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ESCOLA SUPERIOR DE BIOTECNOLOGIA
PORTO

DEVELOPMENT OF A NEW FUNCTIONAL DAIRY PRODUCT ENRICHED IN MICROBIAL BIOACTIVE CONJUGATED FATTY ACIDS THROUGH AN INDUSTRIAL-BASED APPROACH

Thesis submitted to Universidade Católica Portuguesa to attain the degree of PhD in Biotechnology, with specialization in Food Science and Engineering

Ana Luiza Rodrigues Fontes

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December, 2023

Aos meus Pais, Rui e Isaura.
Obrigada por sempre acreditarem em mim,
mesmo quando eu própria não acreditava.

RESUMO

A procura por produtos alimentares inovadores com propriedades de valor acrescentado tem sido uma tendência crescente nos últimos anos. Desta forma, diferentes compostos alimentares com potenciais propriedades bioativas têm sido identificados, como por exemplo o ácido linoleico conjugado (CLA) e o ácido linolénico conjugado (CLNA), os quais têm sido descritos com atividades anti-carcinogénica, anti-obesidade e anti-inflamatória, entre outras. Estes compostos encontram-se naturalmente na carne e no leite de ruminantes ou em óleos vegetais, mas, devido a questões de disponibilidade e concentração, não é exequível obter efeitos benéficos de CLA/CLNA através destas fontes. Alternativamente, tem sido estudado o enriquecimento microbiano *in situ* de matrizes alimentares, uma vez que foram descritas várias estirpes probióticas com capacidade de isomerizar o ácido linoleico (LA) e o ácido α-linolénico (α-LNA) ao correspondente ácido conjugado. Assim, o objetivo deste trabalho foi estudar o desenvolvimento de um novo produto lácteo de valor acrescentado através da produção microbiana *in situ* de CLA e CLNA utilizando óleos vegetais comerciais previamente hidrolisados como fontes de substrato, assim como avaliar possíveis metabolitos de ácidos gordos libertados durante o processo, estudar as propriedades bioquímicas e organoléticas, e avaliar a estabilidade em prateleira do produto lácteo desenvolvido.

Em primeiro lugar, selecionaram-se estirpes produtoras de CLA/CLNA de entre 85 estirpes probióticas através da deteção molecular de genes que codificam enzimas envolvidas na formação de CLA/CLNA, nomeadamente, linoleato isomerase (LAI), antigénio reativo cruzado da miosina (MCRA) e hidratase de ácido gordos (FA-HY). Para tal, usaram-se *primers* descritos na literatura e *primers* desenhados com base em regiões conservadas. Entretanto, determinou-se a tolerância máxima das estirpes ao LA após exposição a 1, 2 e 5 mg/mL. Cerca de 34 estirpes revelaram a presença de pelo menos um dos genes analisados, sendo os *primers* desenhados neste estudo mais eficientes. Contudo não foi encontrada qualquer associação entre a sua tolerância ao LA e o potencial de produção de CLA. Além disso, apenas 4 estirpes genotipicamente positivas revelaram a capacidade de converter 0,5 mg/mL de LA em isómeros de CLA. Estas foram ainda testadas para a produção de CLNA a partir de 0,5 mg/mL de α-LNA. A estirpe *Bifidobacterium breve* DSM 20091 demonstrou os melhores valores de produção de isómeros de CLA e CLNA (>50% de conversão do substrato), sendo selecionada para os ensaios seguintes.

De seguida, foi estudada a utilização de óleos vegetais comerciais como fontes de substrato para o desenvolvimento de um leite fermentado enriquecido em CLA/CLNA. Para aumentar a biodisponibilidade de LA e α-LNA nos óleos selecionados, isto é, óleo de soja (43,7 g LA/100 g de óleo) e de linhaça (41,3 g α-LNA/100 g de óleo), foram testadas diferentes lípases. A lípase de *Candida rugosa* apresentou os melhores rendimentos para qualquer um dos óleos testados (>90% de hidrólise). A *B. breve* DSM 20091 foi depois testada em leite contendo 0,5 mg/mL de LA e/ou α-LNA com os óleos hidrolisados. Os resultados mostraram que esta estirpe não foi capaz de produzir CLA simultaneamente com CLNA na mesma extensão. Por conseguinte, foram testadas concentrações de substrato mais elevadas (α-LNA) apenas com óleo de linhaça hidrolisado. Os

melhores rendimentos foram obtidos a 2 mg/mL de α-LNA, tendo-se registado cerca de 1 mg/g de isómeros de CLNA maioritariamente.

Para avaliar possíveis metabolitos de ácidos gordos, foi efetuada uma análise por cromatografia gasosa acoplada a espectrometria de massa (GC-MS) ao leite fermentado com LA puro e/ou α-LNA puro ou óleo de linhaça hidrolisado. Não foram encontrados outros compostos de ácidos gordos que pudessem resultar da metabolização do LA ou α-LNA; no entanto, foram descobertos dois isómeros adicionais de CLNA nunca descritos para as Bifidobactérias.

As propriedades bioquímicas e organoléticas do leite fermentado enriquecido com CLNA foram depois avaliadas através da análise do teor de açúcares e ácidos orgânicos, acidez titulável, pH, composição nutricional, perfil de compostos voláteis e propriedades sensoriais. O produto desenvolvido apresentou características comparáveis a outros produtos semelhantes, no entanto, faltaram-lhe propriedades organoléticas importantes, prevalecendo o amargor e a adstringência.

Finalmente, foi avaliada a estabilidade do leite fermentado enriquecido com CLNA durante 28 dias de armazenamento refrigerado (4 ºC). Para obter uma via alternativa de administração dos isómeros de CLNA, o leite fermentado enriquecido foi depois liofilizado, sendo também avaliada a sua estabilidade durante o armazenamento (12 semanas à temperatura ambiente). Em ambos os produtos observou-se ao longo do tempo uma perda de ácidos gordos saturados livres e um aumento da quantidade de isómeros conjugados.

Em conclusão, a identificação de estirpes potencialmente produtoras de CLA/CLNA não pode basear-se apenas em técnicas de seleção genotípica e/ou de tolerância ao substrato. Apesar disso, foi possível desenvolver um leite fermentado enriquecido em ácidos gordos conjugados com a *B. breve* DSM 20091 utilizando óleo de linhaça hidrolisado, e esta estirpe mostrou uma preferência para produzir isómeros de CLNA. Além disso, foram descobertos dois isómeros adicionais de CLNA nunca descritos. As características bioquímicas e nutricionais do leite fermentado enriquecido com CLNA foram aceitáveis, mas há propriedades organoléticas a serem melhoradas. Embora os isómeros conjugados nos produtos lácteos desenvolvidos tenham aumentado durante o armazenamento, as alterações nos ácidos gordos saturados livres sugeriram a ocorrência de processos de oxidação.

Palavras-chave: Ácido linoleico conjugado; Ácido linolénico conjugado; *Bifidobacterium breve*; óleo de linhaça; leite fermentado.

ABSTRACT

The search for innovative food products with added-value properties has been an increasing tendency in the last years. Accordingly, different food compounds with potential bioactive properties have been identified, such as, conjugated linoleic acid (CLA) and conjugated linolenic acid (CLNA), which have been described with anti-carcinogenic, anti-obesity and anti-inflammatory activities, among others. These compounds are naturally found in meat and milk of ruminants or vegetables oils, but, due to availability and concentration concerns, it is not feasible to attain beneficial effects from CLA/CLNA through these sources. Alternatively, *in situ* microbial enrichment of food matrices has been studied, since several probiotic strains have been described with the capacity to isomerize linoleic acid (LA) and α-linolenic acid (α-LNA) into their corresponding conjugated forms. Thus, the objective of this work was to study the development of a new added-value dairy product through *in situ* microbial production of CLA and CLNA using previously hydrolyzed commercial vegetable oils as substrate sources, so as to evaluate possible side fatty acid (FA) metabolites released during the process, study the biochemical and organoleptic properties, and assess the shelf-life stability of the developed dairy product.

First the selection of CLA/CLNA-producing strains was performed from among 85 probiotic strains through molecular detection of genes encoding enzymes involved in CLA/CLNA formation, namely, linoleate isomerase (LAI), myosin-cross-reactive antigen (MCRA) and fatty acid-hydratase (FA-HY), using reported primers and primers designed based on conserved motifs. Meanwhile, it was determined the strains' maximum tolerance to LA after exposure to increasing concentrations thereof, namely 1, 2 and 5 mg/mL. About 34 strains revealed the presence of at least one of the screened genes, where the designed primers were more effective, but no association was found between their LA-tolerance and the CLA production potential. Moreover, only 4 genotypically-positive strains revealed the capacity to convert 0.5 mg/mL LA into CLA isomers. These were further tested for CLNA production from 0.5 mg/mL α-LNA. The strain *Bifidobacterium breve* DSM 20091 demonstrated the best yields of CLA and CLNA isomers (>50% of substrate conversion), being selected for the following assays.

In a second phase, the utilization of commercial vegetables oils as substrate sources for the development of a CLA/CLNA-enriched fermented milk was studied. To increase the bioavailability of LA and α-LNA present in the selected oils, i.e., soybean (43.7 g LA/100 g oil) and flaxseed (41.3 g α-LNA/100 g oil) oils, different lipases were tested. The *Candida rugosa* lipase showed the best yields for all of the tested oils (>90% of hydrolysis). *Bifidobacterium breve* DSM 20091 was thereafter assayed in milk containing 0.5 mg/mL of LA and/or α-LNA obtained from the hydrolyzed oils. Results showed that this strain was not able to produce CLA simultaneously with CLNA to the same extent. Therefore, higher substrate concentrations (i.e., α-LNA) were further tested with only hydrolyzed flaxseed oil. The best yields were attained at 2 mg/mL α-LNA, registering ca. 1 mg/g of mainly CLNA isomers.

To evaluate possible FA metabolites, a gas chromatography-mass spectrometry (GC-MS) analysis of milk fermented with pure LA and/or α-LNA or hydrolyzed flaxseed oil was performed. No

further FA compounds were found that could result from LA or α-LNA metabolization; however, two additional CLNA isomers never reported before for bifidobacteria strains were discovered.

The biochemical and organoleptic properties of the developed CLNA-enriched fermented milk were thereafter evaluated through analysis of sugars and organic acids content, titratable acidity, pH, nutritional composition, volatile compounds profile and sensory properties. The developed product showed comparable compositional properties; however, it lacked important flavor contributors, and bitterness and astringency prevailed.

Finally, the stability of the CLNA-enriched fermented milk was assessed throughout 28 days of refrigerated (4 ºC) storage. To obtain an alternative delivery system of CLNA isomers, the enriched fermented milk was further lyophilized, and its stability during storage (12 weeks at room temperature) was also evaluated. For both products a free saturated FAs loss and an increment of conjugated isomers content was observed.

In conclusion, the selection of potential CLA/CLNA-producing strains cannot rely solely on genotypic and/or substrate tolerance screening techniques. Even though, it was possible to obtain a fermented milk enriched in conjugated FAs with *B. breve* DSM 20091 using hydrolyzed flaxseed oil, and this strain showed a preference to produce CLNA isomers. Moreover, two additional CLNA isomers never reported before were discovered. The biochemical and nutritional characteristics of the CLNA-enriched fermented milk were acceptable, but there are organoleptic features that remain to be tackled. Although conjugated isomers in the developed dairy products increased throughout storage, the alterations in free saturated FA suggested the occurrence of oxidation processes.

Keywords: Conjugated linoleic acid; Conjugated linolenic acid; *Bifidobacterium breve;* flaxseed oil; fermented milk.

ACKNOWLEDGMENTS

First, I would like to thank Escola Superior de Biotecnologia of Universidade Católica Portuguesa for giving me the opportunity to take my PhD in Biotechnology. To Centro de Biotecnologia e Química Fina of ESB-UCP and Associated Laboratory for Green Chemistry – LAQV-REQUIMTE of Universidade de Aveiro for providing all the needed resources for the development of this project. To Fundação para a Ciência e Tecnologia of Portuguese government for the granted PhD scholarship (SFRH/BD/117721/2016). To Pro-TECh-CLnA project (PTDC/AGR-TEC/2125/2014) for all the support during this research work.

Then, I would like to thank Prof. Ana Maria Gomes for believing in me once more by accepting to supervise this project. To my co-supervisor Dr. Luís Rodríguez-Alcalá who has been tireless since day one and with who I have learn so much and grown as a researcher. To my co-supervisor Prof. Maria do Rosário Dominges who, even without knowing me, believed in this project and always gave readily support, especially during my stay in Mass Spectrometry Center of Universiade de Aveiro.

To Dr. Lígia Pimentel, who embraced this project from the first day, giving essential input and support. Thank you for your constant kindness and all I could learn with you.

To Beatriz Cardoso, Ana Sofia Salsinha, Erdem Carsanba and Célia Costa, research fellows of the Pro-TECh-CLnA project, who gave major support during the first stage of this research work. Within this former project, thanks are due to Prof. José Andrade from CESPU as well.

To Javier Calzada and Prof. Juan Arqués from Instituto Nacional de Investigación y Tecnología Agraria y Alimentaria (INIA), and Prof. Juan Miguel Rodríguez from Universidad Complutense de Madrid, for the provided bacterial strains, the primers for genotypic screening, and the valuable scientific knowledge contribution to this project.

To Sérgio Sousa, who is always prompted to help the others. Thanks for the hand in the HPLC-UV/RI analyses. To Ana Soares, who gave major help in the UV analysis part (thanks to you we could convince the reviewer and our paper be accepted!). To Prof. Maria João Monteiro, for the crucial support in the sensory analysis part, so as to all elements of the trained panel for the valuable contribute. To Bruna Neves, Tiago Conde and Daniela Couto, thanks for the huge support in the GC-MS analysis part, and for receiving me so kindly in your laboratory.

To CINATE, for the support in the physico-chemical analysis part and for the uncountable times your collaborators kindly let me use your analytical scale for the preparation of analytical standards. To Paulo Filipe Costa, Ana Martins, Susana Xis, Joaquim Cunha, Raquel Afonso, Fernanda Ferreira, Marta Guimarães, Cristina Santos, Ricardo Gonçalves and Rui Magalhães, thank you for all the technical support provided during this research work. To the security officers of Escola Superior de Biotecnologia for guaranteeing my security during the weekends, holidays and after-hours I had to be in the laboratory working.

To Bioactives and Bioproducts and Food and Health research groups of CBQF, for all the knowledge, support and memories shared in the laboratories. To my PhD colleagues, AKA “Amigos do Mateus”, Tatiana Vilela, Marta Mendes, Nádia Silva and Gianuario Fortunato, thank you for the

amazing moments spent together, thank you for your friendship in the bad and the good days. To the fantastic Lipidomicas Team, thank you for your strength and companionship.

To Juliana Ferreira and Andreia Ferreira, the wonderful friends “R/C Esquerdo” brought into my life. Thank you for your loving friendship, for the extensive collection of memories, and for your constant support in so many ways throughout all this path.

To my brothers João and Paulo, thank you for giving me the strength to become a good example for you. To my family in Azores, distance has made some days very hard, but you were always with me in thoughts and heart. To my grandfather Manuel Fontes, my guiding star. To my boyfriend’s family, Dona Paula, Sr. Zé and Sandrinha, thank you for receiving me so many weekends in your house where I could disconnect from everything else and focus on my PhD thesis, thank you for treating me so kindly.

To my dearest Diogo, you came into my life when I needed the most, you were my rock during the hardest part of this path. Thank you for being so supportive, understanding, encouraging, gentle and mindful. There are not enough words to describe the amazing boyfriend you are. I love you with all my heart!

And finally, but not least, I would like to thank my parents Rui and Isaura, to whom I dedicate all this work, because I would not have made this far if it was not for your effort and support. Thank you for the valuable principles instilled, which are so scarce these days, thank you for the exemplary education, thank you for your love and care. My main concern was always to made you proud, in return of everything you have given to me. I hope I have done it well. You are my heroes!

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LIST OF ABBREVIATIONS

CFA	Conjugated fatty acid
CFU	Colony forming unit
CLA	Conjugated linoleic acid
CLNA	Conjugated linolenic acid
CRL	*Candida rugosa* lipase
EFA	Esterified fatty acid
FA	Fatty acid
FA-HY	Fatty acid-hydratase
FAME	Fatty acid methyl ester
FFA	Free fatty acid
FSO	Flaxseed oil
HSO	Hempseed oil
JA	Jacaric acid
LA	Linoleic acid
LAB	Lactic acid bacteria
LAI	Linoleate isomerase
MCRA	Myosin-cross-reactive antigen
MRS	De Man–Rogosa–Sharpe
MUFA	Monounsaturated fatty acid
PFL	*Pseudomonas fluorescens* lipase
PPAR-γ	Peroxisome proliferator-activated receptor gamma
PPL	Pancreatic porcine lipase
PSO	Pomegranate seed oil
PUA	Punicic acid
PUFA	Polyunsaturated fatty acid
RA	Rumenic acid
RLA	Rumelenic acid
SBO	Soybean oil
SFA	Saturated fatty acid
TNF-α	Tumour necrosis factor alpha
α-ESA	alpha-Eleostearic acid
α-LNA	alpha-Linolenic acid

THESIS OUTLINE

This PhD thesis is divided into five chapters, as follows:

- Chapter 1 – Introduction;
- Chapter 2 – Materials and Methods;
- Chapter 3 – Results and Discussion;
- Chapter 4 – General Conclusions;
- Chapter 5 – Future Prospects.

