves indicate that the melting temperature of the PCR product for all three strains is the same, hence the PCR product must be the same. Few studies have been performed for the detection of LAI gene in *Lactobacillus* strains and only Gorissen *et al*. (2011) were able to also detect the presence of this gene in other strains of *Lactobacillus plantarum*. Macouzet *et al*. (2010) were able to detect the LAI gene in *Lactobacillus acidophilus* strains, however the primers used were based on a putative gene sequence of other *Lactobacillus* species (i.e. *Lactobacillus reuteri* LI (EC 5.2.1.5)), whereas the primers used by Gorissen *et al*. (2011), and those used in our study, were constructed based on the LAI gene sequence of *Lactobacillus sakei* 23K. These findings suggest that the LAI gene sequence may be different between *Lactobacillus* species, not existing a single pair of specific primers applicable for all *Lactobacillus* strains. However, more studies should be undertaken on this subject in order to validate this hypothesis.

To the best of our knowledge, no other studies have described the presence of LAI gene in *Lactobacillus brevis* strains, so this is the first study to accomplish such a feat.

Lactobacillus plantarum D36 and *L. brevis* D24, as they were positive for the LAI gene presence, were chosen for the following set of experiments where it was tested their ability to isomerize LA. *Lactobacillus plantarum* 299v was rejected because although the molecular screening had given a positive result, in the optimization of bacterial growth conditions experiment (section 3.1) it was observed that the concentration of CLA formed was too low in comparison to the percentage of LA reduction. The fact that *L. plantarum* 299v is LAI gene-

positive and LA is decreased but CLA production remains low, may suggest that LA is being absorbed or converted into other products not yet described by the strain. Nevertheless, it cannot be overlooked that the fact that the strain is LAI gene-positive does not necessarily imply LA conversion into CLA if the enzyme is not expressed by the strain under the growth conditions applied.

3.3. CLA production with selected strains

CLNA isomers are chemically similar to CLA isomers and previous studies (Gorissen *et al*., 2010; Hennessy *et al*., 2012; Villar-Tajadura *et al*., 2014) have seen that the majority of the strains that were able to produce CLA could also form CLNA isomers. Therefore, in order to test strains CLNA producing ability, it was first tested their capacity to form CLA isomers.

According to the results in the molecular detection of the LAI gene in *Lactobacillus* strains (section 3.2), the gene positive strains *L. brevis* D24 and *L. plantarum* D36 were selected to test their capability in producing CLA isomers. Although no molecular screening could be applied for the *Bifidobacterium* strains, it was important to also include these bacteria in this study. *B. breve* NCIMB 702258 was chosen since *B. breve* strains have demonstrated CLA-producing capacity in other studies (Coakley et al., 2009; Gorissen et al., 2010; Hennessy et al., 2012) and *B. lactis* B94 was included because at the time there were no more *Bifidobacterium* strains available in stock.

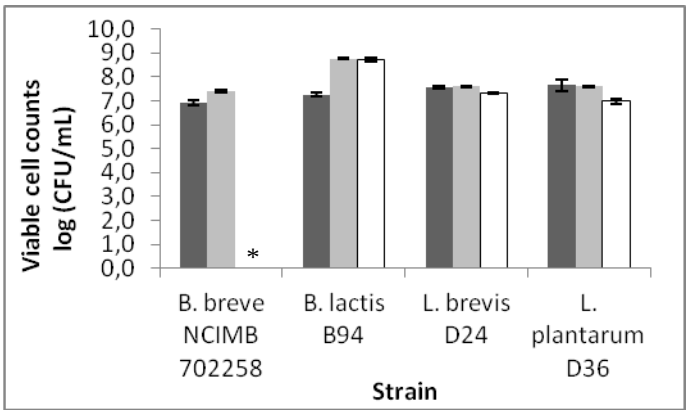


Figure 3.5 Bacterial growth of *B. breve* NCIMB 702258, *B. lactis* B94, *L. brevis* D24 and *L. plantarum* D36 at 0 (■), 24 (▣) and 48 h (□) of incubation in the presence of LA (0.5 mg/mL). Results are expressed in log (CFU/mL).

* No significant growth was detected.

Concerning bacterial growth (Figure 3.5), with exception to *B. breve* NCIMB 702258 that did not grow after 48 h no significant differences ($p>0.05$) were observed between 0, 24 and 48 h of incubation for each strain, although *B.lactis* B94 viable cell numbers tended to be higher after 24 h of incubation.

Table 3.2 LA reduction (%) and CLA formation (µg/mL) by selected strains in the culture supernatant collected after 24 and 48 h of incubation.

Strain	Incubation time (h)	LA reduction (%)*	CLA (µg/mL)*
B. breve NCIMB 702258	24	66.13 ± 1.18^{b}	155.83 ± 5.00^{a}
	48	71.14 ± 1.76^{a}	139.30 ± 5.28^{b}
B. lactis B94	24	40.36 ± 1.24^{b}	ND
	48	49.98 ± 2.36^{a}	ND
L. brevis D24	24	57.47 ± 0.54	0.93 ± 0.05^{a}
	48	58.26 ± 0.44	0.74 ± 0.04^{b}
L. plantarum D36	24	59.07 ± 0.75^{b}	1.18 ± 0.06^{a}
	48	63.00 ± 0.29^{a}	ND^{b}

* Average value ± standard deviation.

a, b - Different superscript letters stand for significant differences ($p<0.05$) between 24 h vs. 48h.

ND = not detected.

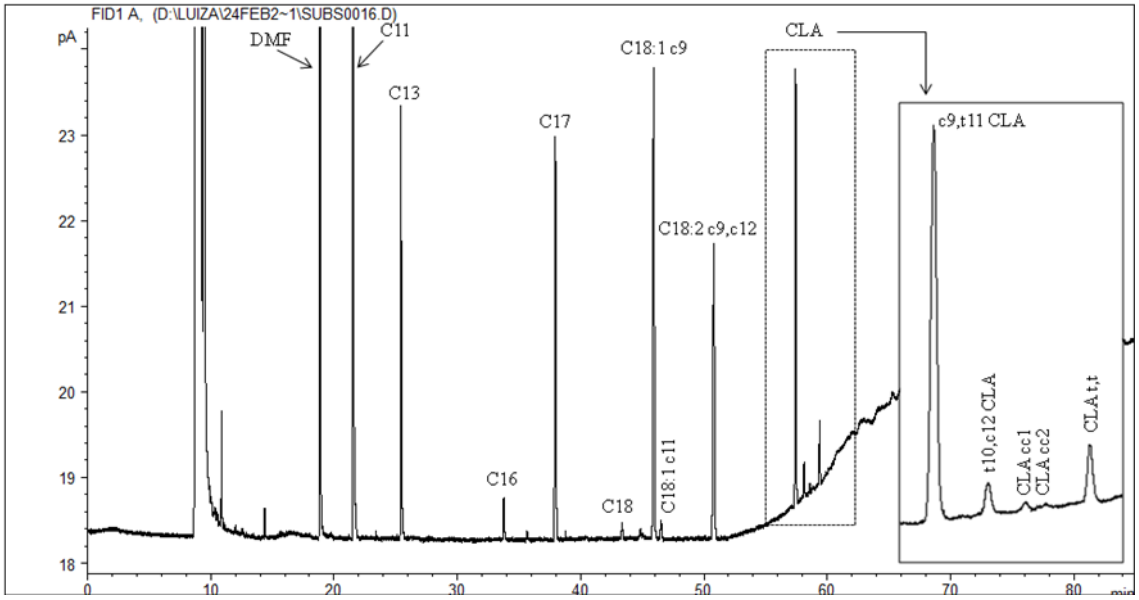
Analysis of supernatants collected from each culture (Table 3.2) revealed overall LA reduction percentages above 40%. These values were significantly higher ($p<0.05$) after 48 h for all strains, except for *L. brevis* D24 whose LA reduction percentages were not different ($p>0.05$) between both incubation times. In what concerns CLA production, values were significantly higher after 24 h of incubation for all the strains tested, except for *B. lactis* B94 for which no CLA production was detected. *Bifidobacterium breve* NCIMB 702258 showed the best CLA-producing ability with values ranging from 139.30 µg/mL at 48 h to 155.83 µg/mL at 24 h (Table 3.2), being C18:2 c9,t11 the major isomer formed. Its capacity to form CLA isomers is proved by the peaks present in the corresponding chromatograms shown in Figure 3.6. Indeed, other studies have also demonstrated *B. breve* strains to be good CLA producers. In their study with other *B. breve* strains (NCFB 2257, NCFB 2258, NCTC 11815, NCIMB 8815 and NCIMB 8807) grown at 37 ºC for 48 h with 0.55 mg/mL of LA, Coakley *et al*. (2003) were also able to detect evident amounts of CLA (122.9-364.0 µg/mL), specifically C18:2 c9,t11. Other isomers such as C18:2 t10,c12 and C18:2 t9,t11 were also identified but at lower concentrations. When grown at 37 ºC for 24 h with 0.5 mg/mL of LA, *B. breve*

ZL12-28, *B. breve* 29M2 and *B. breve* M7-70 also showed ability to produce CLA in Villar-Tajadura *et al*. (2014) study, having reached values of 170.6, 158.7 and 170.3 µg/mL, respectively. The main isomer identified was C18:2 c9,t11, but C18:2 t10,c12 and C18:2 t9,t11 were also detected. In another research work (Hennessy *et al*., 2012), that also studied *B. breve* NCIMB 702258, it was observed a production of 259.0 µg/mL of CLA when the strain was grown at 37 ºC for 72 h with 0.45 mg/mL of LA. The main isomers identified was C18:2 c9,t11, but C18:2 t9,t11 was also present.

No other studies have tested *B. lactis* strains ability to produce CLA isomers, although in the present study it was observed that with *B. lactis* B94 no CLA was detected at both incubation times, even though LA reduction was considerably high. A similar scenario was observed for *L. brevis* D24 and *L. plantarum* D36, which although LAI gene-positive could not produce significant amounts of CLA, similarly to what had been observed with *L. plantarum* 299v (section 3.1). On the other hand, previous reports (Kishino *et al*., 2002b; Xu *et al*., 2008) were able to detect CLA production by *L. brevis* and *L. plantarum* strains, and Kishino *et al*. (2002b) also found HFAs as intermediate metabolites that we have not been able to identify.

In this research, negative controls (with substrate but no strain) were also incubated throughout 24 and 48 h. Fatty acid analysis revealed an LA reduction between 10% and 20% in these controls (data not shown), but in the presence of any strain, those values were much higher (Table 3.2). Therefore, it was hypothesized that it was the presence of the bacteria that caused LA reduction and so we further analyzed the pellets to test whether the substrate was being used by the bacteria.

(a)



(b)

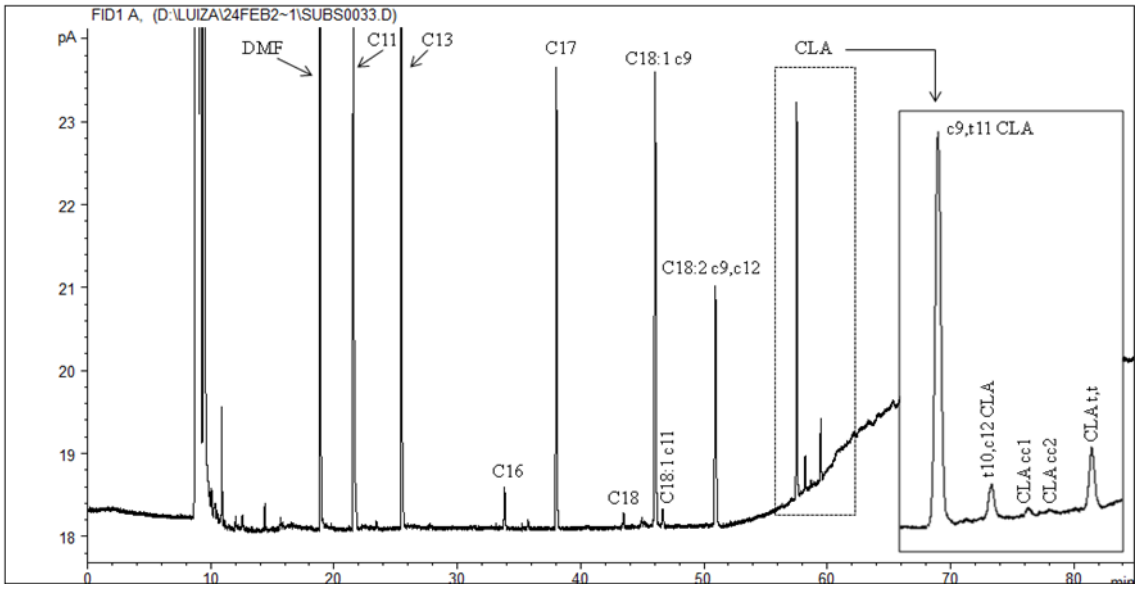


Figure 3.6 Chromatogram profile assessed by gas chromatography of the fatty acids present in the culture supernatant of *B. breve* NCIMB 702258 grown with LA (0.5 mg/mL) for 24 (a) and 48 h (b).

Table 3.3 Fatty acids composition (μg/mg) of selected strains' pellets after 24 and 48 h of incubation in the presence of LA (0.5 mg/mL).

	Strains							
	B. breve NCIMB 702258		*B. lactis* B94		*L. brevis* D24		*L. plantarum* D36	
Fatty acid*	24 h	48 h	24 h	48 h	24 h	48 h	24 h	48 h
C4	2.29 ± 0.11[a]	0.77 ± 0.09[b]	0.44 ± 0.04	0.32 ± <0.01	0.27 ± 0.01	0.28 ± 0.02	0.26 ± <0.01	0.27 ± 0.01
C14	1.25 ± 0.06[a]	0.43 ± 0.03[b]	0.25 ± <0.01	0.21 ± 0.02	0.07 ± <0.01	0.08 ± <0.01	0.07 ± 0.01	0.08 ± <0.01
C16	6.69 ± 0.33[a]	2.28 ± 0.04[b]	1.82 ± 0.02	1.66 ± 0.12	0.65 ± 0.03	0.68 ± 0.08	0.57 ± 0.07[b]	0.69 ± 0.02[a]
C18	0.55 ± 0.03[a]	0.18 ± 0.03[b]	0.16 ± 0.03	0.16 ± 0.01	ND	ND	0.05 ± <0.01	0.06 ± 0.01
C18:1 c9	7.06 ± 0.35[a]	2.47 ± 0.03[b]	1.33 ± 0.05	1.36 ± <0.01	0.63 ± 0.03	0.73 ± 0.08	0.55 ± 0.07[b]	0.73 ± 0.04[a]
C18:1 c11	1.38 ± 0.07[a]	0.46 ± 0.02[b]	0.10 ± 0.01	0.10 ± 0.01	0.11 ± <0.01	0.12 ± 0.01	0.10 ± 0.01	0.13 ± 0.01
C18:2 c9,c12	7.73 ± 0.37[a]	2.68 ± 0.05[b]	2.05 ± 0.11	2.13 ± 0.12	3.94 ± 0.14	4.14 ± 0.31	3.59 ± 0.27[b]	3.99 ± 0.13[a]
C18:2 c9,c15	0.65 ± 0.03[a]	0.21 ± 0.02[b]	ND	ND	ND	ND	ND	ND
cyc 19:0(c9)	0.65 ± 0.03[a]	0.24 ± 0.02[b]	0.81 ± 0.02	0.76 ± 0.04	1.19 ± 0.05	1.20 ± 0.10	1.06 ± 0.11	1.25 ± 0.06
C18:2 c9,t11	6.16 ± 0.31[a]	2.02 ± 0.03[b]	ND	ND	ND	ND	ND	ND
C18:2 CLA tt	0.85 ± 0.04[a]	0.32 ± 0.05[b]	ND	ND	ND	ND	ND	ND
Σ CLA	7.01 ± 0.35[a]	2.34 ± 0.02[b]	ND	ND	ND	ND	ND	ND
Σ FA	35.27 ± 1.76[a]	12.06 ± 0.11[b]	6.97 ± 0.28	6.72 ± 0.29	6.87 ± 0.26	7.23 ± 0.60	6.27 ± <0.01[b]	7.21 ± 0.29[a]

* Average value ± standard deviation.

a, b - Different superscript letters for significant differences ($p<0.05$) between 24 h vs. 48 h.

c = *cis* double bound; t = *trans* double bound; cyc = cyclopropane fatty acid; ∑ = total; CLA = conjugated linoleic acid; FA = fatty acids; ND = not detected.