Chapter 1 contextualizes the problem addressed in this research work, presenting a comprehensive review of the available data, which enabled the formulation of the hypothesis proposed for this PhD work and further outline the objectives to prove it.

Chapter 2 describes the experimental work performed, designed in accordance with the main goals outlined for this PhD work, and is divided into five sequential parts, as shown below:

- Part I – Identification of CLA/CLNA-producing strains;
- Part II – Study of the utilization of commercially available substrate sources to obtain a microbial CLA/CLNA-enriched fermented milk;
- Part III – Evaluation of fatty acid metabolites in microbial CLA/CLNA-enrichment of milk;
- Part IV – Biochemical and organoleptic properties of microbial CLNA-enriched fermented milk;
- Part V – Stability assessment of developed CLNA-enriched dairy products throughout storage.

Chapter 3 displays all the results obtained within the different research experiments performed, their critical analysis and interpretation and provides a detailed discussion, showing the main scientific findings within each of the above-mentioned parts. In Part I, besides the optimization assays, the results from genotypic, substrate tolerance and phenotypic screenings that enabled the selection of a probiotic strain capable of producing conjugated linoleic (CLA) and conjugated linolenic (CLNA) isomers are targeted. In Part II the results regarding the commercial vegetable oils hydrolysis with lipases, and the CLA/CLNA yields from the selected strain in milk with hydrolyzed vegetable oils as substrate sources are described, enabling the selection of the best lipase for vegetable oils hydrolysis, and the identification of the best conditions (i.e., which vegetable oil and at which maximum concentration) for the development of a fermented milk enriched in microbial conjugated isomers. Part III covers the results related with the comprehensive fatty acid analysis performed in milk fermented with the selected *Bifidobacterium* strain and pure precursor substrates (i.e., LA and α-LNA) or the selected hydrolyzed vegetable oil, to determine if other side fatty acid metabolites were being produced as well. In Part IV the results related with the nutritional, biochemical and organoleptic evaluation of the developed fermented milk enriched in microbial conjugated fatty acids are presented and discussed in terms of its suitability for consumption. Finally, in Part V is reported the results from the storage stability assessment of the developed dairy products enriched in microbial conjugated isomers, thus showing if those products' microbial and fatty acid composition would not change during shelf-life. In page XXIX is presented a scheme that portrays the overall

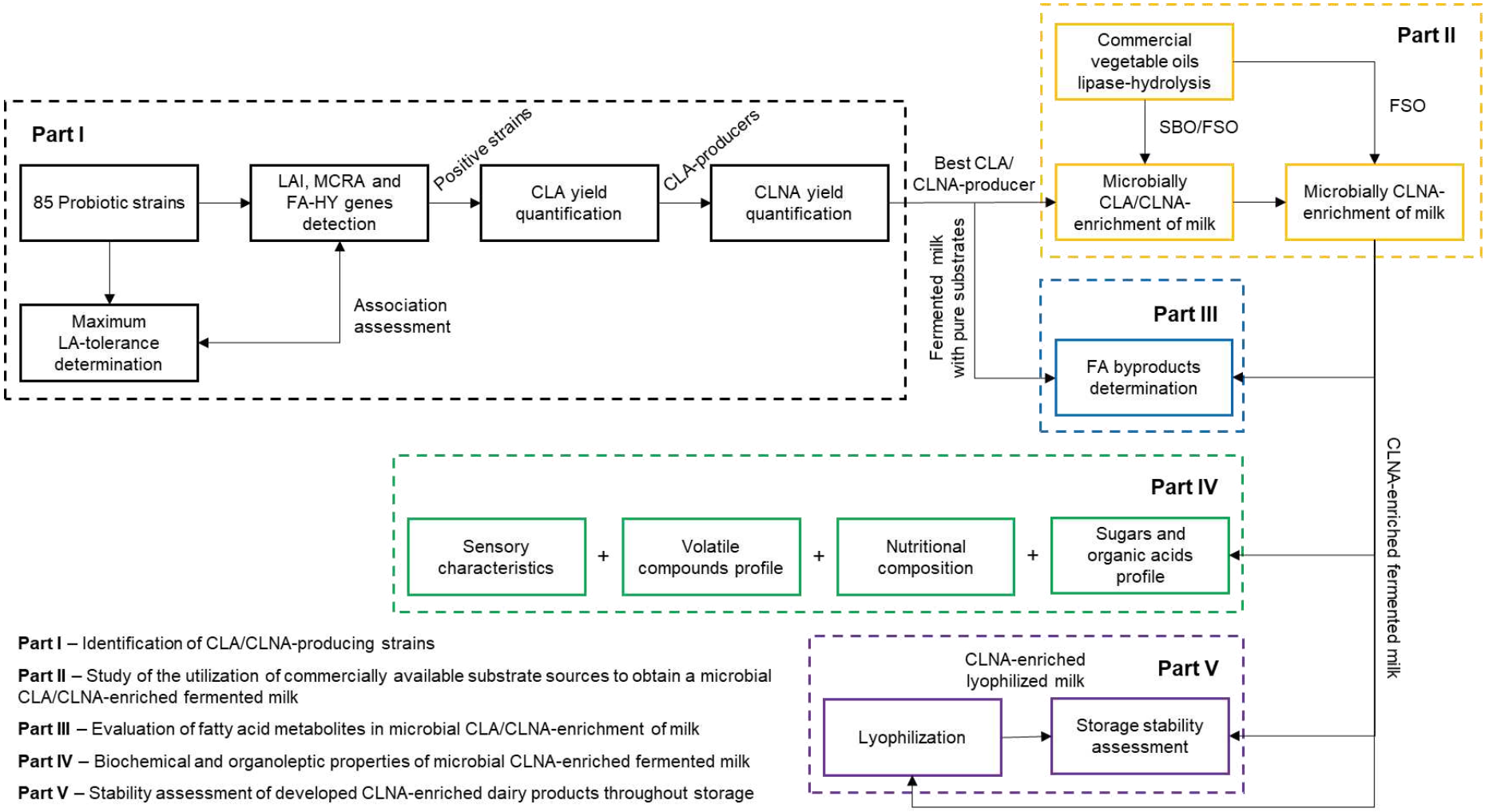
experimental work performed within this PhD thesis highlighting the articulation between the different parts.

Chapter 4 presents the main conclusions drawn from the research work performed within the framework of this PhD program.

The final **Chapter 5** depicts the experimental work that could be done in the future, in order to increment the scientific knowledge regarding the subject addressed in this PhD work, as well as, to improve the issues found with the development of a fermented milk enriched in microbial conjugated fatty acids.

The findings from this PhD work were presented in seven poster communications at national and international conferences. Furthermore, results were also disseminated through one oral communication at a national conference and two more at institutional workshops. Moreover, this PhD work resulted in four research articles published in peer-reviewed journals with impact factor and one conference proceeding with DOI, as presented below:

- Fontes, A.L., Pimentel, L.L., Monteiro, M.J., Domingues, M.R., Rodríguez-Alcalá, L.M., Gomes, A.M. (2024). Microbial conjugated linolenic acid-enriched fermented milk using lipase-hydrolyzed flaxseed oil: biochemical, organoleptic and storage traits. *Foods*, 13 (1), 21: 1-15. DOI: 10.3390/foods13010021. (IF: 5.2; Q1 – Food Science & Technology; Rank JCI: 40/170).
- Fontes, A.L., Neves, B., Conde, T., Couto, D., Pimentel, L.L., Rodríguez-Alcalá, L.M., Domingues, R., Gomes, A.M. (2024). Short-communication: Study of fatty acid metabolites in microbial conjugated fatty acids-enrichment of milk and discovery of additional undescribed conjugated linolenic acid isomers. *Food Chemistry*, 432, 137276: 1-7. DOI: 10.1016/j.foodchem.2023.137276. (IF: 8.8; Q1 – Food Science & Technology; Rank JCI: 5/170).
- Fontes, A.L., Pimentel, L. Calzada, J., Salsinha, A.S., Rodriguez, J.M., Gomes, A.M., Arques, J.L., Rodríguez-Alcalá, L. (2023). Study of the association between genotypic potential and linoleic acid tolerance with microbial production of conjugated linoleic acid. *ACS Food Science & Technology*, 38 (12): 2199-2207. DOI: 10.1021/acsfoodscitech.3c00399. (IF: 2.3; Q3 – Food Science & Technology; Rank JCI: 113/170).
- Fontes, A.L., Pimentel, L.L., Soares, A.M.S., Domingues, M.R., Rodríguez-Alcalá, L.M., Gomes, A.M. (2023). Study of the viability of using lipase-hydrolyzed commercial vegetable oils to produce microbially conjugated linolenic acid-enriched milk. *Food Chemistry*, 413, 135665: 1-8. DOI: 10.1016/j.foodchem.2023.135665. (IF: 8.8; Q1 – Food Science & Technology; Rank JCI: 5/170).
- Fontes, A.L., Pimentel, L.L., Rodríguez-Alcalá, L.M., Gomes, A.M. (2021). Stability of a fermented milk enriched with microbial CLA/CLNA. *Proceedings of the 7th World Congress on New Technologies*: 7-8. DOI: 10.11159/icbb21.231.



Outline scheme of the experimental work performed within the present PhD program.

LA – Linoleic acid, LAI – Linoleate isomerase, MCRA – Myosin-cross-reactive antigen, FA-HY – Fatty acid hydratase, CLA – Conjugated linoleic acid, CLNA – Conjugated linolenic acid, SBO – Soybean oil, FSO – Flaxseed oil, FA – Fatty acid.

CHAPTER 1

Introduction

CHAPTER 1 – INTRODUCTION

1.1. Context

According to the World Health Organization, non-communicable diseases are responsible for 74% of global deaths each year. Cardiovascular diseases account for most deaths (17.9 million/year), followed by cancer (9.3 million/year) (World Health Organization, 2023). People are aware that diet influences the development of such health conditions, so the search for healthier and innovative food products with functional properties that could prevent or counteract those conditions has been an increasing tendency in the last years. In accordance, several studies have focused on the identification of compounds in food with added-value properties, including those found among the lipid moieties. Thus, different lipids have been described with potential bioactive properties, namely conjugated linoleic acid (CLA) and conjugated linolenic acid (CLNA) isomers. Conjugated fatty acids (CFA) consist of positional and geometric isomers of polyunsaturated fatty acids (PUFA) with conjugated double bonds, i.e., two carbon double bonds separated by only one carbon single bond (Liu & Leung, 2015c). In this case, CLA is a group of isomers of linoleic acid (LA; C18:2 c9c12) and CLNA of alpha-linolenic acid (α-LNA; C18:3 c9c12c15) (Białek et al., 2017).

Below is described the evidences available on the beneficial health properties of CLA and CLNA isomers and the possibilities of their application in new functional food products, thus establishing the rationale for the research project presented herein.

1.2. Natural sources of CLA/CLNA isomers

Conjugated linoleic acid is naturally present in the milk of different ruminants, such as, cows, sheep, goats and buffaloes (**Table 1.1**). Regarding cows, CLA values can range from ~0.39 to 2.00 g/100 g FA (He et al., 2005; Zhang et al., 2021), or approximately 5.18 mg/g fat (Giesy et al., 2002). As for sheep, contents between 0.41 and 2.35 g/100 g FA have been reported (Addis et al., 2005; Toral et al., 2022). In goats' milk, CLA content can reach 0.57 g/100 g FA (Li et al., 2012) and in buffaloes up to 16.9 mg/g fat (Tyagi et al., 2007). The main CLA isomer present in ruminants' milk is C18:2 c9t11 (Rumenic acid – RA), but other minor isomers are usually also detected, such as, C18:2 t10c12 or C18:2 t11t13 (**Table 1.1**). Variations in CLA contents can be attributed to animals' breed, type of diet and/or year season (pasture conditions).

Besides milk, CLA can also be found in ruminants' meat (**Table 1.2**). In cows, it has been described RA contents between 0.23 and 0.66 g/100 g FA (Dhiman et al., 2005; Madron et al., 2002), or 6.88 mg/g fat of CLA isomers (Lorenzen et al., 2007), while in sheep, RA values can attain 1.08 g/100 g FA (Wachira et al., 2002). As for goats, about 0.40 g/100 g FA of CLA have been detected in its meat (Roy et al., 2013). Moreover, CLA isomers have also been found in chicken meat, at up to 0.47 g/100 g FA (Suksombat et al., 2007).

Table 1.1 Conjugated linoleic acid content found in ruminants' milk.

Animal	Diet	Content	Isomer(s)	Reference
Holstein cows	Alfalfa and oat pastures, corn and corn silage	1.47 g/100 g FA	c9t11	Gagliostro et al. (2020)
Holstein cows	Corn silage, corn and alfalfa hay	2.01 g/100 g FA	c9t11, t10c12	Zhang et al. (2021)
Cows	Barley silage, barley, corn silage and corn	0.389 g/100 g FA	c9t11, t10c12	He et al. (2005)
Holstein cows	Pasture, corn and soybean meal	0.95 g/100 g FA	c9t11, c9c11, t11t13, other t,t	AbuGhazaleh (2008)
Brown Swiss cows	Grass silage and maize silage supplemented with different plants	0.46-0.67 g/100 g FA	c9t11	Birkinshaw et al. (2020)
Holstein cows	Alfalfa hay, corn and barley	5.18 mg/g fat	c9t11, t10c12	Giesy et al. (2002)
Spanish Assaf ewes	Cereals (corn, barley and soybean meal) and dehydrated alfalfa hay	1.17 g/100 g FA	c9t11, t9c11, t10c12, t11c13, t12t14, t11t13, other t,t	Gómez-Cortés et al. (2008)
Sheep	*Undefined*	1.767 g/100 g FA	c9t11, t10c12	Nudda et al. (2005)
Sarda ewes	Ryegrass forage	1.20-1.43 g/100 g FA	c9t11	Addis et al. (2005)
	Sulla forage	1.12-1.25 g/100 g FA		
	Burr medic forage	1.65-2.30 g/100 g FA		
	Daisy forb forage	2.33-2.35 g/100 g FA		
Karagouniko sheep	Pasture	0.95-1.46 g /100 g FA	c9t11, t10c12	Govari et al. (2020)
Assaf ewes	Dehydrated alfalfa, soybean meal and corn	0.412 g/100 g FA	c9t11, c11t13, t10c12, t11c13, t11t13, t12t14	Toral et al. (2022)
Murciano-Granadina goats		0.458 g/100 g FA		
Saanen goats	Cereals (corn, barley and wheat) and alfalfa hay	0.57 g/100 g FA	c9t11, t10c12	Li et al. (2012)
Murrah buffaloes	Concentrate mix (maize, groundnut cake, wheat) and wheat straw	7.8 mg/g fat	c9t11, t10c12	Tyagi et al. (2007)
	Concentrate mix (maize, groundnut cake, wheat), wheat straw and berseem	13.4 mg/g fat		
	Wheat straw and berseem	16.9 mg/g fat		

FA – Fatty acid; *c* – *cis* double bond; *t* – *trans* double bond.

Table 1.2 Conjugated linoleic acid content found in animal meat.

Animal	Diet	Muscle tissue	Content	Isomer(s)	Reference
Angus x Hereford steers	Corn and corn silage	Loin, chuck and round	0.31 g/100 g FA	c9t11	Griswold et al. (2003)
Steer calves	Alfalfa hay and pasture	Longissimus and semitendinosus	0.042 g/100 g FA	t10c12	Poulson et al. (2004)
Angus steers	Corn and corn silage	Rib longissimus, eye of round and chuck	0.66 g/100 g FA	c9t11	Madron et al. (2002)
Steers	Corn	Longissimus and semitendinosus	0.23 g/100 g FA	c9t11	Dhiman et al. (2005)
British x Continental steers	Pasture	Longissimus lumborum, Triceps brachii and semimembranosus	6.88 mg/g fat	c9t11, t10c12, c9c11, t9t11	Lorenzen et al. (2007)
Suffolk x Lleyn lambs	Dried grass	Longissimus dorsi	0.88 g/100 g FA	c9t11	Wachira et al. (2002)
British Friesland x Lleyn lambs			1.01 g/100 g FA		
Soay lambs			1.08 g/100 g FA		
Black Bengal goats	Berseem and maize	Longissimus dorsi	0.40 g/100 g FA	c9t11, t10c12	Roy et al. (2013)
Arbor Acres broilers	Corn and soybean meal	Tight	0.47 g/100 g FA	c9t11, t10c12	Suksombat et al. (2007)
		Drumstick	0.17 g/100 g FA		

FA – Fatty acid; *c* – *cis* double bond; *t* – *trans* double bond.