The analysis to strains' pellet (Table 3.3) showed that LA (C18:2 c9,c12) was present in all of them, being its concentration significantly higher ($p<0.05$) after 48 h in *L. plantarum* D36. Amounts of C16 and C18:1 c9 were also significantly higher in *L. plantarum* D36 after 48 h, which led to a significantly higher total amount of FA (7.21 µg/mg). Although not significant ($p>0.05$) and of low order of magnitude, an increment in LA amount was also observed in *B. lactis* B94 and *L. brevis* D24 after 48 h. This may suggests that the LA added to the medium was absorbed by these strains over time.

Few studies have focused on pellets' analysis, but Jenkins and Courtney (2003) also detected the accumulation of LA in *Lactobacillus* strains, more precisely at the cell membrane, when grown in the presence of this fatty acid. Polyunsaturated fatty acids (PUFAs) like LA are toxic to bacteria (Maia *et al*., 2010; Zheng *et al*., 2005) and LA isomerization consists of a detoxification mechanism (Jiang *et al*., 1998). Rosberg-Cody *et al*. (2007) claimed that the incorporation of PUFAs, namely at the cell membrane, could be part of that mechanism. Therefore, a hypothesis could be that LA is being absorbed into the membranes, isomerized and delivered to the medium. However, CLA isomers were only detected at considerable amounts for *B. breve* NCIMB 702258. Nevertheless, further studies should be undertaken to assure that the strains tested in this work are actually incorporating LA into the cell membrane, since a separation of cell structures was not applied for the lipids analysis. The determination of whether the substrate is being absorbed in a free form or not, would also be of interest.

Bifidobacterium lactis B94, *L. brevis* D24 and *L. plantarum* D36 have revealed LA reduction percentages above 40%, which are equivalent to more than 200 µg/mL of the substrate added to the medium. However, the amount of LA detected in these strains' pellet is very low (between 2.05 and 4.14 µg/mL), therefore the LA reduction verified cannot be explained only by substrate's absorption hypothesis. Besides isomerization, saturation metabolism of PUFAs may also include conversion into other metabolites such as HFAs or oxo fatty acids and partially saturated *trans* fatty acids (Kishino *et al*., 2013). So, it is possible that LA is being transformed into other unknown compounds instead, such as HFAs. Some studies (Kim *et al*., 2003; Kishino *et al*., 2002b) have detected the presence of HFAs with lactic acid bacteria grown in the presence of LA. Ogawa *et al*. (2001) referred that HFAs could be intermediates of LA conversion to CLA. However, in our work, those compounds were not detected.

Usually CLA quantification is performed analyzing the culture supernatant, however, CLA isomers were also identified in the pellet of *B. breve* NCIMB 702258 while none were

detected in the other strains. However, it is unknown if those isomers were absorbed after LA isomerization or if they are naturally present in *B. breve* NCIMB 702258. Therefore, in order to better evaluate LA's influence on fatty acids composition, an analysis of the strains' pellet grown without any substrate was performed in the following experiments.

3.3.1. Addition of LA to the pre-inocula and inocula

Since no significant amount of CLA was being produced by the expected strains, two strategies were undertaken to optimize CLA production using *B. breve* NCIMB 702258, *B. lactis* B94 and *L. brevis* D24. *Lactobacillus plantarum* D36 was excluded because although it had a similar CLA production to *L. brevis* D24 at 24 h, no production was detected after 48 h. *Lactobacillus plantarum* 299v and *B. animalis* subsp. *lactis* Bb12 were also included since their behavior in the presence of linoleic acid was well-known from the optimization of bacterial growth conditions (section 2.3). In the first strategy, beside the inocula, the substrate was also added during pre-inocula preparation at a concentration of 0.05 mg/mL. The purpose was to provide an adaption time to LA before inoculation, so that its presence would not negatively affect strains' activity and LA metabolic conversion could be improved.

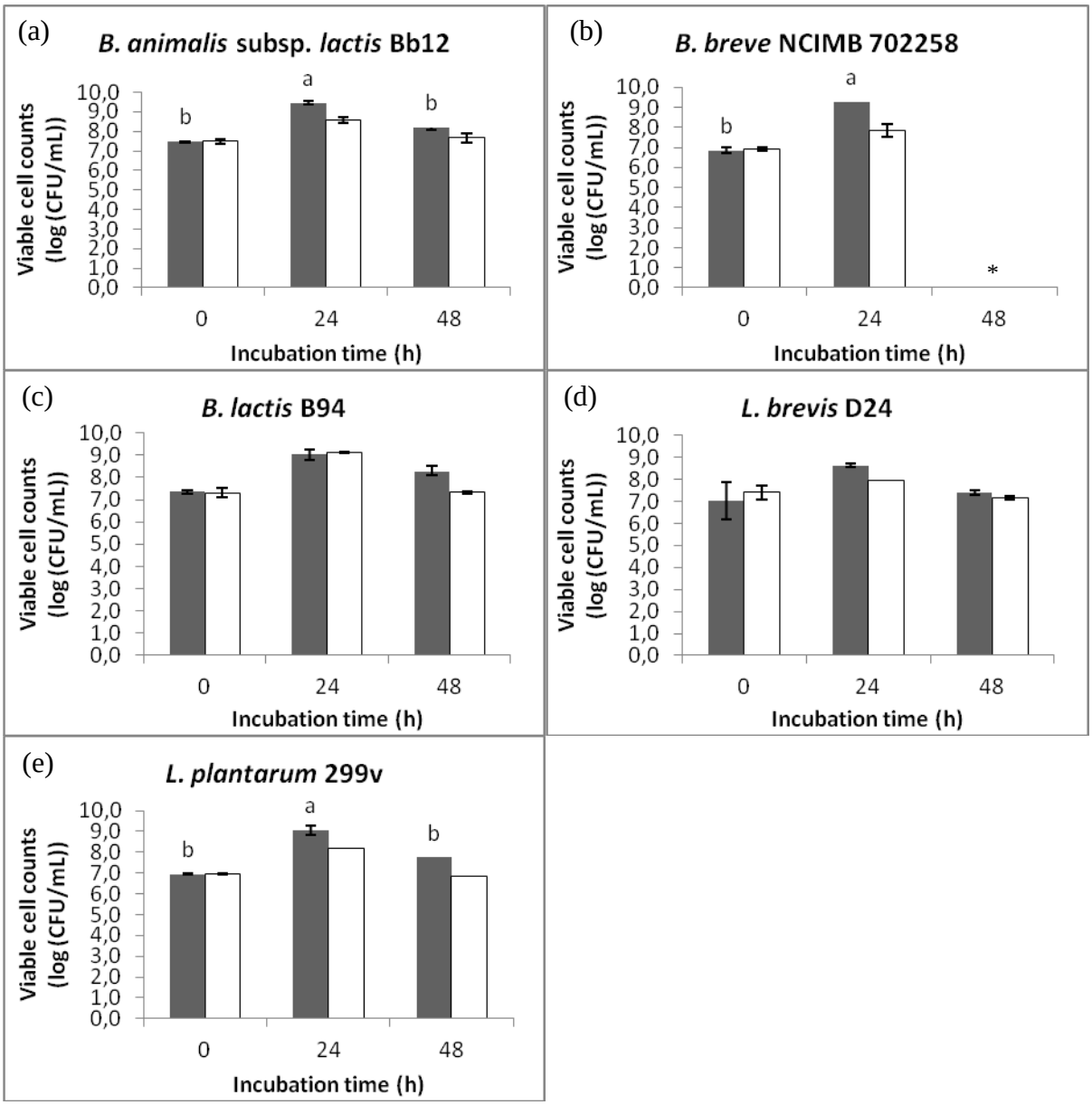


Figure 3.7 Bacterial growth of *B. animalis* subsp. *lactis* Bb12 (a), *B. breve* NCIMB 702258 (b), *B. lactis* B94 (c), *L. brevis* D24 (d) and *L. plantarum* 299v (e) at 0, 24 and 48 h of incubation in the absence (■) and presence (□) of LA added to the pre-inocula (0.05 mg/mL) and inocula (0.5 mg/mL). Results are expressed in log (CFU/mL). Different letters for significant differences ($p<0.05$) between 0 h vs. 24 h vs. 48 h.

* No significant growth was detected.

In the absence of LA, *B. animalis* subsp. *lactis* Bb12, *B. breve* NCIMB 702258 and *L. plantarum* 299v revealed significantly higher ($p<0.05$) viable cell numbers after 24 h of incubation (Figure 3.7), yet after 48 h viable cell numbers were significantly lowered. *B. lactis* B94 and *L. brevis* D24 cell counts tended to be higher after 24 h of incubation. When

LA was added to the culture medium, no differences ($p>0.05$) were observed for all strains assayed, although *B. lactis* B94 viable cell numbers tended to be higher after 24 h. Viable cell numbers were slightly lowered in the presence of LA after 24 and 48 h of incubation for all strains but *B. lactis* B94 at 24 h, which revealed similar cell counts. *Lactobacillus plantarum* 299v and *B. animalis* subsp. *lactis* Bb12 revealed a similar behavior to the one observed in the optimization of bacterial growth conditions (section 3.1). For example, in the case of *B. animalis* subsp. *lactis* Bb12, after 24 h of incubation in the optimization experiment, viable cell counts were of 8.43 and 9.42 log (CFU/mL) (with and without LA, respectively). In this experimental strategy where LA was also added during pre-inocula preparation, the values observed were almost the same, i.e. 8.58 and 9.46 log (CFU/mL) (with and without LA, respectively). Thus, the strategy undertaken in this experiment was not truly successful as far as bacterial growth is concerned.

Table 3.4 LA reduction (%) and CLA formation (µg/mL) by selected strains grown in the presence of LA added to the pre-inocula (0.05 mg/mL) and inocula (0.5 mg/mL) in the culture supernatant collected after 24 and 48 h of incubation.

Strain	Incubation time (h)	LA reduction (%)*	CLA (µg/mL)*
B. animalis subsp. *lactis* Bb12	24	45.90 ± 0.97	0.99 ± 0.05[a]
	48	55.85 ± 1.98	ND[b]
B. breve NCIMB 702258	24	61.25 ± 2.04[b]	145.85 ± 20.65
	48	65.25 ± 1.11[a]	151.10 ± 3.13
B. lactis B94	24	41.64 ± 0.42[b]	0.64 ± 0.03[a]
	48	57.57 ± 0.05[a]	0.40 ± 0.02[b]
L. brevis D24	24	59.09 ± 1.38	ND
	48	60.98 ± 1.26	ND
L. plantarum 299v	24	31.97 ± 2.86[b]	1.69 ± 0.56[a]
	48	42.23 ± 6.28[a]	0.65 ± 0.03[b]

* Average value ± standard deviation.

a, b - Different superscript letters for significant differences ($p<0.05$) between 24 h vs. 48h.

ND = not detected.

The analysis of each culture supernatant (Table 3.4), revealed that the LA was reduced at least by 30% by 24 h incubation and that overall values were significantly higher ($p<0.05$) after 48 h of incubation. CLA production capacity, independently of the order of magnitude, was either similar between both sampling points (in the case of *B. breve* NCIMB 702258) or significantly higher by 24 h in comparison to the values reported by 48 h. The ability of *B. breve* NCIMB 702258 to produce CLA isomers was once more proved in this experiment, as

the concentration of CLA produced ranged from 145.85 µg/mL at 24 h to 151.10 µg/mL at 48 h, being C18:2 c9,t11 the major isomer identified (the corresponding peaks may be seen in the chromatograms of Figure 3.8). In comparison with the results obtained before in the experiments of optimization of bacterial growth conditions (section 3.1) and of CLA production by selected strains (section 3.3), LA reduction percentages and CLA production were not improved in this experiment. In fact, CLA production levels in *L. plantarum* 299v were lowered and *L. brevis* D24 did not produce any amount of CLA, while previously some production had been detected, even though it was not to a considerable extent. The strategy undertaken in this experiment seemed to only work with *B. lactis* B94, as LA reduction was slightly enhanced and the production of CLA isomers was detected, unlike what had been observed in section 3.3.

Providing an adaptation to LA period did not reflect in an improvement of CLA production; indeed, with the exception of *B. breve* NCIMB 702228, a considerable high LA reduction percentage and a low CLA concentration persisted.

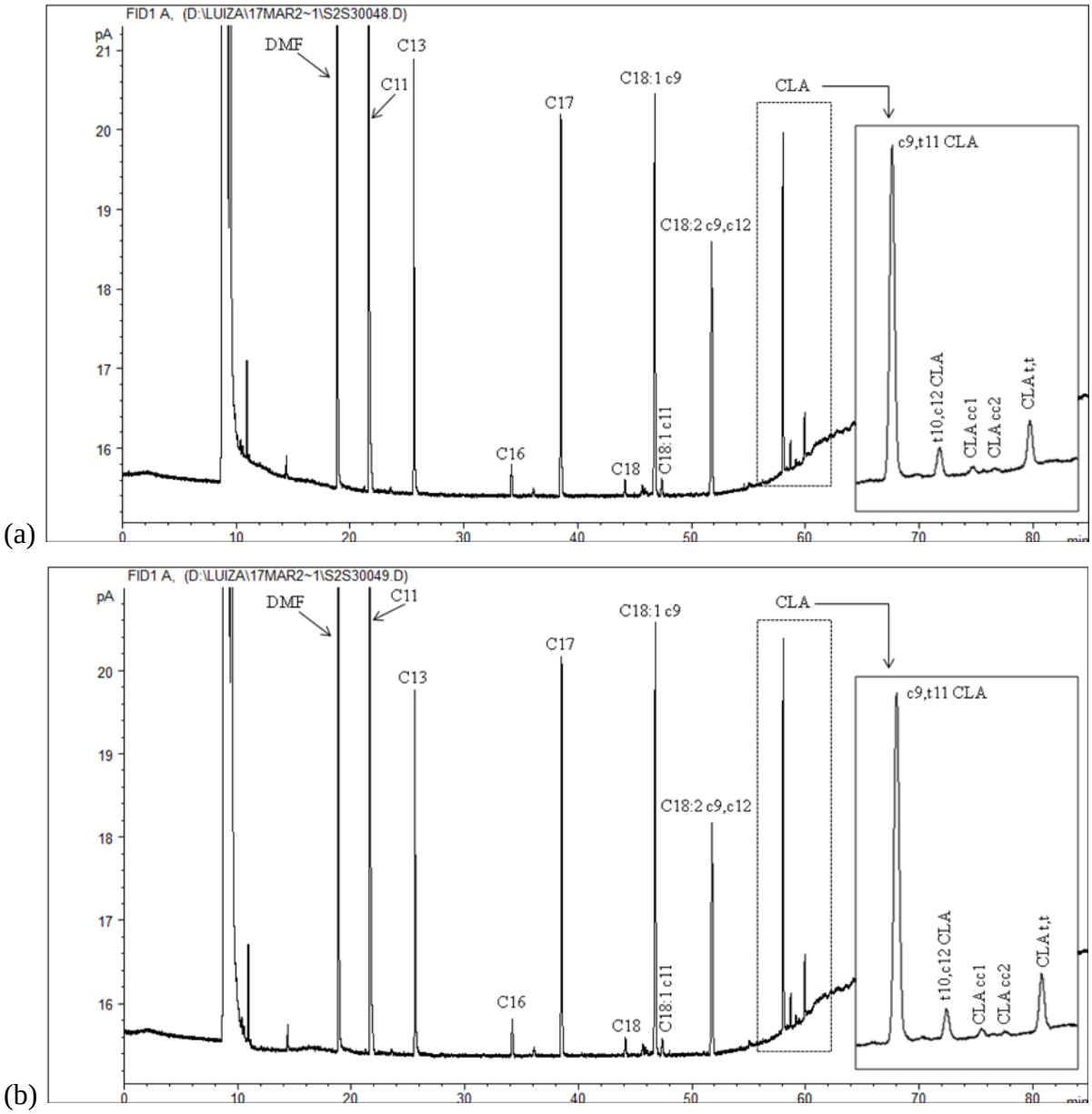


Figure 3.8 Chromatogram profile assessed by gas chromatography of the fatty acids present in the culture supernatant of *B. breve* NCIMB 702258 grown for 24 (a) and 48 h (b) with LA added to the pre-inocula (0.05 mg/mL) and inocula (0.5 mg/mL).