Regarding CLNA isomers, these are mainly found in the seed oils from several plant species (**Table 1.3**). This includes, pomegranate (*Punica granatum* L.; Punicaceae), a deciduous tree originally from the region across Iran to the Himalayas in northern India, but widely distributed nowadays, being India, Iran, Turkey, China, and the United States of America its principal producers (Hip Kam & Neergheen, 2021). The pomegranate seed oil (PSO) is mainly composed by Punicic acid (PUA; C18:3 c9t11c13), with contents between ~51.1 and 80.7 g/100 g FA, depending on the extraction method yield (Silva et al., 2019; Zakeri et al., 2019). The seed oils from *Trichosanthes anguina* L. and *Trichosanthes kirilowii* Maxim constitute other sources of PUA, with contents around 30-40 g/100 g FA, approximately (Saha & Ghosh, 2013; Yang et al., 2012). The *T. angina* (Cucurbitaceae) is an annual climbing plant native to India, but also distributed in humid tropical regions of many countries, whose fruit shape made it commonly known as snake gourd (Layek et al., 2022; Song et al., 2022). The *T. kirilowii* (Cucurbitaceae) consists of a perennial vine, with a long history in China, whose fruits, pericarp, seeds and roots are commonly applied in traditional Chinese medicine (Xu et al., 2022; Zhang et al., 2019).

Jacaranda (*Jacaranda mimosifolia* D.Don; Bignoniaceae) is a deciduous ornamental tree indigenous to Brazil, Bolivia, and Argentina, but distributed across tropical and subtropical regions worldwide (Naz et al., 2020; Zhao et al., 2019). To the best of our knowledge, Jacaric acid (JA; C18:3 c8t10c12) can only be found in Jacaranda seed oil, constituting up to 32.2 g/100 g FA (Van Nieuwenhove et al., 2022). In the same way, α-Calendic acid (α-CDA; C18:3 t8t10c12) and β-Calendic acid (β-CDA; C18:3 t8t10t12) have been reported only for Pot marigold (*Calendula officinalis* L.; Asteraceae) seed oil, at 18.3 and 11.2 g/100 g FA, respectively (Özgül-Yücel, 2005). Pot marigold is a common garden plant, endemic to Central Europe and the Mediterranean region, used in Europe, China, United States, and India for medicinal purposes (Givol et al., 2019; Silva et al., 2021).

Catalpic acid (CPA; C18:3 t9t11c13) can be found in American catalpa (*Catalpa bignonioides* Walt.; Bignoniaceae), at 14.9 g/100 g FA (Özgül-Yücel, 2005), but contents in Chinese catalpa (*Catalpa ovata* G.Don; Bignoniaceae) are reportedly higher, ca., 40.2 g/100 g FA (Suzuki et al., 2006). American catalpa, also known as cigar tree or India bean tree, is a deciduous ornamental tree native to southeastern North America, mainly grown in some regions of Canada and central and southern United States (Kim et al., 2022; Quan et al., 2022). The Chinese catalpa is a tree similar to American catalpa, but, as its common name reveals, it is indigenous to China, being distributed all across Korea, China and Japan (Olsen & Kirkbride, 2017; Park et al., 2010).

Bitter gourd (*Mormodica charantia* L.; Cucurbitaceae), also acknowledged as bitter melon, or karela, constitutes a tropical and subtropical vine original from southern India, widely cultivated nowadays in Asia, Africa, and the Caribbean for its edible fruit (Khan et al., 2022; Liu et al., 2021). Its seed oil is composed by up to 52.3 g/100 g FA of α-Eleostearic acid (α-ESA; C18:3 c9t11t13) (Saha & Ghosh, 2013). Another source of α-ESA is Mahaleb cherry (*Prunus mahaleb* L.; Rosaceae) seed oil, containing up to 38.3 g/100 g FA (Sbihi et al., 2014). This deciduous tree, also recognized as English cherry, Rock cherry or St. Lucie cherry, is native to Europe and Western Asia, being

widely grown in warm climate of Central and South-Europe, Central Asia and Northern Africa (Gerardi et al., 2019; Mariod et al., 2010).

Table 1.3 Conjugated linolenic acid content found in vegetable seed oils.

Plant	Content	Isomer	Reference
Pomegranate (*Punica granatum* L.)	57.3 g/100 g FA	c9t11c13 (PUA)	Özgül-Yücel (2005)
	60.6-80.7 g/100 g FA		Silva et al. (2019)
	77.2 g/100 g FA		Emami et al. (2016)
	51.06 g/100 g FA		Zakeri et al. (2019)
	57.65 g/100 g FA		Van Nieuwenhove et al. (2019)
Snake gourd *(Trichosanthes anguina L.)*	40.1 g/100 g FA		Saha and Ghosh (2013)
Trichosanthes kirilowii Maxim	33.09-39.15 g/100 g FA		Yang et al. (2012)
Jacaranda (*Jacaranda mimosifolia* D.Don)	31.2 g/100 g FA	c8t10c12 (JA)	Van Nieuwenhove et al. (2019)
	32.2 g/100 g FA		Van Nieuwenhove et al. (2022)
Pot marigold (*Calendula officinalis* L.)	18.3 g/100 g FA	t8t10c12 (α-CDA)	Özgül-Yücel (2005)
	11.2 g/100 g FA	t8t10t12 (β-CDA)	
American catalpa (*Catalpa bignonioides* Walt.)	14.9 g/100 g FA	t9t11c13 (CPA)	Özgül-Yücel (2005)
Chinese catalpa (*Catalpa ovata* G.Don)	40.2 g/100 g FA		Suzuki et al. (2006)
Bitter gourd (*Momordica charantia* L.)	49.9 g/100 g FA	c9t11t13 (α-ESA)	Özgül-Yücel (2005)
	52.3 g/100 g FA		Saha and Ghosh (2013)
Mahaleb cherry (*Prunus mahaleb* L.)	21 g/100 g FA		Özgül-Yücel (2005)
	38.32 g/100 g FA		Sbihi et al (2014)

FA – Fatty acid; *c* – *cis* double bond; *t* – *trans* double bond; PUA – Punicic acid; JA – Jacaric acid; α-CDA – alfa-Calendic acid; β-CDA – beta-Calendic acid; CPA – Catalpic acid; α-ESA – alfa-Eleostearic acid.

Similarly to CLA, CLNA is present in ruminants' milk and meat as well, however, at lower amounts (**Table 1.4**). In cows' milk, values can range from about 0.06 to 0.39 g/100 g FA (Lerch et al., 2012; Plourde et al., 2007). As for sheep milk, it has been described with 0.022 g/100 g FA and goat milk with 0.013 g/100 g FA of CLNA isomers (Toral et al., 2022). Regarding meat, CLNA has been found at 0.34 g/100 g FA in cows (Plourde et al., 2007) and 0.31 g/100 g FA in goats (Ebrahimi et al., 2014). The main CLNA isomers detected are C18:3 c9t11c15 (Rumelenic acid – RLA) and C18:3 c9t11t15.

Table 1.4 Conjugated linolenic acid content found in milk and meat of ruminants.

Animal	Diet	Food	Content	Isomer(s)	Reference
Simmental x Hereford cows	Pasture	Milk	0.39 g/100 g FA	c9t11c15	Plourde et al. (2007)
Holstein cows	Grass silage and hay	Milk	0.064-0.105 g/100 g FA	c9t11c15, c9t11t13, c9t11t15	Lerch et al. (2012)
	Pasture		0.145-0.172 g/100 g FA		
Assaf ewes	Dehydrated alfalfa, soybean meal and corn	Milk	0.022 g/100 g FA	c9t11c15, c9t11t15	Toral et al. (2022)
Murciano-Granadina goats			0.013 g/100 g FA		
Angus steers	Grass silage	Longissimus muscle	0.34 g/100 g FA	c9t11c15, c9t13c15	Plourde et al. (2007)
Boer goats	Oil palm, palm kernel cake, corn and soybean meal	Semitendinosus muscle	0.31 g/100 g FA	c9t11c15, c9t11t15	Ebrahimi et al. (2014)

FA – Fatty acid; *c* – *cis* double bond; *t* – *trans* double bond.

1.3. Bioactive properties of CLA/CLNA isomers

1.3.1. Anti-carcinogenic

Anti-cancer activity of CLA and CLNA isomers has been reported for several cancer models, namely, breast (Moon et al., 2010), prostate (Hagen et al., 2013), colorectal (Kim et al., 2015), liver (Lu et al., 2015), ovarian (Thuillier et al., 2013), glioma (Silva-Ramirez et al., 2018), endometrial (Wang et al., 2013), osteosarcoma (Wong et al., 2015), pheochromocytoma (Cao et al., 2014), colon (Coakley et al., 2009), cervix (Eom et al., 2010), choriocarcinoma (Li et al., 2013), leukemia (Yamasaki et al., 2013) and bladder (Sun et al., 2012) cancers.

The main *in vitro* effects related to CLA/CLNA toxicity in cancer cell lines have been cell proliferation inhibition, cell viability decrease and cell motility and migration reduction (El Roz et al., 2013; Moon et al., 2010), which were associated to cell cycle arrest, autophagy or apoptosis. Cell cycle arrest has been evidenced by an increase of cells in G0/G1, G1 or G2/M phases and decrease in the S phase (Grossmann et al., 2009; Liu & Leung, 2014; Shahzad et al., 2018). Increment of autophagosomes and LC3 I conversion to LC3 II (autophagosome-associating protein) have proved autophagy occurrence (Eom et al., 2010; Shahzad et al., 2018). Detected apoptosis hallmarks have been essentially DNA damage, cellular morphology alteration (cell shrinkage, chromatin condensation, nuclei diameter reduction and cell membrane blending), mitochondrial membrane potential decrease and phosphatidylserine (PS) externalization (Li et al., 2013; Liu & Leung, 2015b; Wang et al., 2013). Moreover, anti-apoptotic proteins (Bcl-xL, Bcl-2 and Bcl-w) have been down-regulated and pro-apoptotic proteins (caspase-3,-9, BAD, BAX, BAK, BIM and BID) up-regulated (Li et al., 2013; Lu et al., 2015).

Rodents with chemically induced cancer or injected with cancer cells, have been the animal models applied to study CLA/CLNA *in vivo* anti-cancer activity. In these models CFA toxicity has been evidenced by a reduction of cancer incidence, reflected as the decrease of tumor number and size (volume and weight) (Białek et al., 2014; Stawarska et al., 2015; Thuillier et al., 2013).

In humans, a mixture of CLAs (C18:2 c9t11 [RA] + C18:2 t10c12 [1:1]) has been tested in women with invasive non-metastatic breast cancer. Participants received 7,5 g/day of CLA for 10 days before tumor resection, but were allowed to take CLA for up to 28 days (McGowan et al., 2013). In breast cancer, *de novo* fatty acid (FA) synthesis reveals to be crucial for tumor progression, so lipid metabolism becomes a potential therapeutic target (Hilvo et al., 2011). Although there was a decrease in S14 protein expression (lipogenic enzymes regulator) after CLA treatment in the above-mentioned study, FASN (FA-synthesizing protein) and LPL (FA uptake enzyme) expression levels did not change. Moreover, cleaved caspase-3 (apoptosis hallmark) did not differ before and after treatment. Authors pointed out small sample size (n = 23) and short treatment duration as limitations of their research work. In fact, the administered dose was even higher than the recommended 3-6 g/day of CLA to exert beneficial effects (Fuke & Nornberg, 2017) and it showed to be safe in short-term consumption, with minor adverse events like nausea and fatigue, which did not require pharmacological intervention, dose maintenance or reduction.

Concerning the CLA/CLNA degree of toxicity in cancer, there seems to be differences depending on the number and configuration of double bonds in the FAs. First of all, CLA and CLNA have shown to be more toxic than their precursor FAs (i.e., LA and α-LNA, respectively) (Rakib et al., 2013; Sun et al., 2012; Wong et al., 2015). Furthermore, more potent effects have been achieved with CLNA isomers containing a conjugated triene comparing to CLA, which possess a conjugated diene (Degen et al., 2011; Yamasaki et al., 2013). However, the number of double bonds per se appears to also have influence on cancer toxicity, because Grossmann et al. (2009) observed that, although α-LNA anti-proliferative effect was weak, it was slightly higher than CLA in breast cancer MDA-Era7 and MDA-MB-231 cells. Another interesting fact observed in several studies (Liu & Leung, 2014; Shahzad et al., 2018; Thuillier et al., 2013), was that the CFAs exhibiting the strongest outcome against cancer contained *trans*-10,*cis*-12 configuration , whether being CLA or CLNA. Moreover, all *trans* configurations showed to be more effective in CLA isomers (Degen et al., 2011; El Roz et al., 2013). Furthermore, cancer type and exposure conditions also seem to have impact on CLA/CLNA degree of toxicity. In a different cancer cell line, namely the endometrial cancer RL95-2 cells – C18:2 t10c12 was not the most effective CLA isomer (Wang et al., 2013). Furthermore, its inhibitory activity was not superior to that of LA at 160 μM, since effects were observed at 40 μM. In another study, the antiproliferative efficacy of CLNA isomers with double bonds at 9,11 and 15 positions on SW480 colon cancer cells was reported to be similar to that of α-LNA (Hennessy et al., 2016). On the other hand, Coakley et al. (2009) observed that the same CLNA isomers used against the same cancer cell line had a stronger effect than α-LNA. These studies differed in terms of exposure conditions, more precisely, 200 μM for 72 h *vs*. 50 μg/mL for 120 h, respectively.

The evidence on CLA and CLNA anti-cancer potential is considerable, however, it would be critical if its toxicity is not cancer-selective affecting normal cells as well. Therefore, several studies have also assayed with normal cells and most observed that CLA/CLNA did not interfere with its proliferation and viability (Hagen et al., 2013; Hennessy et al., 2016; Silva-Ramirez et al., 2018). The only CFA that does not seem safe is α-ESA, because, when it was tested in human normal liver L02 cells, although the proliferation index increased and the apoptosis index was not positively affected, the cell viability was significantly reduced to approximately 20%, after 120 h at 80 μM (Zhang et al., 2012). Moreover, α-ESA suppressed vascular endothelial growth factor (VEGF)-induced angiogenesis in human umbilical vein endothelial cells cocultured with human diploid fibroblasts (Tsuzuki & Kawakami, 2008). The VEGFs play an important role in formation, function and maintenance of vasculature (Simons et al., 2016), but are also produced by tumor cells and promote angiogenesis in tumors (Yadav et al., 2015). However, in the above-mentioned study, the VEGF did not come from tumor cells, it was added by the researchers. So, if α-ESA can inhibit angiogenesis independently of its origin, it could be critical for normal blood vessel formation.

The mechanisms behind CLA/CLNA anti-cancer action are not fully clarified since there are diverse proposals. However, many of them associate CLA/CLNA inhibitory effect with activation of peroxisome proliferator-activated receptor gamma (PPAR-γ) (Lu et al., 2015; Sun et al., 2012; Zhang et al., 2012) or with oxidation mediated-mechanisms (Liu & Leung, 2015b; McGowan et al., 2013; Yamasaki et al., 2013).

1.3.2. Anti-inflammatory

In vitro and *in vivo* studies have reported that CLA and CLNA possess the ability to modulate inflammatory and immunity markers towards an anti-inflammatory response. In fact, the increase of eosinophil and monocyte activities and the increment of migratory macrophages level, caused by chemically induced mucositis and colitis in mice, have been reduced by CLA isomers. Moreover, monocyte accumulation has been prevented and resident macrophages level enhanced (de Barros et al., 2018; Moreira et al., 2018). Furthermore, Boussetta et al. (2009) observed that CLNA reverted enhanced neutrophil infiltration and activation, and the Myeloperoxidase (MPO; enzyme released by neutrophils) activity in rats with TNBS-induced colitis. In the case of Moreira et al. (2018) study, where CLA was tested in mice with DSS-induced colitis, no changes were detected in neutrophil level, but MPO activity was also reduced. Conjugated linoleic acid isomers have also revealed capacity to enhance Secretory Immunoglobulin A (SIg A) level in mice with 5-FU-induced mucositis (de Barros et al., 2018). The SIg A consists of an important line of defense in the intestinal mucosal surface against enteric toxins and pathogenic microorganisms (Ren et al., 2016). Concerning to pro-inflammatory cytokines and chemokines, CLA isomers have demonstrated capacity to reduce the levels of Interleukin (IL)-1β, IL-6, IL-8, Tumour necrosis factor alpha (TNF-α), Interferon (IFN)-γ, CC chemokine ligand (CCL)2, CCL5, CCL20 and CXC chemokine ligand (CXCL)1, key markers of an inflammatory status. On the other hand, anti-inflammatory cytokine IL-10 levels have been enhanced by this CFA (de Barros et al., 2018; Huang et al., 2016; Ma et al., 2019).

The efficacy of CLA has also been evidenced from CLA-enriched food products. For instance, Bergamo et al., (2017), who tested milk enriched in C18:2 c9t11 (RA) + C18:2 t10c12 on mice with DSS-induced colitis, observed a decrease on expression levels of cytokines enhanced by colitis, including Transforming growth factor (TGF)-β, which is described as having either pro- or anti-inflammatory properties (Travis & Sheppard, 2014). As for Huebner et al. (2016), where mice with collagen-induced arthritis were fed a butter enriched in RA, although anti-inflammatory cytokine IL-10 maintained decreased levels, pro-inflammatory cytokines were reduced to levels similar or lower than a pure CLA-supplemented diet.

The anti-inflammatory ability of CLA and CLNA has also been observed in physiological terms, by reduction of pathological symptoms, disease activity and incidence in animal models (Bassaganya-Riera et al., 2011; Bergamo et al., 2017; Muhlenbeck et al., 2017). In mucositis and colitis models, ileum and colon mucosal architecture have been preserved in animals treated with CLA/CLNA (Bassaganya-Riera et al., 2011; de Barros et al., 2018; Moreira et al., 2018). Moreover, CLA/CLNA-supplemented/enriched diets have ameliorated colon shortening and inflammatory infiltration and reduced edema in animals with colitis (Bergamo et al., 2017; Boussetta et al., 2009; Moreira et al., 2018). Administration of CLA has also led to decreased paw swelling in mice with arthritis (Huebner et al., 2016).

Some studies have also evaluated if CLA and/or CLNA do not compromise host immunity when no inflammation is occurring. Indeed, under normal conditions, CLA isomers have not revealed any negative effect on mice intestinal macroscopic or morphological parameters (Bergamo et al., 2017; de Barros et al., 2018). Moreover, CLNA did not exhibit cytotoxicity towards mast cells (≤8

mM) (Liu & Leung, 2015a) and was only slightly toxic (<10%) to macrophages at up to 100 mM (Liu & Leung, 2015c). In other studies, several inflammatory and immunity markers have not been affected by CLA isomers in mice (de Barros et al., 2017, 2018), but Bergamo et al. (2017) observed a decrease in IL-1β (pro-inflammatory) expression level in mice fed CLA-enriched butter, while TGF-β (pro- and anti-inflammatory) and IL-10 (anti-inflammatory) levels were not altered.

In humans, CLA has been tested in healthy young volunteers (Penedo et al., 2013), where participants first went into a 56-day CLA-depletion period (regular consumed dairy products substituted by low-fat dairy) and then a 56-day CLA-repletion period (return to regular dairy consumption plus 20 g/day of CLA-enriched butter). The level of C-reactive protein (CRP), a robust clinical inflammatory biomarker (Shrivastava et al., 2015), was not modified on repletion comparing to depletion period, so as IL-4, while TNF-α, IL-2 and IL-8 (pro-inflammatory) were decreased and IL-10 (anti-inflammatory) increased. The above-mentioned observations suggest that CLA exhibits anti-inflammatory effects at normal state as well.

Most of the studies focusing on CLA/CLNA anti-inflammatory activity have been carried out with only one or a mixture of isomers, which hampers the determination of whether there is any relation between isomer type and its effectiveness against inflammation processes. Nevertheless, the decrease of pro-inflammatory cytokines expression level has been higher for certain CLA isomers than for LA in an inflamed state model, where C18:2 t10c12 has not decreased IL-10 expression level so pronouncedly as RA (C18:2 c9t11) (Dipasquale et al., 2018).

Concerning the CLA/CLNA mechanism of action in inflammation, few reports have brought any insights. Nevertheless, it is suggested that CLA and CLNA act through inhibition of MAPK and NFkB signaling pathways (Huang et al., 2016; Ma et al., 2019), mediated by PPAR-γ and -δ (Bassaganya-Riera et al., 2011), respectively.

1.3.3. Anti-obesity

High triglyceride (TAG) and/or high glucose administration has been applied to induce an obesity state in *in vitro* and *in vivo* studies testing CLA/CLNA anti-obesity properties. Main beneficial effects observed from these CFA have been reduction of body weight and weight gain (den Hartigh et al., 2018; Furlan et al., 2013), reduction of epididymal, retroperitoneal and inguinal adipose tissues weights (Chen et al., 2012; Shen et al., 2015), reduction of cellular and circulating TAG levels (Chen et al., 2016; Farmawati et al., 2016) and reduction of circulating glucose levels (Cho et al., 2016; Malinska et al., 2015). Furthermore, decrease of insulin and leptin circulating levels and increase of circulating adiponectin content have validated CLA/CLNA anti-obesity capacity (Chen et al., 2012; Malinska et al., 2015). Insulin is proportionally secreted to increasing plasma glucose levels and is responsible for its normalization to physiological levels by promoting glucose entry into cells (Yaribeygi et al., 2019). As for leptin, this hormone regulates energy homeostasis, being secreted at amounts directly proportional to organism's degree of adiposity (Izquierdo et al., 2019). On the other hand, adiponectin, which normally circulates at very high levels, playing a critical role in whole body homeostasis, is secreted at concentrations inversely proportional to body fat mass (Maeda et al., 2020).

Beyond CLA/CLNA capability to treat obesity, these CFA could also be applied in a preventive way, since at a normal state it has been observed reduction of body weight gain (Shen et al., 2013), reduction of adipose tissue weight (Lam et al., 2008; Qi et al., 2014; Yuan, Sun, et al., 2009), reduction of adipocyte area, viability and number (Chou et al., 2012; Ramiah et al., 2014) and reduction of circulating TAG (Chinnadurai et al., 2013; Shagholian et al., 2019; Shinohara, Ito, et al., 2012). Very few studies have stated adverse effects, only being reported for RA; for instance, in mice models of genetic induced hyperlipidemia, it has been observed an increment of body weight after RA administration (Franczyk-Zarów et al., 2015). Furthermore, rats fed a high-fat diet plus RA, revealed increased circulating TAG level (de Almeida, Luquetti, et al., 2015). On the other hand, other studies have not seen any alteration on obesity parameters by CLA/CLNA, whether at an obesity state (Miranda et al., 2009, 2011, 2013) or not (Castellano et al., 2014). Researchers have suggested that discrepancies could be due to variations in experimental design. Indeed, there are differences in terms of animal model type, CFA isomers, isomers mixture *vs*. individual isomers, CFA dose and treatment duration.

Concerning humans, some studies have tested the CLA isomers effects on overweight or obese subjects. When a mixture of CLA isomers (C18:2 c9c12 + C18:2 t10c12, 1:1) was administered (3 g/day) to overweight volunteers, along with exercise, for 4 weeks, a reduction of circulating TAG was observed. Moreover, waist and hip circumferences and fat mass were reduced, although not significantly (Bulut et al., 2013). Following a similar design, but with no exercise and extended treatment time to 24 weeks, no differences were detected in body weight, body mass index (BMI), fat mass or circulating TAG in López-Plaza et al. (2013) research work. However, with no exercise and at a much lower dose (0.4 g/day) of the same CLA mixture, Madry et al. (2016) could observe a reduction in hip and waist circumferences in overweight subjects after 12 weeks. These differences in CLA anti-obesity effect could be a matter of gender, as the first study was performed with only men and the last one with only women. Furthermore, when the above-mentioned CLA mixture was tested in obese volunteers at 3 g/day for 12 weeks, trunk fat decreased, comparing to placebo, but no effect was verified in body weight, BMI, waist circumference or body fat mass (Shahmirzadi et al., 2019). Moreover, when 3.2 g/day of CLA mixture, together with exercise, was administered to obese women for 8 weeks, no alterations were verified in body weight, fat distribution or circulating TAG level (Ribeiro et al., 2016). At similar conditions, but with a slightly lower CLA mixture dose (3 g/day), and combining a weight loss diet instead of exercise, the decrease in fat mass and circulating TAG was more accentuated in CLA treatment than weight loss diet alone in obese subjects. Body weight, BMI and waist and hip circumferences were also reduced comparing to baseline, however not different between tested groups (Ebrahimi-Mameghani et al., 2016). This suggests that obese subjects may benefit from CLA anti-obesity properties when combined with caloric restriction.

Regarding CLNA, only PUA has been tested, and in healthy young volunteers for 28 days at 3 g/day, where no changes were observed towards body weight or BMI (Yuan, Wahlqvist, et al., 2009).

Small sample size, short follow-up period, impossibility to confirm the actual CFA daily intake (lack of supervision during administration) and lack of extrapolation of results to other types of populations or treatment periods, were stated as limitations by some of the above-mentioned studies

testing CLA/CLNA on humans. Furthermore, it has been pointed out several aspects that may implicate CLA/CLNA anti-obesity activity divergency between animal models and humans: dose levels (10-50x higher in animals), age (young and growing subjects in animal experiments), rate of body fat turnover (faster metabolism in small animals), stored body fat amount and regional deposition (heterogenous in humans while well controlled in animals), dietary regimens (*ad libitum* in animal models *vs*. energy restriction in human trials) and body fat measurement accuracy (superior in animals) (Marques et al., 2015; Ribeiro et al., 2016).

Regarding the influence of CLA/CLNA isomers configuration in anti-obesity potency, a study performed with mouse 3T3-L1 adipocytes observed that CLA isomers were able to decrease TAG content, while LA increased such lipids (Martins et al., 2017). Furthermore, using the same cell line, a study revealed that although TAG content was not altered by CLNA, LNA also increased their concentration (Chou et al., 2012). When comparing different CLA isomers, several *in vivo* studies have observed that while C18:2 t10c12 was able to improve body and adipose tissue weights, RA (C18:2 c9t11) was ineffective (Cordoba-Chacon et al., 2019; den Hartigh et al., 2018; Yeganeh et al., 2017). In line with this, when mice were treated with only C18:2 t10c12 or a mixture of C18:2 c9t11 (RA) + C18:2 t10c12 (1:1), those above-mentioned parameters, as well as circulating TAG levels, were equally reduced by both CLA treatments, so, as stated by the authors, inclusion of RA (C18:2 c9t11) did not bring further anti-obesity abilities (Shen et al., 2013). Moreover, when C18:2 t10c12 was compared with C18:2 t8c10 in 3T3-L1 cells, C18:2 t10c12 was more effective in decreasing TAG content (Martins et al., 2017). Furthermore, after C18:2 t10c12 administration together with RA (C18:2 c9t11), at equal proportions, the adipose tissue weight was reported to be reduced in different animal models, without observed effects for α-ESA (C18:3 c9t11t13), PUA (C18:3 c9t11c13) and LNA also (Lam et al., 2008; Yuan, Sun, et al., 2009). Taken all together, it seems that C18:2 t10c12 possesses a structural configuration that somehow confers effective and stronger anti-obesity properties.

In terms of the anti-obesogenic mode of action of CLA/CLNA, there are several proposed hypotheses. Few authors suggest reduction of fat accumulation through down-regulation of lipogenesis and lipolysis, up-regulation of FA oxidation or stimulation of energy expenditure and browning of white adipose tissue (WAT) (Cordoba-Chacon et al., 2019; Shen et al., 2013, 2015). The term ‘browning’ refers to the emergence of beige adipocytes in WAT. White adipocytes, which store chemical energy as lipids and release them as FA during a lack of food, contribute to metabolic disease in obesity, while beige adipocytes, which normally arise in adaptation to increased energy expenditure (i.e. cold and exercise), may counteract obesity (Bartelt & Heeren, 2014). Other authors have suggested that CLA/CLNA act by adipocyte differentiation inhibition and adipocyte apoptosis, being this last one through mitochondrial signaling (intrinsic pathway) and TNF-α activation of death receptor signaling (extrinsic pathway) or via extracellular signal-regulated kinases (ERK) and PPAR-γ signaling (Chou et al., 2012; Qi et al., 2014; Ramiah et al., 2014).

1.3.4. Anti-cholesterolemic

Cholesterol is crucial for animal cell membranes' structure, but also constitutes a precursor for bile acid, steroid hormones and vitamin D synthesis. Cholesterol is transported through the blood stream by lipoproteins and there are two main types: low-density lipoprotein (LDL) and high-density lipoprotein (HDL). The LDL, also called "bad cholesterol", takes cholesterol from liver to body cells, while HDL, the "good cholesterol", transfers it back to the liver where it is wrecked or secreted from the body (Narwal et al., 2019). However, it is the LDL that carries most of the circulating cholesterol (60 – 70% of total cholesterol) (Engeda et al., 2018).

Animal models fed high-fat and/or high-cholesterol diets revealed that CLA/CLNA could increase HDL cholesterol (de Almeida, de Souza, et al., 2015; de Almeida, Luquetti, et al., 2015; Rodríguez-Alcalá et al., 2013), decrease LDL cholesterol (Cho et al., 2016; Farmawati et al., 2016) and lower non-HDL cholesterol (Miranda et al., 2009; O'Reilly et al., 2020; Sengupta et al., 2013). Non-HDL is calculated by subtracting total cholesterol by HDL cholesterol and corresponds to the cholesterol carried by all other circulating lipoproteins, i.e., LDL, very low density lipoprotein (VLDL), intermediate density lipoprotein (IDL) and lipoprotein (a) [Lp (a)], at a fasting state, otherwise it also includes the cholesterol in chylomicron remnants (Langlois & Nordestgaard, 2018). In most of the above-mentioned studies, the reduction of LDL or non-HDL was followed by the lowering of total plasma cholesterol. Lower hepatic cholesterol levels have also been associated to CLA/CLNA administration (O'Reilly et al., 2020; Sengupta et al., 2013). Anti-cholesterol effects have been observed when CLA/CLNA were administered to healthy animals as well (Chinnadurai et al., 2013; Lam et al., 2008; Shagholian et al., 2019). This suggests that such CFA could be applied not only to reverse abnormal cholesterol levels but also to improve regular lipid profiles. Few adverse effects have been reported and only for CLA isomers, more precisely, C18:2 c9t11 (RA) + C18:2 t10c12 (1:1). In the research work from de Castro et al. (2014) an increment in rats' serum total cholesterol was verified without affecting HDL cholesterol, which means that the cholesterol transported by other lipoproteins, such as LDL, was increased. According to Farmawati et al. (2016), they noticed a decrease in HDL cholesterol, but, in this case, LDL cholesterol and LDL/HDL cholesterol ratio were reduced as well. In other cases, CLA/CLNA isomers where not able to cause any effect on cholesterol parameters, whether at an abnormal condition or not (Franczyk-Zarów et al., 2015; Miranda et al., 2013; Yuan, Sun, et al., 2009). In these former studies different CFA were tested, but PSO, which is rich in PUA, was common to all, being administered *ad libitum* to animals at 6.6-10.0 g/Kg diet. On the other hand, in Shagholian et al. (2019) research work, where rats were fed with a specific and controlled dose of 1.5 g PSO/Kg body weight per day, PSO treatment exhibited anti-cholesterol effects.

In humans, only CLA isomers have been tested. When a CLA mixture (C18:2 c9t11 [RA] + C18:2 t10c12 [1:1]) was administered to overweight men at 3 g/day for 4 weeks, along with exercise; it was possible to verify a decrease in LDL cholesterol (Bulut et al., 2013). On the other hand, López-Plaza et al. (2013), who tested the same CLA mixture and dose on overweight subjects, and even for longer period (24 weeks), did not detect any differences, however, no exercise was applied here. Nevertheless, when obese women received a similar dose of the CLA mixture (3.2 g/day) for 8 weeks, along with exercise, no alterations were reported (Ribeiro et al., 2016). Yet, Ebrahimi-

Mameghani et al. (2016) could observe a reduction in LDL/HDL cholesterol ratio in obese subjects when a weight loss diet was coupled to a similar CLA mixture dose (3 g/day) during equal treatment period. Thus, for an obesity state, CLA effect seems to depend on caloric restriction rather than exercise as for overweight. Different limitations have been stated in the above-mentioned studies that could explain the short anti-cholesterol evidences, however, it must not be forgotten the aspects mentioned in the previous **Section 1.3.3 Anti-obesity** (in **1.3 Bioactive properties of CLA/CLNA isomers** of Introduction) that could bias the translation of effects from animals to humans.

Regarding CLA/CLNA isomers, their anti-cholesterolemic properties have not been properly established. For instance, between a CLNA mixture (α-ESA [C18:3 c9t11t13] + β-ESA [C18:3 t9t11t13] 6:1), and a CLA mixture (C18:2 c9t11 [RA] + C18:2 t10c12 [1:1]), Lam et al. (2008) observed that CLA exerted the best results towards different parameters, namely, lower plasma total cholesterol, lower non-HDL/HDL cholesterol ratio, lower cholesterol retained/cholesterol intake ratio and lower hepatic cholesterol. On the other hand, when JA (C18:3 c8t10c12) and C18:2 t10c12 were compared, their effectiveness was similar (Miranda et al., 2009). Similarly, Shagholian et al. (2019) did not detect any difference between the above-mentioned CLA isomers mixture and a PUA (C18:3 c9t11c13) enriched source (PSO).

The mechanisms behind CLA/CLNA anti-cholesterol activity have not been fully explored. Some reported results suggest that their action is related to up-regulation of cholesterol homeostasis and metabolism and down-regulation of cholesterol biosynthesis (Nicod et al., 2015; O'Reilly et al., 2020). Other findings indicate that it is mediated by down-regulation of intestinal acyl coenzyme A: cholesterol acyltransferase (ACAT) activity and consequent reduction of cholesterol absorption in intestine (Lam et al., 2008; Nicod et al., 2015). The ACAT is responsible for cholesterol conversion into cholesterol esters, its storage form (Xu et al., 2019). If cholesterol is absorbed less in the intestine, then more can be excreted in feces, but there is a lack of reports about this matter.