The analysis of the fatty acid composition of the *B. animalis* subsp. *lactis* Bb12 pellet (Table 3.5) revealed that there was an accumulation of LA (C18:2 c9,c12) at both incubation times. Simultaneously, C18:1 c11, cyc 19:0(c9) and cyc 19:0(c11) (lactobacillic acid) were significantly reduced ($p<0.05$) in the presence of LA. After 48 h of incubation with substrate, a significant increase of C18:1 c9, as well of the CLA isomers, such as C18:2 t10,c12 and C18:2 CLA t,t, was observed. The total amount of CLA and FA was significantly higher after 48 h when LA was present in the medium (0.24 µg/mg of CLA and 7.24 µg/mg of FA).

Table 3.5 Fatty acids composition (µg/mg) of *B. animalis* subsp. *lactis* Bb12 pellet after 24 and 48 h of incubation in the absence (LA-) and presence (LA+) of LA added to the pre-inocula (0.05 mg/mL) and inocula (0.5 mg/mL).

	Incubation time			
	24 h		48 h	
Fatty acid*	LA-	LA+	LA-	LA+
C4	0.10 ± 0.05	0.15 ± 0.07	0.12 ± 0.03	0.20 ± 0.02
C14	0.10 ± 0.04	0.07 ± 0.03	0.12 ± 0.03	0.10 ± 0.01
C16	0.39 ± 0.19	0.31 ± 0.15	0.46 ± 0.12	0.48 ± 0.06
C18	0.04 ± 0.03	0.05 ± 0.03	0.07 ± 0.02	0.11 ± 0.02
C18:1 c9	0.74 ± 0.35^{b}	1.31 ± 0.61^{b}	1.11 ± 0.28^{b}	2.41 ± 0.43^{a}
C18:1 c11	0.66 ± 0.30^{a}	0.16 ± 0.07^{b}	0.70 ± 0.17^{a}	0.23 ± 0.04^{b}
C18:2 c9,c12	0.02 ± 0.01^{b}	1.59 ± 0.74^{a}	0.02 ± 0.01^{b}	3.02 ± 0.61^{a}
cyc 19:0(c9)	0.70 ± 0.33^{ab}	0.33 ± 0.14^{bc}	0.78 ± 0.21^{a}	0.44 ± 0.04^{c}
cyc 19:0(c11)	0.17 ± 0.08^{a}	0.02 ± 0.01^{b}	0.18 ± 0.05^{a}	$0.02 \pm <0.01^{b}$
C18:2 c9,t11	0.01 ± 0.01^{a}	0.04 ± 0.02^{a}	0.03 ± 0.01^{a}	0.08 ± 0.02^{a}
C18:2 t10,c12	0.02 ± 0.01^{b}	0.04 ± 0.02^{b}	0.03 ± 0.01^{b}	0.08 ± 0.01^{a}
C18:2 CLA cc1	0.01 ± <0.01	0.01 ± <0.01	0.01 ± <0.01	0.01 ± 0.01
C18:2 CLA cc2	0.01 ± <0.01	0.01 ± <0.01	0.02 ± 0.01	0.02 ± <0.01
C18:2 CLA t,t	0.02 ± 0.01^{b}	0.02 ± 0.01^{b}	$0.02 \pm <0.01^{b}$	0.05 ± 0.01^{a}
Σ CLA	0.07 ± 0.03^{b}	0.12 ± 0.05^{b}	0.10 ± 0.03^{b}	0.24 ± 0.06^{a}
Σ FA	3.00 ± 1.39^{b}	4.11 ± 1.90^{b}	3.66 ± 0.96^{b}	7.24 ± 1.29^{a}

* Average value ± standard deviation.

a, b, c - Different superscript letters for significant differences ($p<0.05$) between absence vs. presence of LA.

c = *cis* double bound; t = *trans* double bound; cyc = cyclopropane fatty acid; ∑ = total; CLA = conjugated linoleic acid; FA = fatty acids.

In the presence of the substrate, LA (C18:2 c9,c12) and C18:2 c9,c15 were detected in the *B. breve* NCIMB 702258 pellet (Table 3.6), but not when the strain was grown without LA. At the same time, after 24 h of incubation in the presence of LA, C4 was significantly enhanced (especially after 48 h), while C18 and cyc 19:0(c9) were significantly reduced ($p<0.05$). After 48 h of incubation in the presence of the substrate, there was an accumulation of LA (18:2 c9,c12) in the pellet and the level of C16, C18:2 c9,c15 and each CLA isomer was significantly higher. In fact, in comparison to the pellet without substrate, the total content of CLA and FA in the pellet with substrate was 40-fold (4.19 µg/mg) and 6-fold (19.23 µg/mg) higher, respectively.

Table 3.6 Fatty acids composition (µg/mg) of *B. breve* NCIMB 702258 pellet after 24 and 48 h of incubation in the absence (LA-) and presence (LA+) of LA added to the pre-inocula (0.05 mg/mL) and inocula (0.5 mg/mL).

	Incubation time			
	24 h		48 h	
Fatty acid*	LA-	LA+	LA-	LA+
C4	0.20 ± 0.15^{bc}	0.34 ± 0.08^{b}	0.17 ± 0.05^{c}	1.07 ± 0.05^{a}
C14	0.38 ± 0.25	0.19 ± 0.03	0.30 ± 0.10	0.70 ± 0.04
C16	1.78 ± 0.31^{b}	1.14 ± 0.22^{b}	1.46 ± 0.45^{b}	3.57 ± 0.18^{a}
C18	0.26 ± 0.06^{a}	0.08 ± 0.06^{b}	0.22 ± 0.06^{a}	0.28 ± 0.01^{a}
C18:1 c9	1.64 ± 0.21^{b}	1.10 ± 0.19^{b}	1.30 ± 0.38^{b}	4.00 ± 0.20^{a}
C18:1 c11	0.12 ± 0.08^{b}	0.08 ± 0.02^{b}	0.09 ± 0.03^{b}	0.26 ± 0.01^{a}
C18:2 c9,c12	ND^{c}	1.34 ± 0.27^{b}	ND^{c}	4.71 ± 0.24^{a}
C18:2 c9,c15	ND^{c}	0.05 ± 0.01^{b}	ND^{c}	0.15 ± 0.01^{a}
cyc 19:0(c9)	0.36 ± 0.26^{a}	0.08 ± 0.01^{b}	0.34 ± 0.11^{a}	0.30 ± 0.02^{a}
C18:2 c9,t11	0.03 ± 0.02^{c}	0.90 ± 0.16^{b}	0.03 ± 0.01^{c}	3.44 ± 0.17^{a}
C18:2 t10,c12	0.05 ± 0.03^{b}	0.04 ± 0.01^{b}	0.04 ± 0.01^{b}	0.16 ± 0.01^{a}
C18:2 CLA cc1	0.01 ± 0.01^{c}	$0.03 \pm <0.01^{b}$	$0.01 \pm <0.01^{c}$	$0.08 \pm <0.01^{a}$
C18:2 CLA cc2	0.01 ± 0.01	$0.01 \pm <0.01$	$0.01 \pm <0.01$	ND
C18:2 CLA t,t	0.04 ± 0.03^{c}	$0.13 \pm <0.01^{b}$	0.03 ± 0.01^{c}	0.51 ± 0.03^{a}
Σ CLA	0.14 ± 0.09^{c}	1.11 ± 0.17^{b}	0.11 ± 0.04^{c}	4.19 ± 0.21^{a}
Σ FA	4.87 ± 0.56^{b}	5.50 ± 1.02^{b}	3.97 ± 1.21^{c}	19.23 ± 0.96^{a}

* Average value ± standard deviation.

a, b, c - Different superscript letters for significant differences ($p<0.05$) between absence vs. presence of LA.

c = *cis* double bound; t = *trans* double bound; cyc = cyclopropane fatty acid; ∑ = total; CLA = conjugated linoleic acid; FA = fatty acids; ND = not detected.

The results obtained for *B. lactis* B94 are shown in Table 3.7. Its pellet revealed a significant reduction ($p<0.05$) of C18:1 c11 and cyc 19:0(c11) in the presence of LA at both incubation times, while C4 was increased. When LA was added to the growth medium, the level of C16 in the pellet was significantly enhanced, especially after 48 h where values were two-fold greater. Results also show an accumulation of LA (C18:2 c9,c12) in the strain's pellet, which content was considerably higher ($p<0.05$) after 48 h. These differences account for the fact that total FA content was considerably higher after 48 h of incubation in the presence of LA (7.15 µg/mg). Nonetheless, the same trend was not observed for total CLA content, which besides being of very low magnitude (in comparison to CLA content reported for *B. breve* NCIMB 702258; Table 3.6) was significantly higher after 48 h without the addition of LA (0.15 µg/mg).

Table 3.7 Fatty acids composition (µg/mg) of *B. lactis* B94 pellet after 24 and 48 h of incubation in the absence (LA-) and presence (LA+) of LA added to the pre-inocula (0.05 mg/mL) and inocula (0.5 mg/mL).

	Incubation time			
	24 h		48 h	
Fatty acid*	LA-	LA+	LA-	LA+
C4	0.09 ± 0.04^{b}	0.18 ± 0.08^{a}	$0.15 \pm <0.01^{b}$	0.24 ± 0.06^{a}
C14	0.10 ± 0.04	0.11 ± 0.05	0.15 ± 0.01	0.17 ± 0.05
C16	0.41 ± 0.17^{d}	0.94 ± 0.44^{b}	0.68 ± 0.02^{c}	1.36 ± 0.33^{a}
C18	0.07 ± 0.03^{b}	0.08 ± 0.04^{b}	0.13 ± 0.01^{a}	0.12 ± 0.02^{ab}
C18:1 c9	0.87 ± 0.34^{b}	0.70 ± 0.34^{b}	1.61 ± 0.03^{a}	1.18 ± 0.21^{ab}
C18:1 c11	0.56 ± 0.23^{a}	0.09 ± 0.03^{b}	0.86 ± 0.02^{a}	0.14 ± 0.02^{b}
C18:2 c9,c12	$0.01 \pm <0.01^{c}$	1.21 ± 0.61^{b}	$0.02 \pm <0.01^{c}$	2.66 ± 0.51^{a}
cyc 19:0(c9)	0.68 ± 0.28^{b}	0.76 ± 0.38^{b}	1.06 ± 0.02^{ab}	1.14 ± 0.28^{a}
cyc 19:0(c11)	0.11 ± 0.04^{a}	0.02 ± 0.01^{b}	$0.16 \pm <0.01^{a}$	0.02 ± 0.01^{b}
C18:2 c9,t11	0.02 ± 0.01	0.02 ± 0.01	$0.03 \pm <0.01$	$0.03 \pm <0.01$
C18:2 t10,c12	0.02 ± 0.01^{b}	0.02 ± 0.01^{b}	$0.04 \pm <0.01^{a}$	$0.04 \pm <0.01^{a}$
C18:2 CLA cc1	$0.01 \pm <0.01$	$0.01 \pm <0.01$	$0.01 \pm <0.01$	$0.01 \pm <0.01$
C18:2 CLA cc2	$0.01 \pm <0.01$	0.01 ± 0.01	$0.02 \pm <0.01$	$0.01 \pm <0.01$
C18:2 CLA t,t	0.02 ± 0.01^{b}	$0.02 \pm <0.01^{b}$	$0.05 \pm <0.01^{a}$	$0.02 \pm <0.01^{b}$
Σ CLA	0.07 ± 0.03^{b}	0.08 ± 0.03^{b}	$0.15 \pm <0.01^{a}$	0.11 ± 0.02^{b}
Σ FA	2.97 ± 0.14^{c}	4.17 ± 0.21^{b}	4.98 ± 0.10^{b}	7.15 ± 1.51^{a}

* Average value ± standard deviation.

a, b, c, d - Different superscript letters for significant differences ($p<0.05$) between absence vs. presence of LA.

c = *cis* double bound; t = *trans* double bound; cyc = cyclopropane fatty acid; ∑ = total; CLA = conjugated linoleic acid; FA = fatty acids.

Significant amounts ($p<0.05$) of LA (18:2 c9,c12) were detected in the *L. brevis* D24 pellet when LA was added to the culture medium (Table 3.8). However, other fatty acids were significantly reduced in the presence of LA, such as C16, C18:1 c9, C18:1 c11, cyc 19:0(c9) and cyc 19:0(c11). Total CLA level, although little, was significantly lower in the presence of LA, independently of the incubation time, and total FA content was not significantly different between incubation time and presence/absence of LA, ranging from 5.14 to 5.57 µg/mg.

Table 3.8 Fatty acids composition (µg/mg) of *L. brevis* D24 pellet after 24 and 48 h of incubation in the absence (LA-) and presence (LA+) of LA added to the pre-inocula (0.05 mg/mL) and inocula (0.5 mg/mL).

	Incubation time			
	24 h		48 h	
Fatty acid*	LA-	LA+	LA-	LA+
C4	0.17 ± 0.04	0.22 ± 0.04	0.16 ± 0.01	0.19 ± 0.01
C14	0.11 ± 0.03	0.07 ± <0.01	0.10 ± <0.01	0.05 ± 0.01
C16	1.14 ± 0.31[a]	0.55 ± 0.16[b]	1.10 ± 0.04[a]	0.53 ± 0.01[b]
C18	0.09 ± 0.03	0.07 ± 0.02	0.10 ± 0.01	0.07 ± <0.01
C18:1 c9	0.77 ± 0.22[a]	0.58 ± 0.20[b]	0.88 ± 0.04[a]	0.54 ± <0.01[b]
C18:1 c11	0.42 ± 0.12[a]	0.11 ± 0.03[b]	0.40 ± 0.02[a]	0.09 ± 0.01[b]
C18:2 c9,c12	0.06 ± 0.02[b]	2.90 ± 0.87[a]	0.06 ± <0.01[b]	2.72 ± 0.01[a]
cyc 19:0(c9)	1.84 ± 0.50[a]	0.96 ± 0.30[b]	1.75 ± 0.09[a]	0.86 ± <0.01[b]
cyc 19:0(c11)	0.55 ± 0.15[a]	0.02 ± <0.01[b]	0.51 ± 0.04[a]	0.03 ± <0.01[b]
C18:2 c9,t11	0.05 ± 0.01	0.02 ± 0.01	0.05 ± <0.01	0.02 ± <0.01
C18:2 t10,c12	0.07 ± 0.01	0.03 ± 0.01	0.07 ± <0.01	0.02 ± <0.01
C18:2 CLA cc1	0.01 ± <0.01	0.01 ± <0.01	0.01 ± <0.01	ND
C18:2 CLA cc2	0.02 ± <0.01	0.01 ± 0.01	0.02 ± <0.01	ND
C18:2 CLA t,t	0.04 ± <0.01	0.02 ± <0.01	0.05 ± <0.01	0.02 ± <0.01
Σ CLA	0.19 ± 0.03[a]	0.09 ± 0.02[b]	0.19 ± 0.01[a]	0.07 ± 0.01[b]
Σ FA	5.34 ± 1.44	5.57 ± 1.64	5.26 ± 0.25	5.14 ± 0.01

* Average value ± standard deviation.

a, b - Different superscript letters for significant differences ($p<0.05$) between absence vs. presence of LA.

c = *cis* double bound; t = *trans* double bound; cyc = cyclopropane fatty acid; ∑ = total; CLA = conjugated linoleic acid; FA = fatty acids; ND = not detected.