1.3.5. Anti-diabetic

Diabetes mellitus is characterized by a hyperglycemia state caused by deficiencies in insulin secretion and/or action (Chaudhury et al., 2017). Type 1 diabetes mellitus (T1DM) is associated to insufficient insulin secretion due to pancreatic β-cell destruction. Type 2 diabetes mellitus (T2DM), the most common among diabetes cases, is characterized by impairment of insulin secretion and action, being genetic pre-disposition and environmental conditions involved in its pathogenesis. Unbalanced lifestyles and eating habits potentiate T2DM development as well (Öztürk et al., 2017).

Isomers of CLA/CLNA have been tested in animal models against T2DM or metabolic disorders prone to T2DM development, i.e., obesity or consumption of high-fat and/or high-sucrose diets. Some research works have provided evidences over CLA/CLNA anti-diabetic potential, such as, decrease of fasting glucose and insulin levels, reduction of glucose tolerance and lowering of insulin resistance (Cho et al., 2016; Miranda et al., 2013; Song et al., 2016). Moreover, CLA isomers have reduced post-prandial glucose and insulin concentrations, and incremented glucose oxidation and incorporation into lipids in epididymal adipose tissue, at basal and insulin-stimulated conditions of rats (Malinska et al., 2015). However, CLA/CLNA isomers may not be applied preventively in healthy

conditions, as several studies have reported adverse effects in healthy models, such as, increase of glucose and insulin contents and increment of insulin resistance (Bezan et al., 2018; Cordoba-Chacon et al., 2019; Pini et al., 2016).

With respect to humans, PUA revealed no effect on glucose or insulin parameters after healthy young subjects were treated with 3 g/day for 28 days (Yuan, Wahlqvist, et al., 2009). Even when PSO (rich in PUA) was administered to T2DM patients, at 2 g/day for 8 weeks, no changes were observed (Faghihimani et al., 2016). A CLA mixture of C18:2 c9t11 (RA) + C18:2 t10c12 (1:1) have been tested in obese children at 3 g/day for 16 weeks and, although insulin level and insulin resistance were reduced comparing to baseline, final values were not different from control (Garibay-Nieto et al., 2017). Furthermore, at an equal dose, but during a shorter time period (8 weeks), this CLA mixture, coupled with a weight loss diet, could not decrease insulin level in obese volunteers, not being different from control group (i.e., weight loss diet alone) (Ebrahimi-Mameghani et al., 2016). On the other hand, a similar study carried out for a longer time period (12 weeks) and without any special diet, detected a decrease in glucose content and insulin resistance (Shahmirzadi et al., 2019). Nevertheless, when combined with exercise, 3 g/day of CLA mixture could reduce insulin level of overweight volunteers, even more than exercise alone, after just 4 weeks (Bulut et al., 2013). Insulin resistance was also reduced in this last study, yet not different from control.

1.4. Microbial production of CLA/CLNA isomers

In order to obtain beneficial health effects from CLA and CLNA, it would be needed for a human to consume approximately 3-6 g/day and 2-3 g/day, respectively (Fuke & Nornberg, 2017; Shinohara, Tsuduki, et al., 2012). Given the natural CLA/CLNA contents reported for ruminants' milk or meat (**Section 1.2 Natural sources of CLA/CLNA isomers** of Introduction), which are very low, it would not be feasible to attain those effective doses from these sources. Moreover, very few of the mentioned vegetable oils rich in CLNA isomers (**Section 1.2 Natural sources of CLA/CLNA isomers** of Introduction) are commercially available for consumption. Therefore, strategies to increment CLA/CLNA daily consumption have been addressed in the last years. A possible alternative is through *in situ* microbial enrichment of food products, since CLA and CLNA can be naturally produced from their precursor substrates by certain microorganisms. The following sections outline the available information about CLA/CLNA microbial production.

1.4.1. Rumen biohydrogenation

The CLA and CLNA isomers are naturally formed during LA (C18:2 c9c12) and α-LNA (C18:3 c9c12c15) biohydrogenation process in ruminants, a multi-step pathway carried out by different microorganisms on unsaturated FAs (**Figure 1.1**). Once in the rumen, dietary LA and α-LNA are hydrolyzed by microbial lipases from *Butyrivibrio fibrisolvens* (hydrolyses phospholipids) and *Anaerovibrio lipolytica* (hydrolyses di- and triacylglycerols) into free fatty acids (FFA), for further isomerization and hydrogenation (Buccioni et al., 2012). The biohydrogenation of LA involves two main steps: i) isomerization of LA to C18:2 c9t11 (RA) and then ii) 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). However, other C18:2 c9c11 , C18:2 t8t10 and C18:2 t10c12 isomers are also produced through this pathway (Chilliard et al., 2007). The α-LNA biohydrogenation differs only in the intermediate products, thus, after hydrolysis, this FA is first isomerized at *cis*-12 position producing C18:3 c9t11c15 (RLA). Subsequently, RLA is reduced to C18:2 t11c15 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). Another secondary pathway has been proposed from C18:3 c9t13c15, which is transformed into C18:2 c9t13 and C18:2 t13c15, being this former FA further reduced to C18:1 t13 (Destaillats et al., 2005). Previous works suggest that CLA isomers are also released during α-LNA metabolism, such as, C18:2 c9c11 (among other 9,11-configuration isomers), C18:2 t8t10, C18:2 c10c12 and C18:2 t11t13 (Jouany et al., 2007; Lee & Jenkins, 2011; Loor et al., 2005). All biohydrogenation products 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 of ruminants (Gorissen et al., 2015). However, CLA is also produced at the mammary gland of lactating cows through the conversion of TVA (coming from rumen) by the Δ9-desaturase, which represents the primary source of CLA in bovine milk (64%) (Griinari et al., 2000).

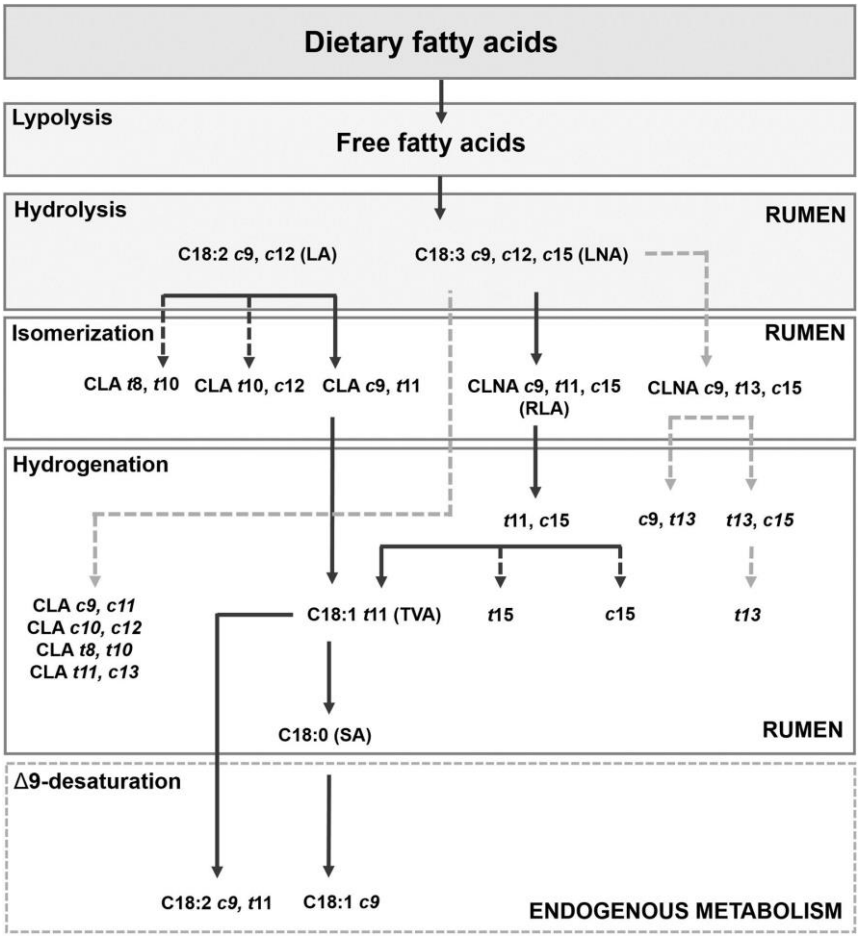


Figure 1.1 Linoleic (LA) and α-linolenic (LNA) biohydrogenation pathways in ruminants (bold arrows - principal pathway; broken lines - alternative pathways). CLA – Conjugated linoleic acid, CLNA – Conjugated linolenic acid, RLA – Rumelenic acid, TVA – *trans*-Vaccenic acid, SA – Stearic acid. [*retrieved from Salsinha et al. (2018)*]

1.4.2. Production by probiotic bacteria

Butyrivibrio fibrisolvens has long been described as the main ruminal bacteria involved in LA and α-LNA biohydrogenation process (Kepler et al., 1966). However, other species than ruminal bacteria have shown the capacity to produce CLA and/or CLNA isomers from their precursor substrates as well, namely, probiotic strains isolated from dairy products and from the human intestinal tract (Fontes et al., 2017; Yang, Gao, et al., 2017). It has been suggested that such ability of probiotics might consist of a detoxifying mechanism from LA/α-LNA (Jiang et al., 1998). In fact, microbial growth is affected by the presence of these FA (Kankaanpää et al., 2001; Mei et al., 2021). Evidence towards CLA/CLNA formation by probiotics and the employment of this feature to develop enriched food sources are discussed below.

1.4.2.1. In vitro

Assays in culture media have demonstrated that CLA and CLNA can be microbiologically produced by different species of bifidobacteria, lactobacilli and propionibacteria. However, not all reported strains are good CLA/CLNA producers, therefore, having in mind the purpose of employing such bacteria for the development of a functional food product enriched in CLA/CLNA isomers, only the strains showing relatively high substrate conversion ability (>40%) were considered for the discussion herein.

Regarding CLA production by bifidobacteria (**Table 1.5**), about 40.7% of 0.5 mg/mL LA could be isomerized by *Bifidobacterium bifidum* LMG 10645 when grown in De Man–Rogosa–Sharpe medium supplemented with cysteine (cys-MRS), at 37 ºC for 72 h (Gorissen et al., 2010). At equal conditions, *Bifidobacterium pseudocatenulatum* MY45B and *Bifidobacterium pseudolongum* subsp. *pseudolongum* LMG 11595 converted 49.9% and 42.2% of the substrate, respectively (Gao et al., 2019; Gorissen et al., 2010). Similarly, *Bifidobacterium longum* DPC6320 transformed 45.5% of 0.45 mg/mL LA into CLA isomers after 72 h at 37 ºC (Hennessy et al., 2012). *Bifidobacterium breve* has been the most studied species on this matter and showing the best CLA yields, with LA conversion percentages that can reach >80% (Gao et al., 2019; Raimondi et al., 2016). Beyond species-specific, CLA-producing capacity has also been shown to be strain-dependent since at equal growth conditions there are discrepancies among the same species. For instance, in cys-MRS at 0.5 mg/mL LA and 37 ºC for 72 h, *B. breve* CCFM683 could convert 85.9% of the substrate (Gao et al., 2019) while *B. breve* LMG 11040 reached 44% transformation (Gorissen et al., 2010). Furthermore, in Raimondi et al. (2016) study, who tested different *B. breve* isolates at similar former conditions, but for 48 h, *B. breve* WC 0421 demonstrated the best LA conversion rate (88.1%), while *B. breve* WC 0422 capacity did not go further than 41.8%.

Concerning lactobacilli and proponibacteria, even though fewer than bifidobacteria, there are some reported strains with good CLA production ability (**Table 1.6**). Namely, *Lactiplantibacillus plantarum* ZS2058 and *Limosilactobacillus reuteri* ATCC55739 have been able to convert 56.9% and 64.1% of 0.55 mg/mL LA, respectively, when cultured in MRS at 37 ºC for 48 h (Yang et al., 2014). At a slightly higher substrate concentration (0.6 mg/mL), *Lactiplantibacillus paraplantarum* BNU105655 transformed 51.6% of the LA into CLA isomers, but *Lactiplantibacillus pentosus*

JBNU105674 revealed the best capacity, by isomerizing the substrate almost completely (99.5%) (Song et al., 2021). *Propionibacterium freudenreichii* subsp. *freudenreichii* Propioni-6 and *Propionibacterium freudenreichii* subsp. *shermanii* 9093, when grown in cys-MRS at 0.45 mg/mL LA and 30 ºC for 72 h, showed conversion percentages of 44.7% and 50.5%, respectively (Hennessy et al., 2012). On the other hand, *Propionibacterium freudenreichii* DSM 20270 revealed a 67% isomerization capacity, but was cultured in synthetic lactate growth medium (SLGM) at 0.4 mg/mL LA and 30 ºC for 70 h (Vahvaselkä et al., 2021).

The CLA isomers produced by the above-mentioned strains were C18:2 c9t11 (RA), C18:2 t9t11, and, in some cases, C18:2 t10c12 (**Table 1.5** and **Table 1.6**).

As for CLNA production, within bifidobacteria (**Table 1.7** and **Table 1.8**), when *B. bifidum* LMG 10645 and *B. pseudolongum* subsp. *pseudolongum* LMG 11595 were grown in cys-MRS at 37 ºC for 72 h, 78.4% and 62.7% of 0.5 mg/mL α-LNA were converted (Gorissen et al., 2010). On the other hand, Yang et al. (2017) observed that *B. pseudocatenulatum* could attain 90.5% of isomerization after 48h at 0.37 mg/mL α-LNA and 37 ºC. This former study was carried out with infant intestinal isolates, where several *B. longum* strains were also identified and tested for CLNA production, which revealed up to 87.7% of α-LNA isomerization capacity. Similarly to what was observed for CLA, *B. breve* strains have been described with the best CLNA outcome, converting >95% of 0.5 mg/mL α-LNA at 37 ºC for 24 h (Villar-Tajadura et al., 2014). Moreover, CLNA yields were also shown to be strain-dependent, since after 72 h at 0.45 mg/mL α-LNA and 37 ºC the conversion ability of *B. breve* strains could vary from 49.7% to 83% (Hennessy et al., 2012). Similarly, the tested *B. longum* strains by Yang et al. (2017), as described above, showed an isomerization range that could decrease to 46.3%.

In contrast to bifidobacteria, very few lactobacilli and propionibacteria have been reported with good CLNA production capacity (**Table 1.8**). *Latilactobacillus sakei* LMG 13558 was able to convert 60.1% of 0.5 mg/mL α-LNA when cultured in MRS at 30 ºC for 72 h (Gorissen et al., 2011). At similar conditions, but in cys-MRS, *P. freudenreichii* subsp. *shermanii* 9093 could transform 53.6% of 0.45 mg/mL α-LNA into CLNA isomers (Hennessy et al., 2012). On the other hand, *P. freudenreichii* DSM 20270 revealed a 82% isomerization capacity, but it was grown in SLGM at 0.4 mg/mL α-LNA and 30 ºC for 70 h (Vahvaselkä et al., 2021).The CLNA isomers produced by the above-mentioned strains were C18:3 c9t11c15 (RLA) and C18:3 t9t11c15.

In comparison to CLA, microbial CLNA yields are generally higher, ca. 72% α-LNA *vs*. 64% LA transformed into conjugated isomers (calculated average from presented data in **Table 1.5** to **Table 1.8**). There are two hypotheses for such finding: i) the α-LNA is more toxic to bacteria than LA, taking into account the detoxification mechanism previously stated (Jiang et al., 1998), or ii) its enzymatic system has a higher specificity for α-LNA. In fact, Meynadier et al. (2018) observed with an enzymatic solution obtained from ruminal bacteria that, although it showed higher affinity for LA (lower K_m value, i.e., saturates more rapidly), the isomerization of α-LNA was more efficient (higher V_{max} value, i.e., higher catalysis rate when saturated).

Table 1.5 Conjugated linoleic acid-producing bifidobacteria, growth conditions, linoleic acid conversion percentages and isomer(s) produced.