The results of *L. plantarum* 299v pellet analysis (Table 3.9) showed a significant reduction ($p<0.05$) of C14 after 24 h of incubation and of C18:1 c9, C18:1 c11 and cyc 19:0(c9) at both incubation times in the presence of LA, whereas C4 was increased. The C16 and LA (C18:2 c9,c12) contents were considerably enhanced ($p<0.05$) in the presence of LA, principally after 48 h of incubation, accounting for the higher total FA content after 48 h in the presence of LA (7.83 µg/mg). The total CLA content detected was not significantly different between incubation times and the presence/absence of LA (0.13-0.20 µg/mg).

Table 3.9 Fatty acids composition (µg/mg) of *L. plantarum* 299v pellet after 24 and 48 h of incubation in the absence (LA-) and presence (LA+) of LA added to the pre-inocula (0.05 mg/mL) and inocula (0.5 mg/mL).

	Incubation time			
	24 h		48 h	
Fatty acid*	LA-	LA+	LA-	LA+
C4	0.13 ± 0.02^{b}	0.20 ± 0.03^{a}	0.15 ± 0.02^{b}	0.27 ± 0.02^{a}
C14	0.14 ± 0.01^{a}	0.08 ± 0.02^{b}	0.16 ± 0.02^{a}	0.13 ± 0.02^{a}
C16	0.52 ± 0.02^{d}	0.84 ± 0.17^{b}	0.62 ± 0.09^{c}	1.07 ± 0.06^{a}
C18	0.07 ± 0.01^{b}	0.06 ± 0.02^{b}	0.09 ± 0.02^{a}	0.09 ± 0.02^{a}
C18:1 c9	1.37 ± 0.04^{a}	0.72 ± 0.10^{c}	1.79 ± 0.26^{a}	1.09 ± 0.04^{b}
C18:1 c11	$0.16 \pm <0.01^{b}$	0.11 ± 0.01^{d}	0.20 ± 0.03^{a}	$0.14 \pm <0.01^{c}$
C18:2 c9,c12	$0.03 \pm <0.01^{c}$	2.81 ± 0.48^{b}	0.04 ± 0.01^{c}	3.66 ± 0.47^{a}
cyc 19:0(c9)	1.34 ± 0.02^{a}	0.96 ± 0.19^{b}	1.57 ± 0.23^{a}	1.19 ± 0.06^{b}
cyc 19:0(c11)	0.03 ± <0.01	0.04 ± <0.01	0.03 ± <0.01	0.04 ± 0.02
C18:2 c9,t11	0.05 ± <0.01	0.03 ± 0.01	0.07 ± 0.01	0.05 ± <0.01
C18:2 t10,c12	0.04 ± <0.01	0.04 ± 0.01	0.05 ± 0.01	0.05 ± <0.01
C18:2 CLA cc1	0.01 ± <0.01	0.01 ± <0.01	0.01 ± <0.01	0.01 ± <0.01
C18:2 CLAcc2	0.02 ± 0.01	0.02 ± <0.01	0.03 ± <0.01	0.02 ± 0.01
C18:2 CLA t,t	0.04 ± <0.01	0.03 ± <0.01	0.04 ± <0.01	0.03 ± 0.01
Σ CLA	0.16 ± 0.02	0.13 ± 0.02	0.20 ± 0.03	0.16 ± <0.01
Σ FA	3.95 ± 0.13^{c}	5.94 ± 1.04^{b}	4.83 ± 0.70^{b}	7.83 ± 0.70^{a}

* Average value ± standard deviation.

a, b, c - Different superscript letters for significant differences ($p<0.05$) between absence vs. presence of LA.

c = *cis* double bound; t = *trans* double bound; cyc = cyclopropane fatty acid; ∑ = total; CLA = conjugated linoleic acid; FA = fatty acids.

In general, when evaluating the results from the pellets' analysis, it is possible to conclude that there was an accumulation of LA in all strains, especially after 48 h of incubation in most cases. This may support the idea made previously (section 3.3) that the strains are absorbing the LA added as a previous step to its isomerization into CLA isomers or alternative metabolic conversion.

On overall, the presence of LA also caused standard changes in certain FA contents, namely, C16 that was systematically enhanced (with exception of *L. brevis* D24) and C18:1 c11 that was consistently reduced, and so were cyc C19:0(9c) (except for *B. lactis* B94) and cyc C19:0(11c). The level of C18:1 c9 was also always affected when LA was present, however the effect was strain-dependent.

In some cases, the total content of CLA isomers was enhanced when LA was added, principally after 48 h, so part of the isomers produced could have been absorbed. The highest levels of CLA isomers (about 20 to 40 times higher) were detected in *B. breve* NCIMB 702258, as expected. The way CLA could be incorporated is unknown, but according to Ogawa *et al*. (2001) CLA is accumulated as intracellular or cell-associated lipids in free form, making it possible for bacteria itself to use it as a CLA source, however, in this case, that would not be viable as the amount of CLA released to the culture medium was much higher. In what concerns *L. brevis* D24 its CLA content was reduced in the presence of LA. What happened to the natural CLA of this strain is unknown, but the fact is that *L. brevis* D24 was really not able to produce any amount of CLA isomers, corroborated through the supernatant analysis.

In what concerns the total FA content, overall it was enhanced in the presence of LA, especially after 48 h, mostly due to the LA accumulated over time.

Taking into account the results obtained from the supernatant and pellets' analyses, it was concluded that the strategy undertaken in this experiment did not improve CLA production. Once more, the results suggested that LA could be being transformed into other unknown compounds, as the amount of LA absorbed (in the pellet) did not correspond to the percentages of LA reduction (in the supernatant) verified.

3.3.2. Addition of LA after 7 h of incubation

As explained in section 3.3.1, two strategies were undertaken in order to tentatively improve CLA production using *B. animalis* subsp. *lactis* Bb12, *B. breve* NCIMB 702258, *B. lactis* B94, *L. brevis* D24 and *L. plantarum* 299v. In the second strategy the LA was only added after 7 h of incubation to minimize the growth inhibition caused by the addition of LA immediately after the inoculation of the strains (Gorissen *et al*., 2010, 2011) and to guarantee that LA is added during a good CLA-producing growth phase, since Van Nieuwenhove *et al*. (2007) observed with dairy bacteria that LA isomerization initiated after the first 4-7 h of incubation and other authors (Coakley *et al*., 2003; Park *et al*., 2009) demonstrated for bifidobacteria strains that CLA formation occurred during the logarithmic to early stationary growth phase (24 h).

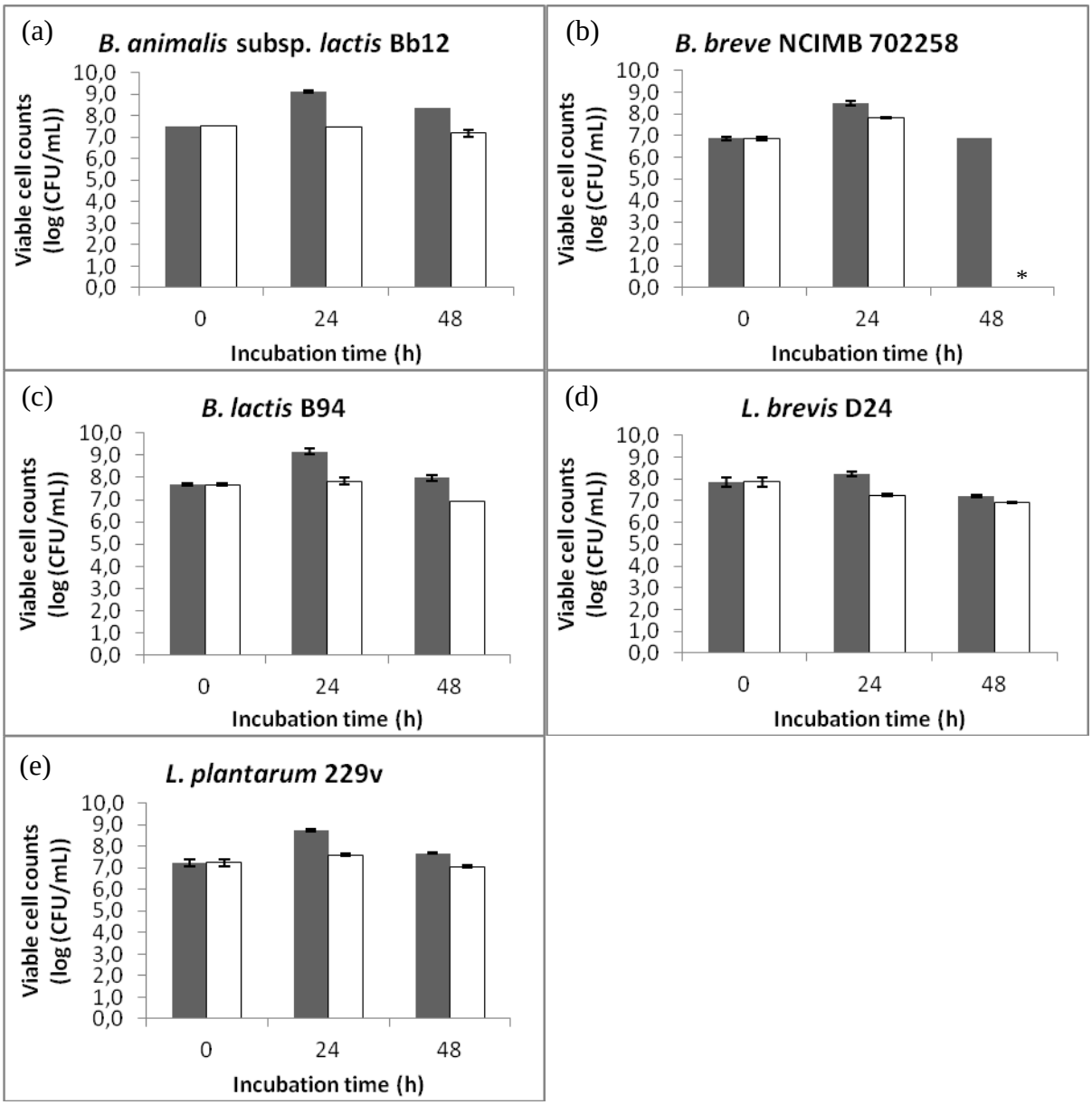


Figure 3.9 Bacterial growth of *B. animalis* subsp. *lactis* Bb12 (a), *B. breve* NCIMB 702258 (b), *B. lactis* B94 (c), *L. brevis* D24 (d) and *L. plantarum* 299v (e) at 0, 24 and 48 h in the absence (■) and presence (□) of LA (0.5 mg/mL) added after 7 h of incubation. Results are expressed in log (CFU/mL).

* No significant growth was detected.

No significant differences ($p>0.05$) in viable cell numbers were detected between 0, 24 and 48 h of incubation for all five strains tested (Figure 3.9), independently of the presence or the absence of LA, with exception of *B. breve* NCIMB 702258, where after 48 h of incubation no significant number of viable cells was detected in the presence of LA. However, in the

absence of LA, bacterial growth tended to be higher after 24 h for all strains but *L. brevis* D24 whose cell counts were similar between incubation times.

Although, in this experiment, LA was only added after 7 h of incubation, there was no improvement on bacterial growth. In fact, comparing to the results obtained in section 3.3.1, where LA was added during pre-inocula preparation, after 24 h bacterial behavior in the presence of LA was similar for both *B. breve* NCIMB 702258 and *L. brevis* D24. Viable cell numbers reported in section 3.3.1 were of 7.85 and 7.97 log (CFU/mL) and in this experiment were of 7.84 and 7.26 log (CFU/mL) for *B. breve* NCIMB 702258 and *L. brevis* D24, respectively. However, in the case of *B. lactis* B94, *B. animalis* subsp. *lactis* Bb12 and *L. plantarum* 299v, viable cell numbers were reduced from 9.10, 8.58 and 8.17 log (CFU/mL) to 7.84, 7.47 and 7.61 log (CFU/mL), respectively. Rosberg-Cody *et al*. (2007) observed with a *L. lactis* strain that even at an advanced growth stage, the strain still showed sensibility to LA, so no matter at what point LA is added bacteria will always be affected by the presence of this fatty acid. This second strategy showed to not be truly effective and when comparing the results, the first strategy (section 3.3.1) showed a better scenario as far as bacterial growth is concerned, so LA should keep being added at least at the beginning of the incubation of the inocula.

Table 3.10 LA reduction (%) and CLA formation (µg/mL) by selected strains grown in the presence of LA (0.5 mg/mL) added after 7 h of incubation in the culture supernatant collected after 24 and 48 h of incubation.

Strain	Incubation time (h)	LA reduction (%)*	CLA (µg/mL)*
B. animalis subsp. *lactis* Bb12	24	44.00 ± 1.33	ND
	48	48.87 ± 3.46	ND
B. breve NCIMB 702258	24	46.69 ± 1.77^{b}	84.30 ± 8.76^{a}
	48	56.67 ± 2.39^{a}	56.55 ± 5.39^{b}
B. lactis B94	24	37.66 ± 2.54	ND
	48	42.42 ± 1.47	ND
L. brevis D24	24	46.49 ± 2.53	ND
	48	49.83 ± 2.12	ND
L. plantarum 299v	24	34.74 ± 2.85	ND
	48	41.57 ± 3.16	ND

* Average value ± standard deviation.

a, b - Different superscript letters for significant differences ($p<0.05$) between 24h vs. 48 h.

ND = not detected.

The analysis of the collected supernatants (Table 3.10) showed an overall LA reduction above 34%, being significantly higher ($p<0.05$) after 48 h for *B. breve* NCIMB 702258. For the remaining strains the values were just slightly higher after 48 h (Table 3.10). In this experiment, only *B. breve* NCIMB 702258 was able to produce CLA at detectable levels. Moreover, the production of *B. breve* NCIMB 702258 was reduced to half of the amount obtained through the first strategy, ranging from 56.55 µg/mL at 48 h to 84.30 µg/mL at 24 h, being C18:2 c9,t11 the major isomer produced (its corresponding peaks can be seen in the chromatograms of Figure 3.10). Therefore, CLA production was not improved through this second strategy, and even LA reduction percentages were lower (34.74-56.67%) when comparing with the results obtained before in the experiments of optimization of bacterial growth conditions (section 3.1) (51.28-69.28%), of CLA production by selected strains (section 3.3) (40.36-71.14%) and even of the first strategy (section 3.3.1) (31.97-65.25%).

For the reasons explained at the beginning of this section, LA was added during the best CLA-producing growth phase, which corresponds to a bacterial stage of highest cell density. Hennessy *et al*. (2009) observed with *B. breve* NCIMB 702258 that a high cell density leads to an increased isomerization capacity, but according to Gorissen *et al*. (2012a) a fast growth is no guarantee for a good conversion of LA into CLA for all strains. In our study, it is possible that the addition of LA only after 7 h of incubation could have provoked an abrupt exposure of this fatty acid to the strains. The initial exposure to LA provided in the previous experiments seems to be essential for the strains, because, even if CLA production was not considerable, with exception of *B. breve* NCIMB 702258, at least they were able to produce CLA isomers, while in this experiment CLA was not detected at all. Therefore, taking into account CLA production and also bacterial growth results from both strategies, LA began to be added simultaneous with the beginning of the incubation of the inocula in the following set of experiments.

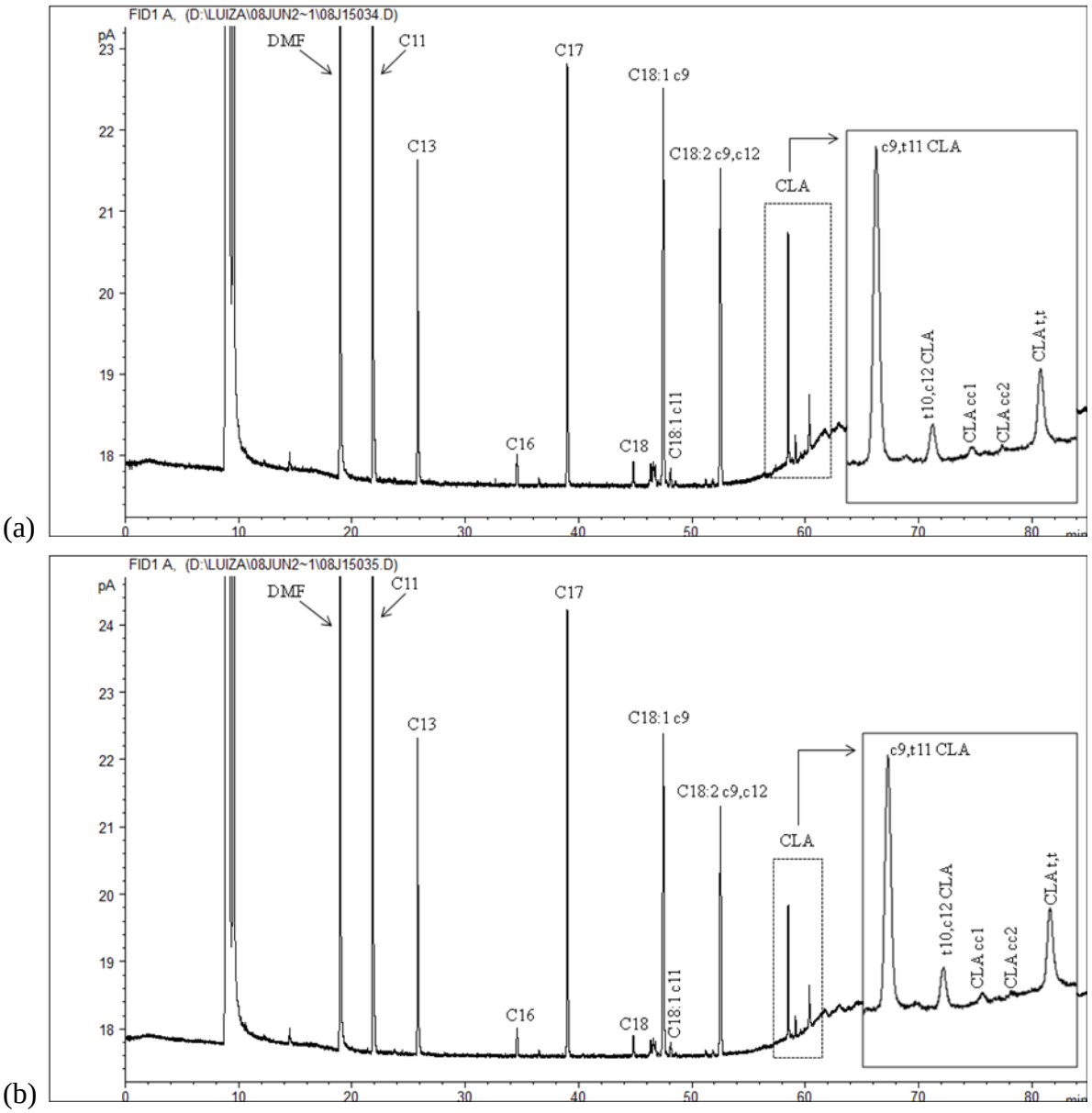


Figure 3.10 Chromatogram profile assessed by gas chromatography of the fatty acids present in the culture supernatant of *B. breve* NCIMB 702258 grown for 24 (a) and 48 h (b) with LA (0.5 mg/mL) added after 7 h of incubation.

Regarding *B. animalis* subsp. *lactis* Bb12 pellet after growth (Table 3.11), in the presence of LA there was a significant increase ($p<0.05$) of C16, C18, C18:1 c9 and C18:1 c11 at both incubation times. Cyc 19:0(c9) level was significantly higher after 24 h in the presence of LA. Linoleic acid (18:2 c9,c12) was detected in the strain's pellet when LA was added to the culture medium and its amount was significantly higher after 48 h. The content of CLA isomers such as C18:2 c9,t11 and C18:2 t10,c12 increased considerably ($p<0.05$) in the presence of LA leading to total CLA values of 0.16 µg/mg at 24 h and 0.19 µg/mg at 48 h.

Total FA content was significantly higher at both incubations times in the presence of added LA (7.03 µg/mg at 24 h and 7.61 µg/mg at 48 h).

Table 3.11 Fatty acids composition (µg/mg) of *B. animalis* subsp. *lactis* Bb12 pellet grown for 24 and 48 h in the absence (LA-) and presence (LA+) of LA (0.5 mg/mL) added after 7 h of incubation.

	Incubation time			
	24 h		48 h	
Fatty acid*	LA-	LA+	LA-	LA+
C14	0.09 ± <0.01	0.15 ± 0.02	0.10 ± 0.02	0.13 ± <0.01
C16	0.40 ± 0.01^{b}	0.70 ± 0.06^{a}	0.50 ± 0.10^{b}	0.71 ± 0.06^{a}
C18	0.09 ± 0.01^{b}	0.18 ± 0.01^{a}	0.11 ± 0.02^{b}	0.20 ± 0.03^{a}
C18:1 c9	0.71 ± <0.01^{b}	1.95 ± 0.23^{a}	1.01 ± 0.23^{b}	2.17 ± 0.19^{a}
C18:1 c11	0.67 ± 0.01^{b}	1.03 ± 0.11^{a}	0.78 ± 0.16^{b}	1.01 ± 0.10^{a}
C18:2 c9,c12	ND^{c}	1.76 ± 0.16^{b}	ND^{c}	2.17 ± 0.22^{a}
cyc 19:0(c9)	0.68 ± 0.01^{b}	0.91 ± 0.09^{a}	0.80 ± 0.19^{a}	0.86 ± 0.06^{a}
cyc 19:0(c11)	0.18 ± 0.01	0.19 ± 0.02	0.21 ± 0.05	0.18 ± 0.01
C18:2 c9,t11	0.01 ± <0.01^{c}	0.04 ± <0.01^{b}	0.02 ± <0.01^{c}	0.06 ± <0.01^{a}
C18:2 t10,c12	0.01 ± <0.01^{b}	0.05 ± <0.01^{a}	0.02 ± <0.01^{b}	0.05 ± <0.01^{a}
C18:2 CLA cc1	0.01 ± <0.01	0.01 ± <0.01	0.01 ± <0.01	0.01 ± <0.01
C18:2 CLA cc2	0.01 ± <0.01	0.02 ± <0.01	0.01 ± <0.01	0.01 ± <0.01
C18:2 CLA t,t	0.02 ± <0.01	0.04 ± 0.01	0.02 ± <0.01	0.06 ± 0.01
Σ CLA	0.05 ± <0.01^{c}	0.16 ± <0.01^{a}	0.08 ± 0.01^{b}	0.19 ± 0.02^{a}
Σ FA	2.87 ± 0.04^{c}	7.03 ± 0.71^{a}	3.59 ± 0.77^{b}	7.61 ± 0.68^{a}

* Average value ± standard deviation.

a, b, c - Different superscript letters for significant differences ($p<0.05$) between absence vs. presence of LA.

c = *cis* double bound; t = *trans* double bound; cyc = cyclopropane fatty acid; ∑ = total; CLA = conjugated linoleic acid; FA = fatty acids; ND = not detected.

The presence of added LA caused a significant increment ($p<0.05$) of C18:1 c9 and C18:1 c11 in *B. breve* NCIMB 702258 pellet (Table 3.12) after 24 and 48h. When LA was added to the culture medium, LA (C18:2 c9,c12) was detected in the strain's pellet. CLA isomers C18:2 c9,t11, C18:2 t10,c12, and C18:2 CLA t,t were significantly enhanced in the presence of LA at both incubation times. Total CLA and FA contents were significantly higher with LA being present (0.99 µg/mg at 24 h and 0.93 µg/mg at 48 h for total CLA; 7.24 µg/mg at 24 h and 7.50 µg/mg at 48 h for total FA).

Table 3.12 Fatty acids composition (µg/mg) of *B. breve* NCIMB 702258 pellet grown for 24 and 48 h in the absence (LA-) and presence (LA+) of LA (0.5 mg/mL) added after 7 h of incubation.

	Incubation time			
	24 h		48 h	
Fatty acid*	LA-	LA+	LA-	LA+
C14	0.16 ± 0.03	0.29 ± 0.01	0.20 ± 0.04	0.29 ± 0.04
C16	1.16 ± 0.25	1.55 ± 0.03	1.42 ± 0.30	1.59 ± 0.21
C18	0.30 ± 0.07	0.37 ± 0.01	0.36 ± 0.08	0.38 ± 0.06
C18:1 c9	1.39 ± 0.28^{b}	2.15 ± 0.07^{a}	1.71 ± 0.31^{ab}	2.23 ± 0.20^{a}
C18:1 c11	0.08 ± 0.02^{b}	0.17 ± 0.01^{a}	0.11 ± 0.02^{b}	0.17 ± 0.04^{a}
C18:2 c9,c12	ND^{b}	1.55 ± 0.08^{a}	ND^{b}	1.73 ± 0.19^{a}
cyc 19:0(c9)	0.21 ± 0.02	0.16 ± 0.01	0.27 ± 0.05	0.16 ± 0.01
C18:2 c9,t11	$0.02 \pm <0.01^{b}$	0.71 ± 0.15^{a}	$0.03 \pm <0.01^{b}$	0.65 ± 0.09^{a}
C18:2 t10,c12	0.03 ± 0.01^{b}	$0.06 \pm <0.01^{a}$	0.03 ± 0.01^{b}	$0.06 \pm <0.01^{a}$
C18:2 CLA cc1	0.01 ± <0.01	0.02 ± <0.01	0.01 ± <0.01	0.02 ± <0.01
C18:2 CLA cc2	0.01 ± <0.01	0.01 ± <0.01	0.02 ± <0.01	0.01 ± <0.01
C18:2 CLA t,t	0.03 ± 0.01^{b}	0.19 ± 0.03^{a}	0.04 ± 0.01^{b}	0.18 ± 0.04^{a}
Σ CLA	0.09 ± 0.01^{b}	0.99 ± 0.20^{a}	0.13 ± 0.02^{b}	0.93 ± 0.13^{a}
Σ FA	3.40 ± 0.69^{b}	7.24 ± 0.25^{a}	4.20 ± 0.83^{b}	7.50 ± 0.86^{a}

* Average value ± standard deviation.

a, b, c,- Different superscript letters for significant differences ($p<0.05$) between absence vs. presence of LA.

c = *cis* double bound; t = *trans* double bound; cyc = cyclopropane fatty acid; ∑ = total; CLA = conjugated linoleic acid; FA = fatty acids; ND = not detected.

In what concerns to *B. lactis* B94 pellet (Table 3.13), the presence of the substrate lead to a significant reduction of cyc 19:0(c11) at both incubation times, while C16, C18 and C18:1 c9 were considerably increased ($p<0.05$) after 48 h. Amounts of LA (C18:2 c9,c12) were detected in strain's pellet, being the value considerably higher after 48 h. Total amount of CLA isomers was not significantly different ($p>0.05$) between incubation times and presence/absence of LA (0.19-0.27 µg/mg), but total FA content was significantly higher with LA, especially after 48 h (9.48 µg/mg).

Table 3.13 Fatty acids composition (µg/mg) of *B. lactis* B94 pellet grown for 24 and 48 h in the absence (LA-) and presence (LA+) of LA (0.5 mg/mL) added after 7 h of incubation.

	Incubation time			
	24 h		48 h	
Fatty acid*	LA-	LA+	LA-	LA+
C14	0.43 ± 0.09	0.43 ± 0.05	0.49 ± 0.12	0.52 ± 0.01
C16	0.79 ± 0.14^{b}	0.72 ± 0.03^{b}	0.92 ± 0.25^{ab}	0.93 ± 0.05^{a}
C18	0.13 ± 0.02^{c}	0.19 ± 0.03^{b}	0.18 ± 0.07^{bc}	0.25 ± 0.04^{a}
C18:1 c9	0.93 ± 0.16^{c}	1.47 ± 0.04^{b}	1.08 ± 0.26^{b}	2.01 ± 0.05^{a}
C18:1 c11	0.24 ± 0.04	0.19 ± 0.01	0.28 ± 0.08	0.24 ± <0.01
C18:2 c9,c12	ND^{c}	2.10 ± 0.05^{b}	ND^{c}	3.06 ± 0.22^{a}
cyc 19:0(c9)	1.73 ± 0.34^{b}	1.74 ± 0.12^{b}	2.00 ± 0.44^{a}	2.15 ± 0.02^{a}
cyc 19:0(c11)	0.13 ± 0.02^{a}	0.04 ± <0.01^{b}	0.15 ± 0.04^{a}	0.05 ± <0.01^{b}
C18:2 c9,t11	0.06 ± 0.01	0.06 ± 0.01	0.07 ± 0.02	0.07 ± <0.01
C18:2 t10,c12	0.05 ± 0.01	0.06 ± <0.01	0.06 ± 0.01	0.07 ± <0.01
C18:2 CLA cc1	0.01 ± <0.01	0.01 ± <0.01	0.02 ± 0.01	0.02 ± <0.01
C18:2 CLA cc2	0.02 ± <0.01	0.02 ± <0.01	0.02 ± 0.01	0.02 ± <0.01
C18:2 CLA t,t	0.05 ± 0.02^{b}	0.06 ± <0.01^{b}	0.05 ± 0.02^{b}	0.09 ± <0.01^{a}
Σ CLA	0.19 ± 0.05	0.20 ± <0.01	0.23 ± 0.07	0.27 ± 0.01
Σ FA	4.56 ± 0.87^{c}	7.08 ± 0.23^{b}	5.33 ± 1.34^{c}	9.48 ± 0.41^{a}

* Average value ± standard deviation.

a, b, c - Different superscript letters for significant differences ($p<0.05$) between absence vs. presence of LA.

c = *cis* double bound; t = *trans* double bound; cyc = cyclopropane fatty acid; ∑ = total; CLA = conjugated linoleic acid; FA = fatty acids; ND = not detected.

In the case of *L. brevis* D24 pellet (Table 3.14), C18:1 c9 was considerably increased ($p<0.05$) in the presence of LA at both incubation times. Results show the detection of LA (C18:2 c9,c12) when the substrate was present. No significant differences ($p>0.05$) were found among the total CLA content, ranging from 0.13 to 0.19 µg/mg, but total FA was considerably higher when the substrate was added (6.77 µg/mg at 24 h and 6.68 µg/mg at 48 h).

Table 3.14 Fatty acids composition (µg/mg) of *L. brevis* D24 pellet grown for 24 and 48 h in the absence (LA-) and presence (LA+) of LA (0.5 mg/mL) added after 7 h of incubation.

	Incubation time			
	24 h		48 h	
Fatty acid*	LA-	LA+	LA-	LA+
C14	0.10 ± 0.01	0.11 ± 0.02	0.10 ± 0.01	0.10 ± 0.01
C16	1.07 ± 0.23	1.15 ± 0.21	1.14 ± 0.03	1.12 ± 0.08
C18	0.12 ± 0.02	0.16 ± 0.04	0.14 ± 0.01	0.16 ± 0.01
C18:1 c9	0.59 ± 0.14^{c}	0.94 ± 0.17^{a}	0.77 ± 0.02^{b}	0.94 ± 0.07^{a}
C18:1 c11	0.40 ± 0.09	0.44 ± 0.07	0.43 ± 0.01	0.43 ± 0.02
C18:2 c9,c12	ND^{b}	1.80 ± 0.48^{a}	ND^{b}	1.81 ± 0.12^{a}
cyc 19:0(c9)	1.46 ± 0.30	1.55 ± 0.29	1.55 ± 0.04	1.51 ± 0.13
cyc 19:0(c11)	0.49 ± 0.11	0.43 ± 0.08	0.51 ± 0.01	0.41 ± 0.03
C18:2 c9,t11	0.04 ± 0.01	0.05 ± 0.01	0.04 ± <0.01	0.05 ± <0.01
C18:2 t10,c12	0.05 ± 0.01	0.06 ± 0.01	0.05 ± <0.01	0.06 ± <0.01
C18:2 CLA cc1	0.01 ± 0.01	0.01 ± <0.01	0.01 ± <0.01	0.01 ± <0.01
C18:2 CLA cc2	0.01 ± 0.01	0.01 ± <0.01	0.01 ± <0.01	0.01 ± <0.01
C18:2 CLA t,t	0.03 ± <0.01	0.06 ± 0.01	0.05 ± 0.02	0.06 ± 0.01
Σ CLA	0.13 ± 0.04	0.19 ± 0.04	0.16 ± 0.02	0.19 ± 0.01
Σ FA	4.35 ± 0.95^{b}	6.77 ± 1.40^{a}	4.80 ± 0.15^{b}	6.68 ± 0.48^{a}

* Average value ± standard deviation.

a, b, c - Different superscript letters for significant differences ($p<0.05$) between absence vs. presence of LA.

c = *cis* double bound; t = *trans* double bound; cyc = cyclopropane fatty acid; ∑ = total; CLA = conjugated linoleic acid; FA = fatty acids; ND = not detected.