Strain	Medium	LA (mg/mL)	T (ºC)	t (h)	LA conv. (%)	Isomer(s)	Reference
Bifidobacterium bifidum LMG 10645	cys-MRS	0.5	37	72	40.7%	c9t11, t9t11	Gorissen et al. (2010)
Bifidobacterium breve 29M2	cys-MRS	0.5	37	24	74.1%	c9t11, t10c12, t9t11	Villar-Tajadura et al. (2014)
Bifidobacterium breve CCFM622	cys-MRS	0.5	37	72	80.7%	*not specified*	Gao et al. (2019)
Bifidobacterium breve CCFM683	cys-MRS	0.5	37	72	85.9%	c9t11, t9t11	Gao et al. (2019)
Bifidobacterium breve CCFM684	cys-MRS	0.5	37	72	83.3%	*not specified*	Gao et al. (2019)
Bifidobacterium breve DPC6330	cys-MRS	0.45	37	72	66.7%	c9t11, t9t11	Hennessy et al. (2012)
Bifidobacterium breve LMG 11040	cys-MRS	0.5	37	72	44.0%	c9t11, t9t11	Gorissen et al. (2010)
Bifidobacterium breve LMG 11084	cys-MRS	0.5	37	72	53.5%	c9t11, t9t11	Gorissen et al. (2010)
Bifidobacterium breve M7-70	cys-MRS	0.5	37	24	77.8%	c9t11, t10c12, t9t11	Villar-Tajadura et al. (2014)
Bifidobacterium breve NCFB 2258	cys-MRS	0.55	37	48	72.4%	c9t11, t9t11	Coakley et al. (2003)
Bifidobacterium breve NCIMB 702258	cys-MRS	0.45	37	72	60.0%	c9t11, t9t11	Hennessy et al. (2012)
Bifidobacterium breve NCIMB 8807	cys-MRS	0.45	37	72	64.3%	c9t11, t9t11	Hennessy et al. (2012)
Bifidobacterium breve NCIMB 8815	cys-MRS	0.55	37	48	44.0%	c9t11, t10c12, t9t11	Coakley et al. (2003)
Bifidobacterium breve WC 0421	cys-MRS	0.5	37	48	88.1%	c9t11, t9t11	Raimondi et al. (2016)
Bifidobacterium breve WC 0422	cys-MRS	0.5	37	48	41.8%	c9t11, t9t11	Raimondi et al. (2016)
Bifidobacterium breve WC 0423	cys-MRS	0.5	37	48	87.6%	c9t11, t9t11	Raimondi et al. (2016)
Bifidobacterium breve WC 0424	cys-MRS	0.5	37	48	77.3%	c9t11, t9t11	Raimondi et al. (2016)
Bifidobacterium breve ZL12-28	cys-MRS	0.5	37	24	74.6%	c9t11, t10c12, t9t11	Villar-Tajadura et al. (2014)
Bifidobacterium longum DPC6320	cys-MRS	0.45	37	72	45.5%	c9t11, t9t11	Hennessy et al. (2012)
Bifidobacterium pseudocatenulatum MY45B	cys-MRS	0.5	37	72	49.9%	c9t11, t9t11	Gao et al. (2019)
Bifidobacterium pseudolongum subsp. *pseudolongum* LMG 11595	cys-MRS	0.5	37	72	42.2%	c9t11, t9t11	Gorissen et al. (2010)

LA – Linoleic acid; LA conv. – Linoleic acid conversion; cys – Cysteine; MRS – De Man–Rogosa–Sharpe; *c* – *cis* double bond; *t* – *trans* double bond.

Table 1.6 Conjugated linoleic acid-producing lactobacilli and propionibacteria, growth conditions, linoleic acid conversion percentages and isomer(s) produced.

Strain	Medium	LA (mg/mL)	T (ºC)	t (h)	LA conv. (%)	Isomer(s)	Reference
Lactobacilli							
Lactiplantibacillus plantarum ZS2058	MRS	0.55	37	48	56.9%	c9t11, t10c12, t9t11	Yang et al. (2014)
Lactiplantibacillus paraplantarum BNU105655	MRS	0.6	37	48	51.6%	c9t11, t10c12	Song et al. (2021)
Lactiplantibacillus pentosus JBNU105674	MRS	0.6	37	48	99.5%	c9t11, t10c12	Song et al. (2021)
Limosilactobacillus reuteri ATCC55739	MRS	0.55	37	48	64.1%	c9t11, t10c12, t9t11	Yang et al. (2014)
Propionibacteria							
Propionibacterium freudenreichii DSM 20270	SLGM	0.4	30	70	67.0%	c9t11, t10c12, t9t11	Vahvaselkä et al. (2021)
Propionibacterium freudenreichii subsp. *freudenreichii* Propioni-6	cys-MRS	0.45	30	72	44.7%	c9t11, t9t11	Hennessy et al. (2012)
Propionibacterium freudenreichii subsp. *shermanii* 9093	cys-MRS	0.45	30	72	50.5%	c9t11, t9t11	Hennessy et al. (2012)

LA – Linoleic acid; LA conv. – Linoleic acid conversion; MRS – De Man–Rogosa–Sharpe; SLGM – Synthetic lactate growth medium; cys – Cysteine; *c* – *cis* double bond; *t* – *trans* double bond.

Table 1.7 Conjugated linolenic acid-producing bifidobacteria, growth conditions, α-linolenic acid conversion percentages and isomer(s) produced.

Strain	Medium	α-LNA (mg/mL)	T (ºC)	t (h)	α-LNA conv. (%)	Isomer(s)	Reference
Bifidobacterium bifidum LMG 10645	cys-MRS	0.5	37	72	78.4%	c9t11c15, t9t11c15	Gorissen et al. (2010)
Bifidobacterium breve 29M2	cys-MRS	0.5	37	24	95.7%	c9t11c15, t9t11c15	Villar-Tajadura et al. (2014)
Bifidobacterium breve C11	cys-MRS	0.37	37	48	84.8%	c9t11c15, t9t11c15	Yang et al. (2017)
Bifidobacterium breve C21	cys-MRS	0.37	37	48	89.7%	c9t11c15, t9t11c15	Yang et al. (2017)
Bifidobacterium breve DPC6330	cys-MRS	0.45	37	72	83.0%	9,11,15	Hennessy et al. (2012)
Bifidobacterium breve LMG 11040	cys-MRS	0.5	37	72	65.5%	*not specified*	Gorissen et al. (2010)
Bifidobacterium breve LMG 11084	cys-MRS	0.5	37	72	72.0%	*not specified*	Gorissen et al. (2010)
Bifidobacterium breve LMG 11613	cys-MRS	0.5	37	72	55.6%	*not specified*	Gorissen et al. (2010)
Bifidobacterium breve LMG 13194	cys-MRS	0.5	37	72	63.3%	*not specified*	Gorissen et al. (2010)
Bifidobacterium breve M7-70	cys-MRS	0.5	37	24	98.7%	c9t11c15, t9t11c15	Villar-Tajadura et al. (2014)
Bifidobacterium breve NCIMB 702258	cys-MRS	0.45	37	72	49.7%	9,11,15	Hennessy et al. (2012)
Bifidobacterium breve NCIMB 8807	cys-MRS	0.45	37	72	68.3%	9,11,15	Hennessy et al. (2012)
Bifidobacterium breve ZL12-28	cys-MRS	0.5	37	24	98.8%	c9t11c15, t9t11c15	Villar-Tajadura et al. (2014)
Bifidobacterium longum C10	cys-MRS	0.37	37	48	71.5%	c9t11c15, t9t11c15	Yang et al. (2017)
Bifidobacterium longum C23	cys-MRS	0.37	37	48	55.0%	c9t11c15, t9t11c15	Yang et al. (2017)
Bifidobacterium longum C5	cys-MRS	0.37	37	48	70.0%	c9t11c15, t9t11c15	Yang et al. (2017)
Bifidobacterium longum C9	cys-MRS	0.37	37	48	75.0%	c9t11c15, t9t11c15	Yang et al. (2017)
Bifidobacterium longum E38	cys-MRS	0.37	37	48	65.2%	c9t11c15, t9t11c15	Yang et al. (2017)
Bifidobacterium longum E41	cys-MRS	0.37	37	48	66.1%	c9t11c15, t9t11c15	Yang et al. (2017)
Bifidobacterium longum GM59	cys-MRS	0.37	37	48	83.5%	c9t11c15, t9t11c15	Yang et al. (2017)
Bifidobacterium longum GN22A2	cys-MRS	0.37	37	48	87.7%	c9t11c15, t9t11c15	Yang et al. (2017)
Bifidobacterium longum GN32A	cys-MRS	0.37	37	48	74.4%	c9t11c15, t9t11c15	Yang et al. (2017)
Bifidobacterium longum GN40A	cys-MRS	0.37	37	48	74.0%	c9t11c15, t9t11c15	Yang et al. (2017)

α-LNA – alpha-Linolenic acid; α-LNA conv. – alpha-Linolenic acid conversion; cys - Cysteine; MRS – De Man–Rogosa–Sharpe; *c* – *cis* double bond; *t* – *trans* double bond.

Table 1.8 Conjugated linolenic acid-producing bifidobacteria (*continued*), lactobacilli and propionibacteria, growth conditions, α-linolenic acid conversion percentages and isomer(s) produced.

Strain	Medium	α-LNA (mg/mL)	T (ºC)	t (h)	α-LNA conv. (%)	Isomer(s)	Reference
Bifidobacteria							
Bifidobacterium longum GN45	cys-MRS	0.37	37	48	72.3%	c9t11c15, t9t11c15	Yang et al. (2017)
Bifidobacterium longum GN57A	cys-MRS	0.37	37	48	56.2%	c9t11c15, t9t11c15	Yang et al. (2017)
Bifidobacterium longum NY26	cys-MRS	0.37	37	48	46.3%	c9t11c15, t9t11c15	Yang et al. (2017)
Bifidobacterium longum NY5	cys-MRS	0.37	37	48	50.0%	c9t11c15, t9t11c15	Yang et al. (2017)
Bifidobacterium longum PA34B	cys-MRS	0.37	37	48	74.4%	c9t11c15, t9t11c15	Yang et al. (2017)
Bifidobacterium longum PA7A	cys-MRS	0.37	37	48	48.8%	c9t11c15, t9t11c15	Yang et al. (2017)
Bifidobacterium pseudocatenulatum PA3	cys-MRS	0.37	37	48	90.5%	c9t11c15, t9t11c15	Yang et al. (2017)
Bifidobacterium pseudocatenulatum PA52	cys-MRS	0.37	37	48	88.7%	c9t11c15, t9t11c15	Yang et al. (2017)
Bifidobacterium pseudolongum subsp. *pseudolongum* LMG 11595	cys-MRS	0.5	37	72	62.7%	*not specified*	Gorissen et al. (2010)
Lactobacilli							
Latilactobacillus sakei LMG 13558	MRS	0.5	30	72	60.1%	c9t11c15, t9t11c15	Gorissen et al. (2011)
Propionibacteria							
Propionibacterium freudenreichii DSM 20270	SLGM	0.4	30	70	82.0%	c9t11c15, t9t11c15	Vahvaselkä et al. (2021)
Propionibacterium freudenreichii subsp. *shermanii* 9093	cys-MRS	0.45	30	72	53.6%	9,11,15	Hennessy et al. (2012)

α-LNA – alpha-Linolenic acid; α-LNA conv. – alpha-Linolenic acid conversion; cys – Cysteine; MRS – De Man–Rogosa–Sharpe; SLGM – Synthetic lactate growth medium; *c* – *cis* double bond; *t* – *trans* double bond.

1.4.2.2. *In situ*

The microbial enrichment with CLA isomers has been tested in several food matrices, including different types of milk, meat and even food residues. Regarding bifidobacteria (**Table 1.9**), when *B. breve* LMC 017 was co-cultured with a yogurt starter in milk containing 0.5% of substrate, it yielded 0.58 mg/mL of CLA after 9 h at 30 ºC (Chung et al., 2008). However, in the same study, skim-milk fermented solely by this strain, at equal conditions, could be enriched with ~1.06 mg/mL of CLA. This shows that the yogurt starter culture interfered with *B. breve* LMC 017 biotransformation capacity, not enabling the development of a yogurt with the best possible CLA content. Furthermore, other investigations in skim-milk showed 0.66 mg CLA/g fat after fermentation with *B. bifidum* 420 at 30 ºC for 24 h (Xu et al., 2004). This former research work was performed with 1% of previously hydrolyzed soy oil, so that the naturally present LA would be bioavailable for microbial isomerization. In fact, when Gorissen et al. (2012) tested different strains from *B. bifidum*, *B. breve* and *B. pseudolongum* subsp. *pseudolongum* species, no conjugated isomers production was detected in milk supplemented with non-hydrolyzed vegetable oils. Moreover, as stated in **Section 1.4.1 Rumen biohydrogenation** (in **1.4 Microbial production of CLA/CLNA isomers** of Introduction), ruminal bacteria hydrolyze the dietary LA/α-LNA before further biohydrogenation, therefore, microbial conjugated isomers can only be produced from free precursor substrates. In the same way, walnut milk was hydrolyzed with lipases prior to fermentation with *B. breve* CCFM683, where after 20 h at 30 ºC was attained a CLA content of 1.29 mg/mL (Mao et al., 2022). On the other hand, *Bifidobacterium animalis* subsp. *lactis* BB12 has been able to double CLA amount in conventional and organic whole milks (from 0.25 to 0.54 g/100 g FA and from 0.48 to 1.04 g/100 g FA, respectively) when fermented for 30 h at 30 ºC without external addition of LA (Moghadam et al., 2022). This suggests that, contrary to other bifidobacteria species, *B. animalis* subsp. *lactis* may possess certain lipolytic activities, releasing free LA from milk for further isomerization. Apart from dairy products, *in situ* CLA production by bifidobacteria has also been assayed in a soybean press residue, where fermentation by *Bifidobacterium lactis* BB04 at 30 ºC for 48 h resulted in 0.18 g/100 g FA of CLA, when initial amount was none (Azari et al., 2022).

Concerning lactobacilli (**Table 1.9** and **Table 1.10**), among different strains of *Lacticaseibacillus casei*, *Lacticaseibacillus rhamnosus*, *Lactiplantibacillus plantarum* and *Lactobacillus acidophilus* tested in skim-milk devoid of CLA with 1% of hydrolyzed soy oil, CLA contents of up to 0.94 mg/g fat were achieved after 24 h or 48 h at 30 ºC (Xu et al., 2004). In whole milk, which contained 0.41 g/100 g FA of CLA, 4 h of fermentation at 30 ºC with *Lactobacillus* sp. 11a and no external substrate, increased CLA content to 0.63 g/100 g FA (Pandit et al., 2012). Even though CLA yield was not so prominent, this study showed that the assayed strain had the capacity to convert some of the milk's natural LA. In fact, lactic acid bacteria (LAB) possess weak lipolytic activity (García-Cano et al., 2019). Furthermore, Ares-Yebra et al. (2019) was able to develop miniature cheeses with 11.1 mg/g fat of CLA with no LA addition as well, by co-culturing *L. plantarum* L200 with the appropriate starter culture for 48 h at 30 ºC. Here the control cheeses (only starter culture) contained 7.1 mg/g fat of CLA, so, apparently, the starter culture did not interfere with *L. plantarum* L200 activity. Microbial CLA-enrichment has also been assessed in meat food products,

particularly with *L. plantarum* strains. In Özer et al. (2016) study, who assayed *L. plantarum* AA1-2 or *L. plantarum* AB20-961 in sucuk at 2% of sunflower oil, CLA content only slightly increased from 4.05 mg/g fat (before fermentation) up to 4.55 mg/g fat. On the other hand, *L. plantarum* DSM 2601 has shown better CLA yields in meat. Namely, in sausage at 5% of hydrolyzed safflower oil, it was attained 7.54 mg/g fat of CLA, while initially the amount was 3.41 mg/g fat (Özer & Kılıç, 2020). Yet, at similar conditions, ground beef CLA value increased from 3.73 mg/g fat (before fermentation) to 42.32 mg/g fat after fermentation (Özer & Kılıç, 2021). This shows that CLA yield is not only strain-dependent but also influenced by the type of food matrix.

In what concerns the *Propionibacterium* genus (**Table 1.11**), within different strains assayed in skim-milk that did not contain any CLA, at 1% of hydrolyzed soy oil, fermentation by *P. freudenreichii* subsp. *shermanii* 51 resulted in the highest CLA-enrichment of 1.64 mg/g fat, after 24 h at 32 ºC (Xu et al., 2004). On the other hand, when propionibacteria strains were co-cultured in milk with yogurt starter, at 5 mg/mL of free LA from hydrolyzed soy oil, CLA amount did not go further than 1 mg/g fat after fermentation, while control yogurt (only starter) contained 0.72 mg/g fat of CLA (Xu et al., 2005). Meanwhile, *in situ* CLA production has also been reported for a previously hydrolyzed blackcurrant press residue. After 48 h at 30 ºC with *P. freudenreichii* DSM 20270, 0.51 mg/mL of CLA were yielded into a 10% blackcurrant press residue slurry (Vahvaselkä et al., 2021).

In comparison to CLA, very few studies have focused on the enrichment of food matrices with microbial CLNA isomers (**Table 1.12**). A study carried out with skim-milk showed that, among the *B. breve* strains tested at 0.5 mg/mL α-LNA, they could yield up to ~0.24 mg/mL of CLNA after 24 h at 37 ºC (Villar-Tajadura et al., 2014). At similar conditions, a further research work observed that semi-skimmed milk, which initially contained 0.007 mg/mL of CLNA, ended up with 0.115 mg/mL after fermentation with *B. breve* NCIMB 702258 (Fontes et al., 2018). On the other hand, Mao et al. (2022) obtained a hydrolyzed walnut milk with about 1 mg/mL of CLNA by fermenting with *B. breve* CCFM683 at 37 ºC for 20 h. Moreover, *P. freudenreichii* DSM 20270 yielded 0.29 mg/mL of CLNA into a 10% hydrolyzed blackcurrant press residue slurry after 48 h at 30ºC (Vahvaselkä et al., 2021).