Concerning to *L. plantarum* 299v pellet (Table 3.15), the presence of LA in the culture medium caused a significant increase of C16. Linoleic acid (C18:2 c9,c12) was detected in the strain's pellet when the substrate was present. Total CLA amount was not significantly different ($p>0.05$) between the situations tested (0.13-0.18 µg/mg), while the level of total FA was considerably higher ($p<0.05$) in the presence of LA (6.23 µg/mg at 24 h and 6.61 µg/mg at 48 h).

Table 3.15 Fatty acids composition (µg/mg) of *L. plantarum* 299v pellet grown for 24 and 48 h in the absence (LA-) and presence (LA+) of LA (0.5 mg/mL) added after 7 h of incubation.

	Incubation time			
	24 h		48 h	
Fatty acid*	LA-	LA+	LA-	LA+
C14	0.14 ± 0.01	0.15 ± 0.01	0.15 ± 0.01	0.16 ± 0.04
C16	0.49 ± 0.04[b]	0.72 ± 0.05[a]	0.53 ± 0.03[b]	0.77 ± 0.14[a]
C18	0.11 ± <0.01	0.16 ± 0.02	0.13 ± 0.01	0.18 ± <0.01
C18:1 c9	1.28 ± 0.11	1.60 ± 0.12	1.50 ± 0.11	1.71 ± 0.27
C18:1 c11	0.16 ± 0.01	0.16 ± 0.01	0.18 ± 0.01	0.17 ± 0.03
C18:2 c9,c12	ND[b]	2.17 ± 0.18[a]	ND[b]	2.27 ± 0.40[a]
cyc 19:0(c9)	1.27 ± 0.13	1.07 ± 0.07	1.34 ± 0.12	1.13 ± 0.20
cyc 19:0(c11)	0.02 ± <0.01	0.03 ± <0.01	0.02 ± <0.01	0.04 ± 0.01
C18:2 c9,t11	0.04 ± <0.01	0.05 ± <0.01	0.05 ± <0.01	0.06 ± 0.02
C18:2 t10,c12	0.03 ± <0.01	0.04 ± <0.01	0.04 ± <0.01	0.04 ± 0.01
C18:2 CLA cc1	0.01 ± <0.01	0.01 ± <0.01	0.01 ± <0.01	0.01 ± <0.01
C18:2 CLA cc2	0.02 ± <0.01	0.02 ± <0.01	0.02 ± <0.01	0.02 ± 0.01
C18:2 CLA t,t	0.03 ± 0.01	0.04 ± <0.01	0.04 ± <0.01	0.05 ± <0.01
Σ CLA	0.13 ± 0.01	0.16 ± 0.01	0.16 ± 0.01	0.18 ± 0.03
Σ FA	3.62 ± 0.31[b]	6.23 ± 0.47[a]	4.03 ± 0.30[b]	6.61 ± 1.11[a]

* Average value ± standard deviation.

a, b - Different superscript letters for significant differences ($p<0.05$) between absence vs. presence of LA.

c = *cis* double bound; t = *trans* double bound; cyc = cyclopropane fatty acid; ∑ = total; CLA = conjugated linoleic acid; FA = fatty acids; ND = not detected.

Evaluating the results from the pellets' analysis, once more the accumulation of LA was observed when such substrate was added to the culture medium, being that value higher after 48 h of incubation for two of the three *Bifidobacterium* strains tested (i.e. *B. animalis* subsp. *lactis* Bb12 and *B. lactis* B94). These results strengthen the hypothesis made from the results of the previous experiments (sections 3.3 and 3.3.1), i.e. LA may be absorbed to be further converted into CLA isomers or other unknown compounds.

Similar to the first strategy (section 3.3.1), in this experiment there was a standard group of certain fatty acids that were generally affected by the presence of LA, namely C16, C18, C18:1 c9 and C18:1 t11, whose values were enhanced. In the first strategy, C19:0(c9) was, in general, reduced when the substrate was added to the culture medium. However, in this experiment, it was not found a standard for the way C19:0(c9) was affected. C19:0(c11) was only significantly ($p<0.05$) reduced for *B. lactis* B94.

Concerning to total CLA content, *B. breve* NCIMB 702258 revealed higher levels when LA was present. Therefore, as in the first strategy, part of the CLA isomers produced were probably absorbed by this strain. However, a substantial amount of CLA continues to be detected in the supernatant. No significant changes ($p>0.05$) on total CLA were observed for *B. lactis* B94, *L. brevis* D24 and *L. plantarum* 299v, but no CLA isomers were detected in the supernatant also. On the other hand, *B. animalis* subsp. *lactis* Bb12, which was also not able to produce CLA at detectable levels in the supernatant, revealed significantly higher amounts of total CLA when LA was present. The possible explanation for this fact is that all LA converted by this strain was absorbed instead of being released to the medium.

Total FA was considerably higher in the presence of LA for all strains, due to the accumulation of the substrate and the associated increment of the fatty acids mentioned above.

Regarding the results from supernatants and pellets' analysis, it is possible to conclude that none of the strategies applied have improved CLA production; indeed, the values from the second strategy were even worse than the first one. If the strains are only able to produce other compounds than CLA, then no matter what strategy it is applied no improvement in CLA production will be seen.

Despite the failure in CLA production optimization, a CLA-producer strain, *B. breve* NCIMB 702258, was found. Therefore, this strain was selected for further studies concerning the potential CLNA production.

3.4. CLA and CLNA production with *Bifidobacterium breve* NCIMB 702258

Among the strains tested in this study, *B. breve* NCIMB 702258 showed the capacity to convert LA into CLA isomers, therefore the following step was to investigate whether this strain could also produce CLNA isomers. Thus, the strain was grown for 24 h in the presence of α-LNA, γ-LNA and LA+α-LNA+γ-LNA.

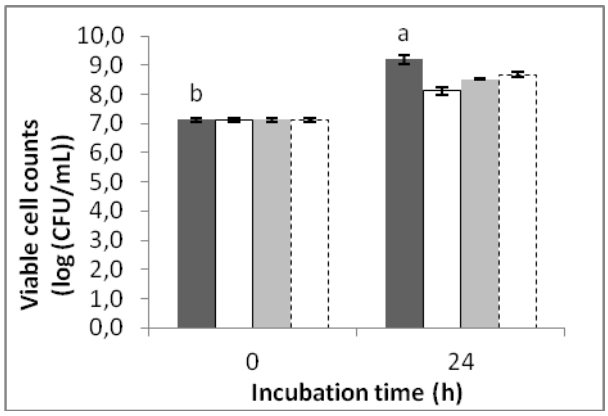


Figure 3.11 Bacterial growth of *B. breve* NCIMB 702258 at 0 and 24 h of incubation in the absence (■) and presence of 0.5 mg/mL of α-LNA (□), γ-LNA (▨) and LA+α-LNA+γ-LNA (1:1:1) (▦) when grown in cys-MRS medium. Results are expressed in log (CFU/mL). Different letters for significant differences ($p<0.05$) between 0 h vs. 24 h.

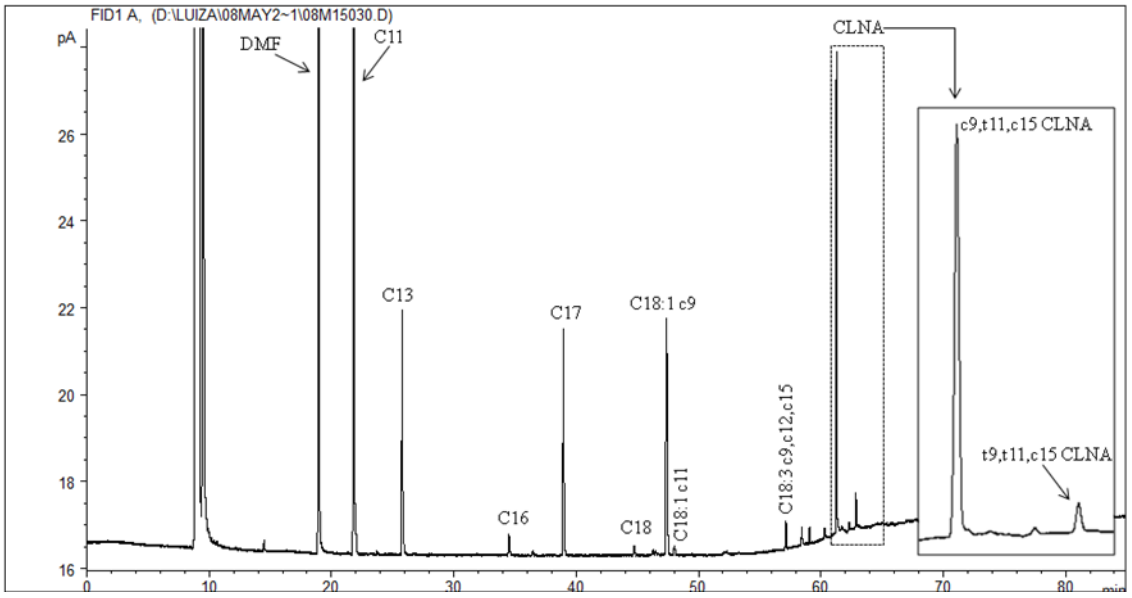
After 24 h of incubation, the number of *B. breve* NCIMB 702258 viable cells was significantly higher ($p<0.05$) in the absence of substrate (9.19 log (CFU/mL)) (Figure 3.11), while in the presence of any of the substrates the values were not significantly different ($p>0.05$), in comparison to those added at the beginning of the incubation (0 h), although in the presence of LA+α-LNA+γ-LNA cell numbers tended to be higher after 24 h. Viable cell numbers were slightly lowered when the precursor substrates were added to the culture medium, specially α-LNA. Villar-Tajadura *et al*. (2014) have also detected some reduction in *B. breve* M7-70 viable cell numbers with 0.5 mg/mL of α-LNA, while Gorissen *et al*. (2010) observed that among 36 *Bifidobacterium* strains, bacterial growth inhibition was only detected in 14 of these strains in the presence of 0.5 mg/mL of α-LNA. Since the α-LNA concentration used was always the same, *Bidifobacterium* strains behavior in the presence of α-LNA seems to differ according to the strain, as it was previously observed for LA.

When *B. breve* NCIMB 702258 was grown with α-LNA, 94.96% of this substrate was reduced and ca. 293.28 μg/mL of CLNA isomers were detected (data not shown), corresponding to C18:3 c9,t11,c15 and C18:3 t9,t11,c15. In the presence of γ-LNA the reduction rate was of 23.48% but no CLNA isomers were detected. When all three substrates were present, the reduction percentages were of 26.01% for LA, 72.61% for α-LNA and 18.95% for γ-LNA, being produced ca. 55.22 μg/mL of CLA (mainly C18:2 c9,t11) and 207.89 μg/mL of CLNA, corresponding to C18:3 c9,t11,c15 and C18:3 t9,t11,c15. *Bifidobacterium breve* NCIMB 702258 capacity to form CLNA isomers can be confirmed by

the peaks revealed in the corresponding chromatograms presented in Figure 3.12. In the study carried out by Coakley *et al*. (2009), *B. breve* NCIMB 702258 also produced high amounts of CLNA (mostly C18:3 c9,t11,c15) when grown at 37 ºC for 42 h with 0.41 mg/mL of α-LNA, where the conversion rate was of 79.1%. Similar results were obtained by Hennessy *et al*. (2012) through the incubation of this same strain at 37 ºC for 72 h with 0.45 mg/mL of α-LNA, where it was detected the production of 199 µg/mL of CLNA isomers, corresponding to C18:3 c9,t11,c15 and C18:3 t9,t11,c15. However, they were also able to detect, at the same conditions, the production of 149 µg/mL of CLNA in the presence of γ-LNA, while in our experiment no isomers were detected using this substrate.

Comparing these results to those obtained in CLA production with selected strains (section 3.3), after 24 h, *B. breve* NCIMB 702258 was able to produce more CLNA (293.28 µg/mL) than CLA (155.83 µg/mL), as LNA reduction rate (94.96%) was also higher than that of LA (66.13%). Moreover, when the three substrates were added together to the culture medium, similar results were observed, i.e. α-LNA reduction rate (72.61%) and CLNA concentration (207.89 µg/mL) were higher than LA reduction rate (26.01%) and CLA concentration (55.22 µg/mL). Other studies (Gorissen *et al*., 2010; Villar-Tajadura *et al*., 2014) have also detected higher conversion rates of LNA than LA when added separately. Jiang *et al*. (1998) have hypothesized that the reaction involved in LA and LNA conversion could be a detoxification mechanism of the bacteria. Therefore the results of this experiment suggest that α-LNA might be more toxic than LA.

(a)



(b)

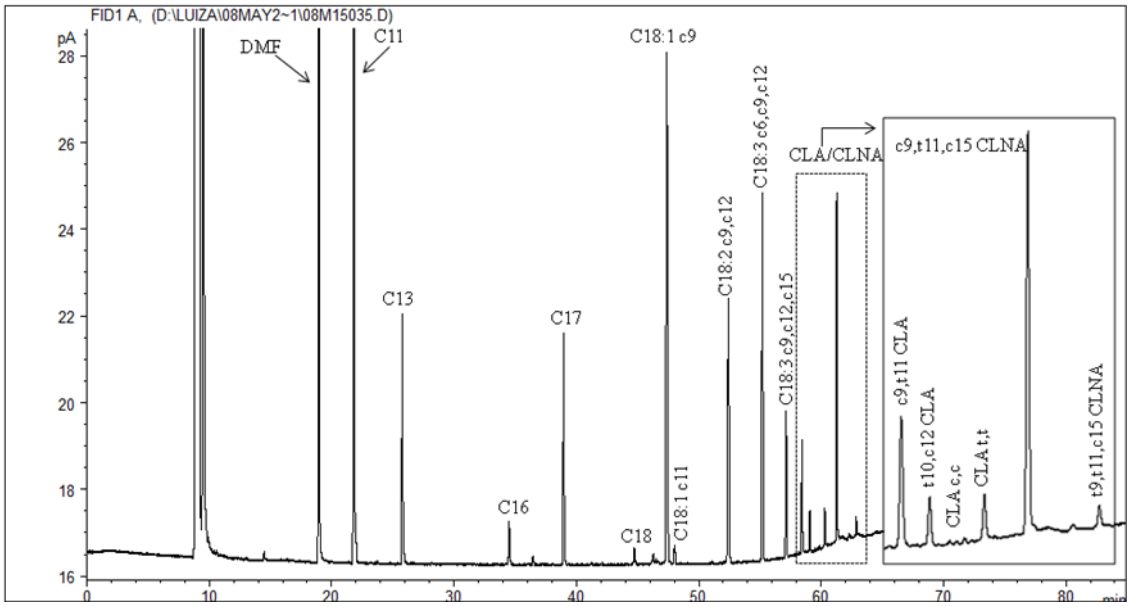


Figure 3.12 Chromatogram profile assessed by gas chromatography of the fatty acids present in the culture supernatant of *B. breve* NCIMB 702258 grown for 24 h with 0.5 mg/mL of α-LNA (a) and LA+α-LNA+γ-LNA (1:1:1) (b).