Taken all together, the above-mentioned research works suggest that it may be possible to enrich food matrices with microbial CLA/CLNA isomers to a certain extent. In addition, some studies have shown that vegetable oils could be applied as substrate sources if previously hydrolyzed, becoming a more cost-effective alternative to pure substrates.

Table 1.9 Conjugated linoleic acid (CLA) production in food matrices by bifidobacteria and lactobacilli, fermentation conditions and obtained CLA content/yield.

Strain	Matrix	Substrate source	[Substrate]	T (ºC)	t (h)	CLA content/yield	Observations	Reference
Bifidobacteria								
Bifidobacterium animalis subsp. *lactis* BB12	Conventional whole milk	Natural LA	*not specified*	30	30	0.54 g/100 g FA	Matrix – 0.25 g/100 g FA of CLA before fermentation	Moghadam et al. (2022)
	Organic whole milk	Natural LA	*not specified*	30	30	1.04 g/100 g FA	Matrix – 0.48 g/100 g FA of CLA before fermentation	Moghadam et al. (2022)
Bifidobacterium bifidum 420	Skim-milk	Hydrolyzed soy oil	1%	30	24	0.66 mg/g fat	Matrix – no CLA before fermentation	Xu et al. (2004)
Bifidobacterium breve CCFM683	Hydrolyzed walnut milk	Natural LA	*not specified*	30	20	1.29 mg/mL		Mao et al. (2022)
Bifidobacterium breve LMC 017	Skim-milk	Monolinolein	0.5%	30	9	1.056 mg/mL	Yielded amount	Chung et al. (2008)
Bifidobacterium breve LMC 017*	Milk	Monolinolein	0.5%	30	9	0.58 mg/mL	Yielded amount	Chung et al. (2008)
Bifidobacterium lactis BB04	Soybean press residue	Natural LA	*not specified*	30	48	0.18 g/100 g FA	Matrix – no CLA before fermentation	Azari et al. (2022)
Lactobacilli								
Lacticaseibacillus casei 163	Skim-milk	Hydrolyzed soy oil	1%	30	24	0.70 mg/g fat	Matrix – no CLA before fermentation	Xu et al. (2004)
Lacticaseibacillus rhamnosus	Skim-milk	Hydrolyzed soy oil	1%	30	48	0.73 mg/g fat	Matrix – no CLA before fermentation	Xu et al. (2004)

*Co-cultured with yogurt starter.

CLA – Conjugated linoleic acid; LA – Linoleic acid; FA – Fatty acid.

Table 1.10 Conjugated linoleic acid (CLA) production in food matrices by lactobacilli (*continued*), fermentation conditions and obtained CLA content/yield.

Strain	Matrix	Substrate source	[Substrate]	T (ºC)	t (h)	CLA content/yield	Observations	Reference
Lactiplantibacillus plantarum AA1-2	Sucuk	Hydrolyzed sunflower oil	2%	24-18	*7 d*	4.44 mg/g fat	Matrix – 4.05 mg/g fat of CLA before fermentation	Özer et al. (2016)
	Sucuk	Hydrolyzed sunflower oil	2%	24-18	*7 d*	4.55 mg/g fat	Matrix – 4.05 mg/g fat of CLA before fermentation	Özer et al. (2016)
Lactiplantibacillus plantarum AB20-961	Ground beef	Hydrolyzed safflower oil	5%	30	78.78	7.65 mg/g fat	Matrix – 3.73 mg/g fat of CLA before fermentation	Özer and Kiliç (2021)
	Sausage	Hydrolyzed safflower oil	5%	30	78.78	4.15 mg/g fat	Matrix – 3.41 mg/g fat of CLA before fermentation	Özer and Kılıç (2020)
Lactiplantibacillus plantarum DSM 2601	Ground beef	Hydrolyzed safflower oil	5%	30	72.57	42.32 mg/g fat	Matrix – 3.73 mg/g fat of CLA before fermentation	Özer and Kiliç (2021)
	Sausage	Hydrolyzed safflower oil	5%	30	72.57	7.54 mg/g fat	Matrix – 3.41 mg/g fat of CLA before fermentation	Özer and Kılıç (2020)
Lactiplantibacillus plantarum L200*	Milk	Natural LA	*not specified*	30	48	11.1 mg/g fat	Control (only starter culture) – 7.1 mg/g fat of CLA after fermentation	Ares-Yebra et al. (2019)
Lactiplantibacillus plantarum L2-1	Skim-milk	Hydrolyzed soy oil	1%	30	48	0.82 mg/g fat	Matrix - no CLA before fermentation	Xu et al. (2004)
Lactobacillus acidophilus 74-2	Skim-milk	Hydrolyzed soy oil	1%	30	48	0.94 mg/g fat	Matrix - no CLA before fermentation	Xu et al. (2004)
Lactobacillus sp. 11a	Whole milk	Natural LA	*not specified*	30	4	0.63 g/100 g FA	Matrix – 0.41 g/100 g FA of CLA before fermentation	Pandit et al. (2012)

*Co-cultured with cheese starter.

CLA – Conjugated linoleic acid; LA – Linoleic acid; d – days; FA – Fatty acid.

Table 1.11 Conjugated linoleic acid (CLA) production in food matrices by propionibacteria, fermentation conditions and obtained CLA content/yield.

Strain	Matrix	Substrate source	[Substrate]	T (ºC)	t (h)	CLA content/yield	Observations	Reference
Propionibacterium freudenreichii DSM 20270	Hydrolyzed blackcurrant press residue	Natural LA	14 mg/g DM (free LA)	30	48	0.51 mg/mL	Yielded amount	Vahvaselkä et al. (2021)
Propionibacterium freudenreichii subsp. *freudenreichii* 23	Skim-milk	Hydrolyzed soy oil	1%	32	24	0.82 mg/g fat	Matrix – no CLA before fermentation	Xu et al. (2004)
Propionibacterium freudenreichii subsp. *freudenreichii* 23*	Milk	Hydrolyzed soy oil	5 mg/mL (free LA)	45	*until pH 4.4-5.0*	0.85 mg/g fat	Control (only starter culture) – 0.72 mg/g fat of CLA after fermentation	Xu et al. (2005)
Propionibacterium freudenreichii subsp. *shermanii* 51	Skim-milk	Hydrolyzed soy oil	1%	32	24	1.64 mg/g fat	Matrix – no CLA before fermentation	Xu et al. (2004)
Propionibacterium freudenreichii subsp. *shermanii* 56	Skim-milk	Hydrolyzed soy oil	1%	32	24	1.17 mg/g fat	Matrix – no CLA before fermentation	Xu et al. (2004)
Propionibacterium freudenreichii subsp. *shermanii* 56*	Milk	Hydrolyzed soy oil	5 mg/mL (free LA)	45	*until pH 4.4-5.0*	1.00 mg/g fat	Control (only starter culture) – 0.72 mg/g fat of CLA after fermentation	Xu et al. (2005)

*Co-cultured with yogurt starter.

CLA – Conjugated linoleic acid; LA – Linoleic acid; DM – dry matter.

Table 1.12 Conjugated linolenic acid (CLNA) production in food matrices by bifidobacteria and propionibacteria, fermentation conditions and obtained CLNA content/yield.

Strain	Matrix	Substrate source	[Substrate]	T (ºC)	t (h)	CLNA content/yield	Observations	Reference
Bifidobacteria								
Bifidobacterium breve 29M2	Skim-milk	Pure α-LNA	0.5 mg/mL	37	24	0.220 mg/mL	Yielded amount	Villar-Tajadura et al. (2014)
Bifidobacterium breve CCFM683	Hydrolyzed walnut milk	Natural α-LNA	*not specified*	37	20	1.01 mg/mL		Mao et al. (2022)
Bifidobacterium breve M7-70	Skim-milk	Pure α-LNA	0.5 mg/mL	37	24	0.210 mg/mL	Yielded amount	Villar-Tajadura et al. (2014)
Bifidobacterium breve NCIMB 702258	Semi-skimmed milk	Pure α-LNA	0.5 mg/mL	37	24	0.115 mg/mL	Matrix - 0.007 mg/mL of CLNA before fermentation	Fontes et al. (2018)
Bifidobacterium breve ZL12-28	Skim-milk	Pure α-LNA	0.5 mg/mL	37	24	0.244 mg/mL	Yielded amount	Villar-Tajadura et al. (2014)
Propionibacteria								
Propionibacterium freudenreichii DSM 20270	Hydrolyzed blackcurrant press residue	Natural α-LNA	5 mg/g DM (free α-LNA)	30	48	0.29 mg/mL	Yielded amount	Vahvaselkä et al. (2021)

CLNA – Conjugated linolenic acid; α-LNA – alpha-Linolenic acid; DM – dry matter.

1.4.3. Linoleate isomerase and other involved enzymes

Linoleate isomerase (LAI) (EC 5.2.1.5) is the enzyme responsible for LA and α-LNA conversion into their conjugated forms and was first described by Kepler and Tove (1967) from ruminal *B. fibrisolvens*. There are two categories of LAIs: i) C12 isomerase that catalyzes the conversion of LA to C18:2 c9t11 and ii) C9 isomerase that catalyzes the conversion of LA to C18:2 t10c12 (Luo et al., 2013). The C12 isomerase has been found in different species of bacteria, including *B. fibrisolvens*, *Clostridium sporogenes* and *L. acidophilus* (Kepler & Tove, 1967; Luo et al., 2013; Peng et al., 2007). Moreover, C12 isomerase activity has been detected in several bifidobacteria and lactobacilli species (Gao et al., 2020; Terán et al., 2015; Yang et al., 2014). The presence of a double bond at the *cis*-15 position in α-LNA has little or no effect on the biohydrogenation process (Fontes et al., 2017), so its isomerization into CLNA, such as RLA (C18:3 c9t11c15), must be catalyzed by a C12 isomerase as well. Ineffective attempts to solubilize this isomerase suggest that it is a membrane-associated enzyme (Kepler & Tove, 1967; Peng et al., 2007). On the other hand, C9 isomerase is a soluble cytoplasmic protein and has been detected in *Propionibacterium acnes*, being also denominated as PAI (Deng et al., 2007).

Different LAI protein sequences have been annotated in GenBank and according to the amino acid identity percentage of the annotated sequences, LAIs can be divided into four groups: i) LAI from *P. acnes*; ii) LAIs from *L. reuteri*, *L. acidophilus* and *Lactococcus lactis* spp. *lactis*; iii) LAIs of *L. plantarum* and *R. erythropolis* and iv) LAIs from *Bifidobacterium 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).

It has been established that the LAIs from *L. acidophilus*, *L. plantarum*, *Lc. lactis* ssp. *lactis*, *B. dentium*, *B. breve*, and *R. erythropolis* have a high sequence identity (97-100%) with their myosin-cross-reactive antigen (MCRA) proteins (Farmani et al., 2010). The MCRA have shown to be involved in bacterial stress tolerance processes (O'connell et al., 2013; O'Flaherty & Klaenhammer, 2010). When the MCRA from probiotic strains was heterologously expressed, hydroxy FAs, such as 10-hydroxy-*cis*-12-octadecenoic acid (10-HOE), were produced from LA, being the protein described as a FAD-containing hydratase (Rosberg-Cody et al., 2011; Yang et al., 2013). Some studies have reported the yield of hydroxy FAs along with CLA isomers by lactobacilli and bifidobacteria strains cultured with LA (Gao et al., 2020; Yang et al., 2014). Moreover, it has been shown that CLA could be produced from 10-HOE (Gao et al., 2019; Kishino, Ogawa, et al., 2011). This suggests that MCRA could be involved in intermediary steps of LA isomerization. Despite this, its high homology with LAI suggests that detection of the MCRA gene may be associated with CFA production.

Hydratases catalyze the hydration/dehydration (i.e., addition/removal of water) of carbon double bonds (Demming et al., 2018). Fatty acid hydratases from *L. acidophilus* NTV001, namely, FA-HY1 and FA-HY2, have been described as responsible for the formation of 13-hydroxy-*cis*-9-octadecenoic acid (13-HOE) or 10-HOE from LA (Hirata et al., 2015). These presented FAD binding motifs at the N-terminal region being classified as belonging to the MCRA family of proteins, which have also shown hydratase activity, as reported above.

Several research works suggest that the conversion of LA into CLA in lactobacilli consists of a multienzymatic system (**Figure 1.2**). Taking into account their previous work (Kishino, Park, et al., 2011), Kishino et al. (2013), who worked with *L. plantarum* AKU 1009a, proposed a pathway that would start with 1) hydration of the carbon–carbon double bond at Δ9 position to generate 10-hydroxy fatty acid, by a hydratase denominated as CLA-HY, followed by 2) dehydrogenation of the hydroxy group at C10, to generate 10-oxo fatty acid, by a dehydrogenase/oxidoreductase referred as CLA-DH, then 3) isomerization of the carbon–carbon double bond at Δ12 to generate the conjugated enone structure 10-oxo-*trans*-11-fatty acid, by an acetoacetate decarboxylase designated as CLA-DC, 4) hydrogenation of the oxo group at C10 to generate 10-hydroxy-*trans*-11-fatty acid, by CLA-DH, and, finally, 5) dehydration of the hydroxy group at C10 to generate CLA with conjugated double bonds at Δ9 and Δ11, by CLA-HY. In fact, only the combined recombinant *E. coli* cells expressing all above-mentioned enzymes, along with FAD and NADH as cofactors, led to the formation of CLA from LA (Kishino, Park, et al., 2011). However, authors further suggested that CLA production process consists of a branched pathway in LA metabolization, where from step 3 there is iv) hydrogenation of the carbon–carbon double bond at Δ11 of 10-oxo-*trans*-11-fatty acid to generate the carbon–carbon single bond, by an enone reductase denominated as CLA-ER, followed by v) hydrogenation of the oxo group at C10 to generate 10-hydroxy fatty acid, by CLA-DH, and vi) dehydration of the hydroxy group at C10 to generate *cis*-9 and *trans*-10 monoenoic FA by CLA-HY (Kishino et al., 2013). Furthermore, an α-enolase from *L. plantarum* CFQ-100 was shown to catalyze the formation of RA from 10-HOE, being proposed that this enzyme would act along with the above-mentioned multienzymatic system during LA isomerization (Ortega-Anaya & Hernández-Santoyo, 2016).

Yang et al. (2014) proposed that CLA production in *L. plantarum* ZS2058 is carried out by a LAI complex composed by MCRA, DH and DC proteins, being MCRA responsible for the hydration/dehydration steps. In fact, further deletion of the *mcra* gene led to no formation of CLA nor 10-HOE from LA, where *dh* deletion mutant could only produce 10-HOE and *dc* deletion mutant produced also 10-oxo-*cis*-12-octadecenoic acid, besides the 10-HOE (Yang, Qi, et al., 2017). Moreover, these authors found that these genes were also present in the genome of other probiotic species, including, *B. bifidum*, *B. breve*, *B. longum*, *L. casei*, *L. plantarum*, *Levilactobacillus brevis*, *Lactobacillus johnsonii*, *L. reuteri*, *L. rhamnosus*, *Lactococcus lactis* and *Lactococcus cremoris*.