Table 3.16 Fatty acids composition (μg/mg) of *B. breve* NCIMB 702258 pellet after 24 h of incubation in the absence and presence of 0.5 mg/mL of α-LNA, γ-LNA and LA+α-LNA+γ-LNA (1:1:1).

	Substrate			
Fatty acid*	Absent	α-LNA	γ-LNA	LA+α-LNA+γ-LNA
C14	0.41 ± 0.02	0.23 ± 0.11	0.09 ± 0.01	0.11 ± 0.04
C16	1.96 ± 0.05^{b}	2.31 ± 0.12^{a}	1.53 ± 0.08^{c}	1.86 ± 0.09^{b}
C18	0.30 ± 0.01	0.21 ± 0.13	0.14 ± 0.01	0.19 ± 0.10
C18:1 c9	2.08 ± 0.13^{a}	1.53 ± 0.75^{a}	0.80 ± 0.04^{b}	0.86 ± 0.38^{b}
C18:1 c11	0.14 ± 0.01^{b}	0.23 ± 0.14^{a}	0.09 ± 0.01^{c}	0.11 ± 0.04^{b}
C18:2 c9,c12	ND	ND	ND	0.78 ± 0.37
cyc 19:0(c9)	0.37 ± 0.02	0.18 ± 0.11	0.15 ± 0.01	0.12 ± 0.06
C18:3 c6,c9,c12	ND^{c}	ND^{c}	0.83 ± 0.02^{a}	0.64 ± 0.03^{b}
C18:3 c9,c12,c15	ND^{b}	0.23 ± 0.11^{a}	ND^{b}	0.24 ± 0.14^{a}
C18:2 c9,t11	0.03 ± 0.01^{b}	ND^{c}	ND^{c}	0.23 ± 0.13^{a}
C18:2 t10,c12	0.04 ± 0.01^{a}	ND^{b}	ND^{b}	0.05 ± 0.03^{a}
C18:2 CLA t,t	0.06 ± 0.01^{b}	ND^{c}	ND^{c}	0.09 ± 0.04^{a}
C18:3 c6,c9,t11	ND	ND	ND	ND
C18:3 c6,t9,t11	ND	ND	ND	ND
C18:3 c9,t11,c15	ND^{c}	1.79 ± 0.78^{a}	ND^{c}	0.55 ± 0.27^{b}
C18:3 t9,t11,c15	ND^{c}	0.25 ± 0.09^{a}	ND^{c}	0.14 ± 0.07^{b}
Σ CLA	0.16 ± 0.02^{b}	ND^{c}	ND^{c}	0.37 ± 0.19^{a}
Σ CLNA	ND^{c}	2.04 ± 0.87^{a}	ND^{c}	0.69 ± 0.34^{b}
Σ FA	5.40 ± 0.26^{a}	6.96 ± 3.35^{a}	3.63 ± 0.02^{b}	5.95 ± 2.84^{a}

* Average value ± standard deviation.

a, b, c - Different superscript letters for significant differences ($p<0.05$) between absence vs. presence of α-LNA, γ-LNA or LA+α-LNA+γ-LNA.

c = *cis* double bound; t = *trans* double bound; cyc = cyclopropane fatty acid; ∑ = total; CLA = conjugated linoleic acid; CLNA = conjugated linolenic acid; FA = fatty acids; ND = not detected.

As it was seen in the previous experiments (sections 3.3.1 and 3.3.2), the levels of certain fatty acids, such as C16, C18:1 c9 and C18:1 c11, were also affected in the presence of the substrates tested in this experiment (Table 3.16). Comparing the fatty acid composition values of *B. breve* NCIMB 702258 grown with no substrate, C16 and C18:1 c11 amounts were significantly enhanced ($p<0.05$) in the presence of α-LNA, while C18:1 c9 content was not different ($p>0.05$). In the presence of γ-LNA, the same fatty acids were significantly reduced. When the three substrates were added together, no significant alterations in C16 and C18:1 c11 levels were observed, while C18:1 c9 content was considerably lower ($p<0.05$).

These results also show an accumulation of each substrate in the strain's pellet. Since *B. breve* NCIMB 702258 demonstrated the ability to produce CLA and CLNA isomers, such absorption must be related to the desintoxification mechanism, as hypothesized by Rosberg-Cody *et al*. (2007).

Comparing the values of *B. breve* NCIMB 702258 pellet fatty acid composition in the absence of substrate, total amount of CLA was significantly increased in the presence of the three substrates (0.37 µg/mg) and the total content of CLNA was significantly enhanced in the presence of α-LNA (2.04 µg/mg). This may suggest that a fraction of the isomers produced was absorbed by the strain. However, the majority of CLA and CLNA isomers formed continues to be released into the culture medium. The total FA content was not significantly different between the conditions tested (5.40-6.69 µg/mg), except when γ-LNA was added individually, where the value was significantly lower (3.63 µg/mg), since not only was there a significant reduction of C16, C18:1 c9 and C18:1 c11 but also no CLA and CLNA isomers were detected.

3.5. CLA and CLNA production in milk with *Bifidobacterium breve* NCIMB 702258

Besides CLA, *B. breve* NCIMB 702258 revealed capacity to produce CLNA isomers. Due to CLA and CLNA bioactive potential as functional ingredients, it was of interest to test if *B. breve* NCIMB 702258 could also produce those isomers in a food matrix. Therefore, this strain was grown in commercial 1.6% reduced-fat milk for 24 h with either LA, α-LNA or both substrates.

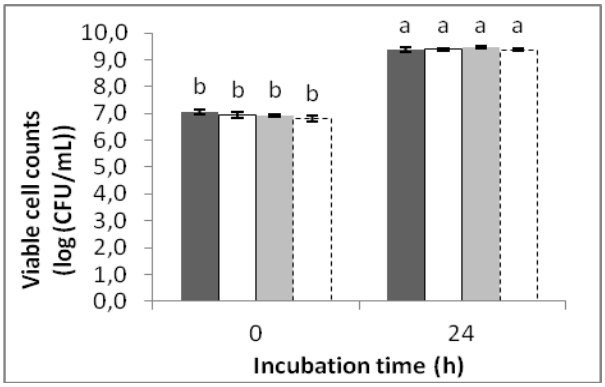


Figure 3.13 Bacterial growth of *B. breve* NCIMB 702258 at 0 and 24 h of incubation in the absence (■) and presence of 0.5 mg/mL of LA (□), α-LNA (▨) and LA+α-LNA (1:1) (⬚) when grown in 1.6% reduced-fat milk. Results are expressed in log (CFU/mL). Different letters for significant differences ($p<0.05$) between 0 h vs. 24 h.

After 24 h of incubation, there was a significant increase ($p<0.05$) of more than two log cycles in the viable cell numbers of *B. breve* NCIMB 702258 independently of the absence or presence of the different substrates added (cell numbers varied from 6.71-7.05 log (CFU/mL) to 9.38-9.47 log (CFU/mL)) (Figure 3.13). Indeed, viable cell numbers after 24 h of incubation were similar between all substrate situations tested. Some other studies (Gorissen *et al*., 2012b; Van Nieuwenhove *et al*., 2007) also observed that the growth of different bifidobacteria strains in milk was not affected by the presence of LA and α-LNA, when comparing to the growth behavior with no substrate added. In the previous experiment (section 3.4), where the strain was grown in cys-MRS, it was detected a slight decrease in cell numbers in the presence of any substrate. Therefore, milk demonstrated to be a more suitable growth medium for this bifidobacteria strain, a somehow expected trait given the protective effect of milk protein on bifidobacteria stability and viability (Gomes and Malcata, 1998; Madureira *et al*., 2011).

Table 3.17 Substrate reduction (%) and CLA/CLNA formation (μg/mL) by *B. breve* NCIMB 702258 grown in 1.6% reduced-fat milk added with either 0.5 mg/mL of LA, α-LNA or both substrates (1:1) for 24 h of incubation.

Substrate	Substrate reduction (%)*	CLA (μg/mL)*	CLNA (μg/mL)*
LA	45.98 ± 3.89	94.86 ± 6.99	ND
α-LNA	53.76 ± 4.20	ND	114.03 ± 9.40
LA + α-LNA	22.15 ± 0.87 / 28.62 ± 1.48	108.88 ± 5.59	61.01 ± 4.12

* Average value ± standard deviation.

ND = not detected.

Evaluating the milk samples collected after 24 h of incubation in the presence of LA (Table 3.17), the substrate reduction rate was of 45.98%, being produced 94.86 μg/mL of CLA. When α-LNA was used as precursor substrate, the percentage of substrate reduction was higher (53.76%) as well as the concentration of CLNA isomers formed (114.03 μg/mL), which may support the previously mentioned hypothesis that α-LNA could be more toxic than LA. However, when both substrates were present, the amount of CLA isomers formed (108.88 μg/mL) was higher than those of CLNA (61.01 μg/mL). The possible explanation for this fact, could be that, in the presence of LA, α-LNA is converted to CLA isomers instead of CLNA counterparts, as already suggested by other studies (Jouany *et al*., 2007; Lee and Jenkins, 2011; Loor *et al*., 2005). Figure 3.14 shows the fatty acids peaks revealed for the milk samples analyzed in this experiment.

Some studies (Chung *et al*., 2008; Van Nieuwenhove *et al*., 2007) were also able to detect CLA production with *B. bidifum* CRL1399 and *B. breve* LMC 520 in milk based media with 0.2 mg/mL and 0.5 mg/mL of LA, respectively. After 24 h of incubation at 37 ºC, *B. bidifum* CRL1399 produced 190.2 µg/mL of CLA and *B. breve* LMC 520 formed 113 µg/mL after 48 h at 37 ºC. Our research is the first to show microbial production of CLA and CLNA in a food matrix in the presence of the corresponding precursor substrates.

Hennessy *et al*. (2009) observed that with *B. breve* NCIMB 702258 the production of CLA in a 20% (w/v) reconstituted skim milk was significantly lower than in cys-MRS culture medium. Similar results were obtained in our study, for both CLA and CLNA, where under the same growth conditions (24 h at 37 ºC with added 0.5 mg/mL of LA or α-LNA), the CLA amount was reduced from 155.83 µg/mL (section 3.3) to 94.86 µg/mL and CLNA from 293.23 µg/mL (section 3.4) to 114.03 µg/mL. Therefore, for a future elaboration of CLA- and CLNA-enriched foodstuffs, the production in milk will need to be improved.

Despite the reduction in CLA and CLNA production values in milk, *B. breve* NCIMB 702258 demonstrated a great potential for the development of enriched food products. However, to obtain an edible product, apart from the sensorial parameters, that need to be optimized, the source of LA and α-LNA must be other than free fatty acids, since pure LA and α-LNA are not allowed as food additives, due to their potential toxicity at high levels (Roden, 2004; Wyne, 2003). Some studies (Choi *et al*., 2008; Chung *et al*., 2008; Xu *et al*., 2004) have tested CLA production in milk-based media using esterified LA, however, in most cases the concentration of CLA isomers detected was lower than with pure LA or an hydrolyzed LA source. Literature studies have included the study of sunflower, canola, castor and safflower oils added to milk-based media as source of LA (Kishino *et al*., 2002a; Rodríguez-Alcalá *et al*., 2011).

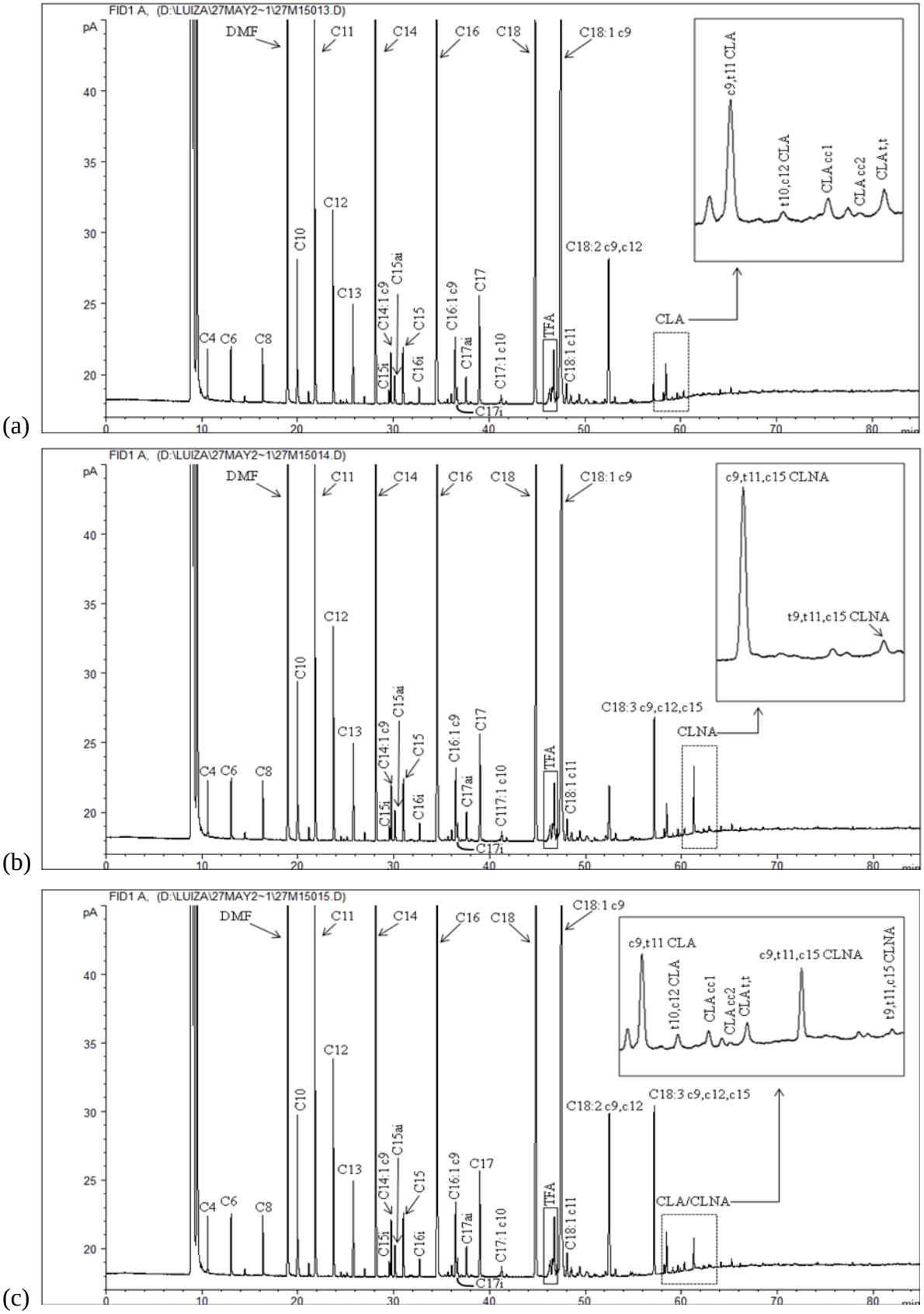


Figure 3.14 Chromatogram profile assessed by gas chromatography of the fatty acids present in the cultures of *B. breve* NCIMB 702258 grown in 1.6% reduced-fat milk for 24 h with 0.5 mg/mL of LA (a), α-LNA (b) or both substrates (1:1) (c).

Table 3.18 Fatty acids composition (μg/mL) of the 1.6% reduced-fat milk used (control) and of the milk cultured with *B. breve* NCIMB 702258 during 24 h in the absence and presence of either 0.5 mg/mL of LA, α-LNA or both substrates (1:1).

	Milk samples				
Fatty acid*	Control	Strain	Strain+LA	Strain+α-LNA	Strain+LA+α-LNA
C4	92.36 ± 0.97^{a}	74.99 ± 5.67^{bc}	66.61 ± 0.96^{c}	70.87 ± 4.70^{bc}	79.14 ± 0.20^{b}
C6	107.78 ± 1.50^{a}	86.67 ± 7.59^{c}	76.80 ± 0.49^{d}	82.88 ± 4.62^{c}	93.74 ± 0.03^{b}
C8	100.24 ± 0.63^{a}	84.06 ± 6.69^{bc}	72.43 ± 2.25^{d}	77.49 ± 6.13^{cd}	85.59 ± 1.18^{b}
C10	314.45 ± 2.25^{a}	263.27 ± 24.93^{bc}	228.80 ± 3.08^{c}	242.19 ± 13.83^{c}	273.03 ± 2.12^{b}
C12	433.39 ± 3.28	361.76 ± 36.17	312.61 ± 7.50	332.61 ± 27.71	371.56 ± 3.31
C14	1549.81 ± 8.32^{a}	1307.93 ± 130.74^{b}	1127.51 ± 30.98^{c}	1197.32 ± 99.76^{c}	1339.43 ± 5.80^{b}
C15i	37.18 ± 1.00	31.87 ± 3.24	27.88 ± 0.47	29.35 ± 3.41	31.86 ± 0.99
C14:1 c9	131.08 ± 2.85^{a}	110.01 ± 11.01^{bc}	96.03 ± 2.22^{c}	102.08 ± 10.33^{bc}	112.24 ± 1.27^{b}
C15ai	81.31 ± 1.25^{a}	68.75 ± 8.29^{bc}	58.34 ± 2.20^{c}	62.94 ± 5.81^{c}	72.12 ± 0.86^{b}
C15	155.16 ± 0.92^{a}	131.87 ± 12.34^{bc}	113.33 ± 2.79^{c}	121.20 ± 11.68^{c}	135.19 ± 0.55^{b}
C16i	43.02 ± 0.26^{a}	36.50 ± 3.61^{b}	31.95 ± 2.71^{c}	34.29 ± 3.72^{bc}	37.26 ± 0.49^{b}
C16	4263.39 ± 26.18^{a}	3619.49 ± 357.14^{bc}	3124.84 ± 83.48^{d}	3314.30 ± 284.04^{cd}	3719.77 ± 15.52^{b}
C16:1 c9	197.31 ± 3.01^{a}	166.07 ± 17.19^{bc}	143.38 ± 5.43^{d}	153.77 ± 13.93^{cd}	173.27 ± 1.68^{b}
C17i	53.46 ± 1.38^{a}	45.39 ± 3.96^{b}	38.38 ± 2.81^{c}	41.40 ± 4.66^{b}	46.57 ± 1.12^{b}
C17ai	89.00 ± 2.82^{a}	75.72 ± 8.90^{bc}	65.57 ± 3.08^{d}	69.37 ± 7.17^{cd}	79.65 ± 0.02^{b}
C17:1 c10	34.69 ± 2.27^{a}	29.19 ± 2.68^{b}	24.45 ± 0.35^{c}	27.95 ± 1.88^{b}	30.92 ± 0.38^{b}
C18	1419.67 ± 9.34^{a}	1202.45 ± 120.57^{bc}	1038.38 ± 28.66^{d}	1101.99 ± 89.23^{cd}	1235.45 ± 2.45^{b}
C18:1 t4	5.44 ± 0.08^{a}	4.57 ± 0.77^{a}	3.23 ± 0.14^{b}	3.93 ± 0.57^{b}	4.95 ± 0.99^{a}
C18:1 t5	7.45 ± 1.36	5.49 ± 1.28	6.15 ± 0.11	5.39 ± 0.85	7.08 ± 1.25
C18:1 t6-t9	76.18 ± 0.75^{a}	69.61 ± 8.99^{ab}	63.99 ± 2.62^{b}	62.76 ± 4.93^{b}	75.67 ± 0.59^{a}
C18:1 t10	55.78 ± 0.99	49.93 ± 6.82	44.51 ± 2.75	45.56 ± 3.74	52.87 ± 1.27

* Average value ± standard deviation.

a, b, c, d - Different superscript letters for significant differences ($p<0.05$) between milk samples.

c = *cis* double bound; t = *trans* double bound; i = iso; ai = anteiso.

Table 3.18 (continued) Fatty acids composition (μg/mL) of the 1.6% reduced-fat milk used (control) and of the milk cultured with *B. breve* NCIMB 702258 during 24 h in the absence and presence of either 0.5 mg/mL of LA, α-LNA or both substrates (1:1).

	Milk samples				
Fatty acid*	Control	Strain	Strain+LA	Strain+α-LNA	Strain+LA+α-LNA
C18:1 t11	213.34 ± 1.84^{a}	180.80 ± 19.64^{bc}	159.57 ± 3.31^{d}	164.92 ± 13.97^{cd}	188.44 ± 0.23^{b}
C18:1 t12	52.70 ± 1.19^{a}	44.94 ± 6.56^{bc}	40.42 ± 2.29^{c}	41.92 ± 2.28^{c}	50.20 ± 0.66^{ab}
C18:1 c9	2760.62 ± 21.02^{a}	2320.69 ± 233.14^{b}	2087.81 ± 59.65^{c}	2227.76 ± 193.05^{b}	2591.34 ± 0.57^{b}
C18:1 t15	28.77 ± 1.00	26.70 ± 5.28	25.65 ± 0.24	25.01 ± 1.16	28.57 ± 1.51
C18:1 c11	81.85 ± 2.61^{a}	67.62 ± 8.75^{b}	63.50 ± 2.12^{b}	66.96 ± 6.53^{b}	78.12 ± 2.55^{a}
C18:2 c9,c12	219.55 ± 1.09^{b}	179.91 ± 18.84^{c}	431.38 ± 15.26^{a}	172.92 ± 11.44^{c}	526.62 ± 3.41^{a}
C18:2 c9,c15	25.02 ± 1.67^{a}	19.82 ± 2.64^{b}	17.94 ± 1.56^{c}	19.04 ± 1.06^{b}	20.46 ± 0.55^{b}
cyc 19:0(c9)	8.60 ± 0.03	10.36 ± 1.34	8.04 ± 2.46	8.94 ± 2.16	9.28 ± 1.41
C18:3 c6,c9,c12	7.74 ± 0.09	7.19 ± 1.14	7.30 ± 1.56	6.83 ± 0.76	6.97 ± 1.14
C18:3 c9,c12,c15	53.59 ± 1.75^{c}	42.88 ± 5.15^{d}	37.90 ± 1.45^{e}	241.33 ± 17.06^{b}	352.83 ± 6.20^{a}
C18:2 c9,t11	82.67 ± 2.55^{a}	61.64 ± 1.26^{d}	72.66 ± 5.09^{bc}	67.34 ± 7.85^{cd}	87.01 ± 4.66^{a}
C18:2 t10,c12	3.63 ± 0.64^{d}	3.96 ± 1.40^{cd}	4.83 ± 0.30^{c}	6.33 ± 0.73^{b}	11.31 ± 1.52^{a}
C18:2 CLA cc1	15.65 ± 0.62	12.51 ± 2.03	11.39 ± 0.63	11.70 ± 0.83	12.88 ± 0.22
C18:2 CLA cc2	2.44 ± 0.08	2.57 ± 0.31	1.83 ± 0.32	2.84 ± 0.52	2.53 ± 0.03
C18:2 CLA t,t	12.70 ± 0.49^{b}	10.18 ± 0.96^{b}	13.81 ± 1.26^{b}	11.89 ± 1.92^{b}	20.39 ± 0.40^{a}
C18:3 c9,t11,c15	4.49 ± 0.24^{c}	3.33 ± 0.17^{d}	4.02 ± 0.53^{c}	106.12 ± 5.83^{a}	53.86 ± 4.34^{b}
C18:3 t9,t11,c15	2.26 ± 0.83^{c}	2.69 ± 0.61^{c}	2.71 ± 0.15^{c}	9.14 ± 3.56^{a}	7.71 ± 0.22^{b}
∑ CLA	117.09 ± 4.38^{b}	90.87 ± 5.95^{c}	104.52 ± 6.99^{c}	100.11 ± 11.86^{c}	134.13 ± 5.59^{a}
∑ CLNA	6.75 ± 0.59^{c}	6.03 ± 0.78^{c}	6.73 ± 0.68^{c}	115.26 ± 9.40^{a}	61.57 ± 4.12^{b}
∑ FA	12823.80 ± 98.44^{a}	10823.38 ± 541.17^{b}	9755.93 ± 277.33^{c}	10370.63 ± 518.53^{bc}	12105.89 ± 2.84^{a}

* Average value ± standard deviation.

a, b, c, d, e - Different superscript letters for significant differences ($p<0.05$) between milk samples.

c = *cis* double bound; t = *trans* double bound; cyc = cyclopropane fatty acid; ∑ = total; CLA = conjugated linoleic acid; CLNA = conjugated linolenic acid; FA = fatty acids.

Concerning the levels of each fatty acid identified in the milk samples (Table 3.18), it is possible to observe that the amount of C4, C6, C8, C10, C14, C14:1 c9, C15ai, C15, c16i, C16, C16:1 c9, C17i, C17ai, C17:1 c10, C18: C18:1 t11, C18:1 c9 and C18:2 c9,c15 is significantly higher ($p<0.05$) in the control. The inoculation with *B. breve* NCIMB 702258 led to the lowering of those values, especially when LA was present (Table 3.18). The possible explanation could be that the strain is retrieving the energy needed for its growth from those fatty acids present in milk. A possibility may be that when LA was added it could have caused a more stressful environment for the strain that made it consume more energy.

It was not possible to separate the supernatant from the pellet, so there is no way to discover if the substrates were being absorbed by *B. breve* NCIMB 702258, as previously observed (sections 3.3.1, 3.3.2 and 3.4). Concerning the CLA and CLNA total contents, the amount of CLA isomers was significantly higher in milk enriched with both substrates (134.13 µg/mL) than with just LA (104.52 µg/mL). However, CLNA concentration was almost double the amount in milk enriched with α-LNA alone (115.26 µg/mL) than with both substrates (61.57 µg/mL). This supports the previously formulated hypothesis: the presence of LA favors α-LNA conversion to CLA isomers instead of to the CLNA counterparts.

The total amount of FA was significantly ($p<0.05$) the highest in the control milk (12823.80 µg/mL), since the presence of *B. breve* NCIMB 702258 resulted in a generally lower fatty acid composition. Among the *B. breve* NCIMB 702258 inoculated milk samples those enriched with both precursor substrates contained the highest total FA content (12105.89 µg/mL), due to the higher content in precursor substrates and conjugated isomers.

4. Conclusions

In order to satisfy the ever-growing consumer demands for healthy functional foods capable of promoting well-being and prevent diseases, scientists are in persistent search for new functional ingredients. The potential bioactivity of the conjugated fatty acids CLA and more recently, CLNA, make them potential candidates even more when theirs *in situ* production may be promoted by adding bacteria able to produce these FA isomers during the culture/manufacturing process. In this work, the identification of CLNA-producing strains as well as the most favourable growth conditions were studied.

Based on the results of this research work, in the experimental conditions used, it can be concluded that:

- Molecular screening was demonstrated to be a useful tool in the identification of potential producers, yet it has to be further combined with production assays in order to test/improve substrate transformation;
- The precursor substrates added to the culture media were being absorbed into the bacteria. The presence of CLA, CLNA or both in the culture supernatant suggests that substrate conversion may occur in the membrane and then the resulting products are released into the culture medium;
- Some bacteria that were LAI-gene positive did not show CLA production, although the precursor substrate was actually reduced. These results suggest that LA was probably being converted into other unexpected compounds, such as HFAs. However, since we were not able to detect those compounds, there must exist an alternative transformation pathway not yet described;
- CLA production demonstrated to be dependent on growth conditions and strain as not all strains were able to isomerize LA into CLA isomers at considerable levels and the changes applied on growth conditions led to different CLA concentration values;
- *B. breve* NCIMB 702258 was identified as a CLA and a CLNA producer. Although it was possible to promote CLA and CLNA production in a food matrix such as 1.6% reduced-fat milk, the organoleptic characteristics of the product obtained were not yet suitable for commercial purposes.

5. Future work

Although the potential of CLA and CLNA producing strains has been demonstrated, challenges remain ahead. Concerning the LAI-gene positive strains, it would be of interest in the future to verify if this gene is actually being expressed when the strain is grown in the presence of the precursor substrate, because, in fact, something in the culture medium could be inhibiting its expression preventing the strains from being able to convert the substrate. LAI-gene primers are specific according to species and none have been described for bifidobacteria strains. Therefore, in order to obtain a high functional screening tool, future researches studies could target the sequencing of bifidobacteria LAI-gene for further construction of the respective primers.

Pellets' analysis demonstrated that the LA added to the cultures medium may be being absorbed by the strains, but the way in which it was being incorporated is unknown so it would be interesting to investigate in which form LA is accumulated and in which part of the cell, whether it be the cytoplasm or the membrane.

High LA reduction percentages and low CLA production were verified in this work, suggesting that the substrate could be being converted into other unknown compounds. Therefore, it would be important to identify which compounds were being generated and by which alternative transformation pathway of the substrates.

A further challenge for the future would be the effective elaboration of enriched foodstuffs with the identified CLNA-producer *B. breve* NCIMB 702258, using a safe substrate source and optimizing the sensorial parameters of the products. The study of CLNA stability during the elaboration and storage of those enriched foodstuffs would also be of interest, in order to verify the profitability and viability of the process with respect to CLNA content. Testing the bioactivity of those products at the human level would also be interesting to investigate if they truly have the capacity to cause any benefit over the long term.

Annex I

Table 1.2 CLNA-producing strains, culture growth conditions (medium, [LNA] (mg/mL), temperature (T; ºC) and time (t; h)), LNA conversion rate (%) and isomer(s) produced.

Strain	Culture medium	[LNA] (mg/mL)	T (ºC)	t (h)	LNA conversion rate (%)	Isomer(s) produced	Reference
Lactobacillus plantarum	MRS	63.00	37	72	40.0	C18:3 c9,t11,c15 C18:3 t9,t11,c15	Kishino *et al*., 2003
	MRS	4.00	37	48	47.0	C18:3 c9,t11,c15 C18:3 t9,t11,c15	Kishino *et al*., 2009
Lactobacillus sakei	MRS	0.50	30	72	60.1	C18:3 c9,t11,c15 C18:3 t9,t11,c15	Gorissen *et al*., 2011
Propionibacterium freudenreichii subsp. *freudenreichii*	BHI	0.02	37	48	30.0	C18:3 c9,t11,c15	Verhulst *et al*., 1987
Propionibacterium freudenreichii subsp. *shermanii*	BHI	0.02	37	48			
Propionibacterium acidipropionici	BHI	0.02	37	48			
Propionibacterium tecbnicum	BHI	0.02	37	48			

Annex II

Table 1.2 (continued) CLNA-producing strains, culture growth conditions (medium, [LNA] (µg/mL), temperature (T; ºC) and time (t; h)), LNA conversion rate (%) and isomer(s) produced.

Strain	Culture medium	[LNA] (µg/mL)	T (ºC)	t (h)	LNA conversion rate (%)	Isomer(s) produced	Reference
Propiobacterium freudenreichii subsp. *shermanii*	cys-MRS	0.45	30	72	53.5	C18:3 c9,t11,c15 C18:3 t9,t11,c15	Hennessy *et al*., 2012
Bifidobacterium breve	cys-MRS	0.24 0.41	37	42	67.6-80.7 49.4-79.1	C18:3 c9,t11,c15 C18:3 t9,t11,c15	Coakley *et al*., 2009
	Skim milk	0.50	37	24	94.0-97.0	C18:3 c9,t11,c15	Villar-Tajadura *et al*., 2014
	cys-MRS	0.50	37	72	55.6-72.0	C18:3 c9,t11,c15 C18:3 t9,t11,c15	Gorissen *et al*., 2010
	Milk	0.75	37	24	ND	ND	Gorissen *et al*., 2012b
Bifidobacterium bifidum	cys-MRS	0.50	37	72	78.4	C18:3 c9,t11,c15 C18:3 t9,t11,c15	Gorissen *et al*., 2010
	Milk	0.75	37	24	ND	ND	Gorissen *et al*., 2012b
Bifidobacterium pseudolongum subsp. *pseudolongum*	cys-MRS	0.50	37	72	62.7	C18:3 c9,t11,c15 C18:3 t9,t11,c15	Gorissen *et al*., 2010
	Milk	0.75	37	24	ND	ND	Gorissen *et al*., 2012b

ND = not detected

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