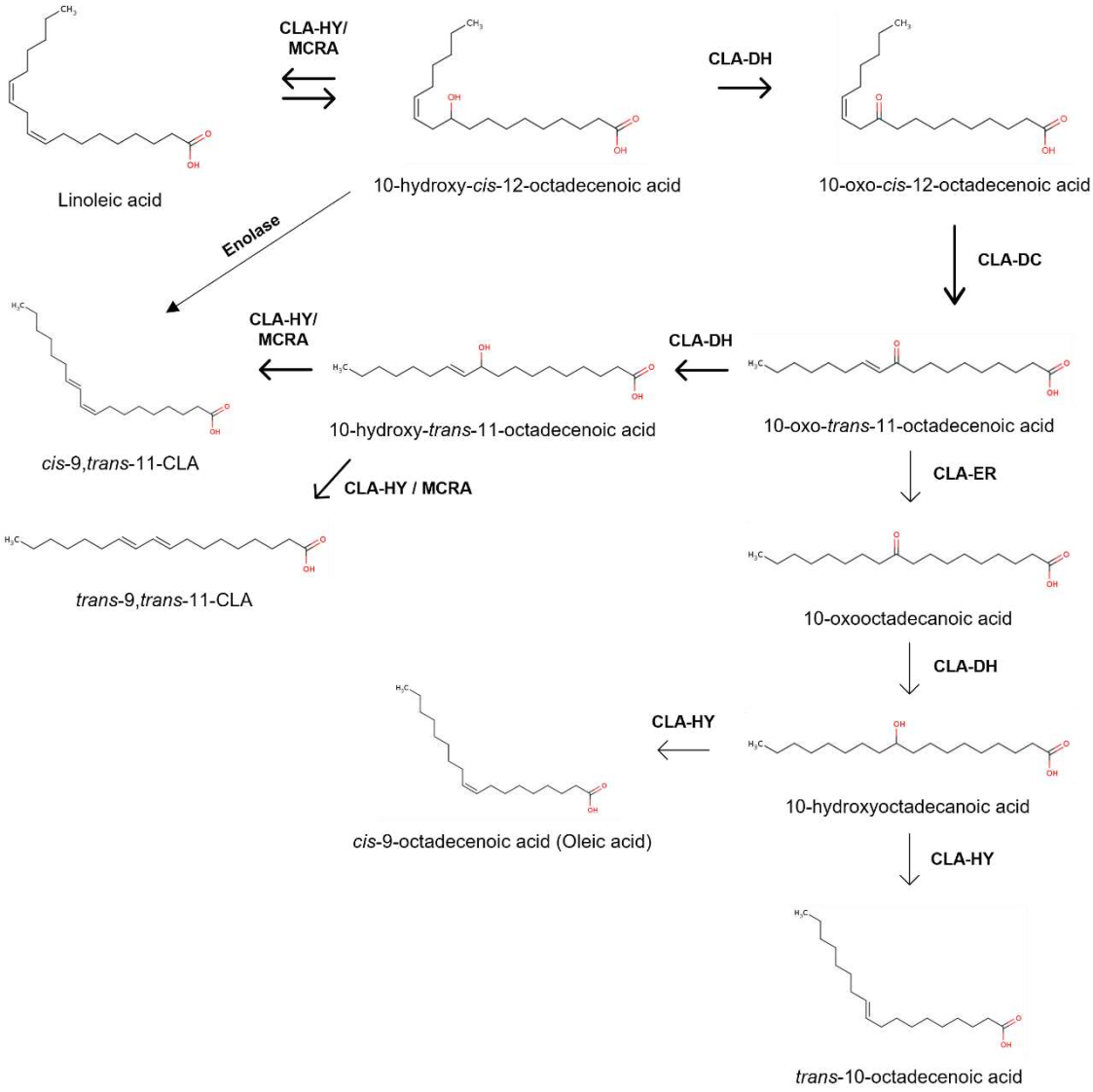


Figure 1.2 Proposed linoleic acid bioconversion pathway according to Kishino et al. (2013), Ortega-Anaya and Hernández-Santoyo (2016) and Yang et al. (2017). Bold arrows indicate the branch of conjugated linoleic acid formation. CLA – Conjugated linoleic acid, HY – Hydratase, MCRA – Myosin-cross-reactive antigen, DH – Dehydrogenase/oxidoreductase, DC – Acetoacetate decarboxylase, ER – Enone reductase.

Regarding the α-LNA isomerization pathway, it has been reported the production of hydroxy FAs, more precisely, 10-hydroxy-*cis*-12,*cis*-15-octadecenoic acid, by LAB grown with α-LNA (Kim et al., 2003; Serra et al., 2020). Moreover, recombinant *E. coli* expressing *mcra* gene from *L. plantarum* ZS2058 has been able to convert α-LNA into 10-hydroxy-*cis*-12,*cis*-15-octadecenoic acid, while with MCRA and DH recombinants together 10-oxo-*cis*-12,*cis*-15-octadecenoic acid would also appear, and adding DC recombinant led to the formation of CLNA isomers, along with the other mentioned products (Yang et al., 2020). Furthermore, in the same study, when any of the genes were knocked out, the mutant failed to produce CLNA. Therefore, the authors hypothesized that α-LNA conversion into CLNA isomers in lactobacilli is catalyzed by the same LAI complex following a similar pathway

to CLA production, as stated above, even though it was not possible to determine the intermediary compounds between 10-oxo-*cis*-12,*cis*-15-octadecenoic and CLNA (**Figure 1.3**).

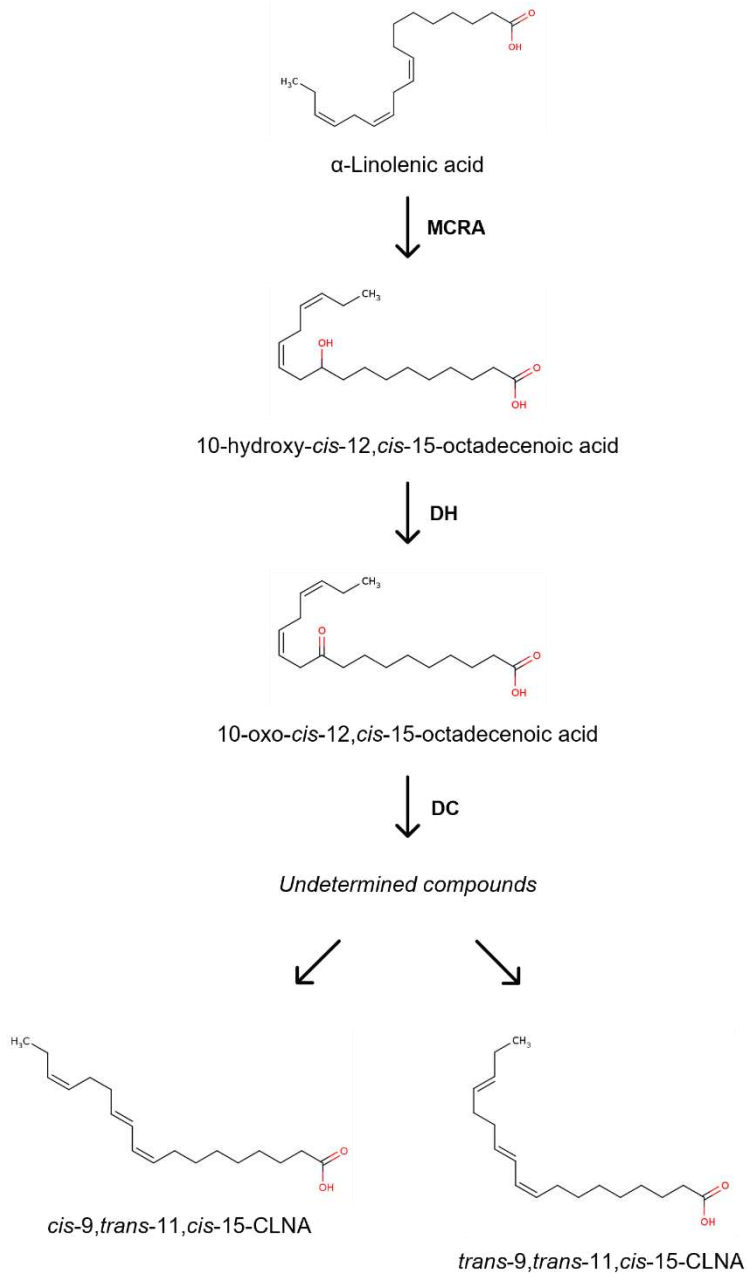


Figure 1.3 Proposed α-linolenic acid bioconversion pathway by Yang et al. (2020). MCRA – Myosin-cross-reactive antigen, DH – Dehydrogenase/oxidoreductase, DC – Acetoacetate decarboxylase, CLNA – Conjugated linolenic acid.

1.5. Objectives

Despite the potential of CLA and CLNA as functional ingredients, there is a lack of investigation focusing on the production of CLA/CLNA-enriched food products, in a way to increase its daily consumption. The development of a new bioactive food product to be assayed in the treatment or prevention of metabolic disorders would have a positive impact on the national

economy, as it has a direct industrial/commercial application and reverts in population's health. Therefore, it was hypothesized that an *in situ* microbial production of CLA and CLNA strategy would enable the increment of these potential bioactive compounds' availability for consumption. Thus, the main objective of this research work was to investigate the impact of CLA and CLNA-producing probiotic strains on the development, with all associated fundamental and applied implications, of a new added-value dairy product microbially enriched in CLA and CLNA isomers, and the resulting gain in technological feasibility and stability. Accordingly, the following specific objectives were pursued:

1. Identification of CLA and CLNA-producing strains through genotypic and phenotypic screening techniques;
2. Study of the viability of using lipase-hydrolyzed commercial vegetable oils as precursor substrates to obtain a microbial CLA/CLNA-enriched fermented milk;
3. Evaluation of FA metabolites in microbial CLA/CLNA-enrichment of milk;
4. Study of biochemical and organoleptic properties of microbial CLNA-enriched fermented milk;
5. Stability assessment of developed CLNA-enriched dairy products.

CHAPTER 2

Materials and Methods

CHAPTER 2 – MATERIALS AND METHODS

Part I - Identification of CLA/CLNA-producing strains

2.1.1. Chemicals

Hexane, methanol, dimethylformamide and acetonitrile were HPLC grade (VWR Chemicals, West Chester, PA). GLC-Nestlé36 FAME mix and glyceryl tritridecanoate (99.9%) were obtained from Nu-Chek Prep, inc. (Elysian, Minnesota, USA), undecanoic acid (99.9%) and N, O-Bis(trimethylsilyl)trifluoroacetamide (BSTFA) from Alfa Aesar (Haverhill, MA, USA) and butterfat CRM-164 (EU Commission; Brussels, Belgium) from Fedelco Inc. (Madrid, Spain). Sulphuric acid was from Fisher Scientific (Hampton, NH, USA), while sodium methoxide was from Acros Organics (Geel, Belgium). Supelco 37 FAME mix, bacterial FAME (BAME mix), LA and α-LNA were purchased from Sigma-Aldrich (St. Louis, MO, USA).

2.1.2. Improvement of linoleic acid emulsion

Solutions of LA were prepared at 15 mg/mL plus 2% (w/v) Tween 80 (Sigma-Aldrich) and subsequently processed by one of the following treatments: i) Filtration at 0.45 µm (Sartorius, Gottingen, Germany); ii) Homogenization with Ultra-Turrax (IKA Works, Inc., Wilmington, NC, USA) at 13 600 rpm for 90 s (three 30 s-intervals separated by 30 s-pauses, to not heat-up the emulsion) followed by filtration at 0.45 µm; iii) Homogenization with Ultra-Turrax at 13 600 rpm for 150 s (five 30 s-intervals separated by 30 s-pauses) followed by filtration at 0.45 µm; iv) Homogenization with Ultrasonic Liquid Processor (Sonics & Materials, Inc., Newtown, CT, USA) of 130 W, 20 kHz for 90 s (three 30 s-intervals separated by 30 s-pauses; 1 s-pulses) followed by filtration at 0.45 µm. Lower filtration diameter was further tested with the selected homogenization conditions as follows: i) Filtration at 0.20 µm (Millipore, Burlington, MA, USA); ii) Homogenization with Ultra-Turrax at 13 600 rpm for 150 s (five 30 s-intervals separated by 30 s-pauses) followed by filtration at 0.20 µm. Samples were collected before and after treatment for further FA analysis (**Sections 2.1.10** and **2.1.11**). All experiments were carried out in duplicate. The LA loss after each treatment was calculated as follows according to values calculated after gas chromatography analyses:

$$LA\ loss\ (\%) = \frac{([LA_i] - [LA_f]) \times 100}{[LA_i]} \qquad \text{Equation 2.1}$$

Being LA_i the amount of LA before treatment and LA_f the amount of LA after treatment.

2.1.3. Culture conditions

A total of 85 strains, belonging to the *Lactobacillus* (reclassified into 25 new genera according to Zheng et al. (2020)), *Bifidobacterium*, *Propionibacterium*, *Lactococcus* and *Enterococcus* genera, (**Table 2.1**, **Table 2.2** and **Table 2.3**), stored at –80 ºC in glycerol 30% (w/w) (Fisher Scientific), were

inoculated at 2% (v/v) in MRS broth (Biokar Diagnostics, Beauvais, France) and incubated overnight at 37 ºC. About 10% (v/v) of the activated culture was then sub-cultured in fresh MRS medium and incubated at 37 ºC for 16 h. Afterwards, 2% (v/v) was spiked into new medium (10 mL) for the subsequent experiments. For bifidobacteria and propionibacteria, the culture medium was supplemented with 0.05% (w/v) L-cysteine-HCl (Sigma-Aldrich, St. Louis, MO, USA) and strains were grown in an anaerobic workstation (Whitley DG 250; Don Withley Scientific, Yorkshire, UK.) under a mixture of 80% nitrogen, 10% hydrogen and 10% carbon dioxide. Lactobacilli, lactococci and enterococci strains were cultured under aerobic conditions.

Table 2.1 Lactobacilli strains assayed in this study and respective sources (all strains are listed according to new taxonomic classification).

Strain	Source
Lacticaseibacillus casei 01	Christian Hansen (HØrsholm, Denmark)
Lacticaseibacillus casei 431	Christian Hansen (HØrsholm, Denmark)
Lacticaseibacillus casei MP10	UCM (Madrid, Spain)
Lacticaseibacillus casei MP11	UCM (Madrid, Spain)
Lacticaseibacillus casei MP8	UCM (Madrid, Spain)
Lacticaseibacillus casei MP9	UCM (Madrid, Spain)
Lacticaseibacillus casei NCTC 6375	NCTC (London, UK)
Lacticaseibacillus paracasei L26	LAFTIÒ DSM (Moorebank, NSW, Australia)
Lacticaseibacillus paracasei subsp. paracasei NCFB 151	NCFB (Reading, UK)
Lacticaseibacillus rhamnosus MP18	UCM (Madrid, Spain)
Lacticaseibacillus rhamnosus R11	Lallemand (Montréal, QC, Canada)
Lactiplantibacillus plantarum 229v	PROBI AB (Lund, Sweden)
Lactiplantibacillus plantarum CECT 7482	CECT (Burjassot, Spain)
Lactiplantibacillus plantarum D36	Persimmon isolate (ESB-UCP, Portugal)
Lactiplantibacillus plantarum DSM 12028	DSMZ (Braunschweig, Germany)
Lactiplantibacillus plantarum DSM 20174	DSMZ (Braunschweig, Germany)
Lactiplantibacillus plantarum DSM 20205	DSMZ (Braunschweig, Germany)
Lactiplantibacillus plantarum MP16	UCM (Madrid, Spain)
Lactiplantibacillus plantarum MP17	UCM (Madrid, Spain)
Lactiplantibacillus plantarum NCFB 1752	NCFB (Reading, UK)
Lactobacillus acidophilus ATCC 4356	ATCC (Manassas, VA, USA)
Lactobacillus acidophilus Ki	CSK (Ede, Netherlands)
Lactobacillus acidophilus L10	LAFTIÒ DSM (Moorebank, NSW, Australia)
Lactobacillus acidophilus LA-5	Christian Hansen (HØrsholm, Denmark)
Lactobacillus acidophilus MP169	UCM (Madrid, Spain)
Lactobacillus acidophilus MP198	UCM (Madrid, Spain)
Lactobacillus acidophilus MP289	UCM (Madrid, Spain)
Lactobacillus acidophilus MP345	UCM (Madrid, Spain)
Lactobacillus acidophilus MP4	UCM (Madrid, Spain)
Lactobacillus acidophilus MP5	UCM (Madrid, Spain)
Lactobacillus acidophilus MP6	UCM (Madrid, Spain)
Lactobacillus acidophilus MP7	UCM (Madrid, Spain)

UCM – Universidad Complutense de Madrid; NCTC – National Collection of Type Cultures; DSM – DSM Food Specialities; NCFB – National Collection of Food Bacteria; CECT – Spanish Type Culture Collection; DSMZ – German Collection of Microorganisms and Cell Cultures GmbH; ATCC – American Type Culture Collection; CSK – CSK Food Enrichment.

Table 2.2 Lactobacilli strains (*continued*) assayed in this study and respective sources (all strains are listed according to new taxonomic classification).

Strain	Source
Lactobacillus delbrueckii subsp. *bulgaricus* DSM 20081	DSMZ (Braunschweig, Germany)
Lactobacillus delbrueckii subsp. *bulgaricus* NCFB 1489	NCFB (Reading, UK)
Lactobacillus gasseri MP199	UCM (Madrid, Spain)
Lactobacillus gasseri MP289	UCM (Madrid, Spain)
Lactobacillus gasseri MP321	UCM (Madrid, Spain)
Lactobacillus gasseri MP551	UCM (Madrid, Spain)
Latilactobacillus curvatus LMG 9198	LMG (Gent, Belgium)
Latilactobacillus sakei CTC 372	CTC (Monells, Spain)
Latilactobacillus sakei DSM 20017	DSMZ (Braunschweig, Germany)
Latilactobacillus sakei Lb 706	UCM (Madrid, Spain)
Lentilactobacillus buchneri NCFB 110	NCFB (Reading, UK)
Lentilactobacillus hilgardii NCFB 264	NCFB (Reading, UK)
Levilactobacillus brevis D24	Persimmon isolate (ESB-UCP, Portugal)
Ligilactobacillus salivarius DSM 20555	DSMZ (Braunschweig, Germany)
Ligilactobacillus salivarius MP143	UCM (Madrid, Spain)
Ligilactobacillus salivarius MP160	UCM (Madrid, Spain)
Ligilactobacillus salivarius MP19	UCM (Madrid, Spain)
Ligilactobacillus salivarius MP20	UCM (Madrid, Spain)
Ligilactobacillus salivarius MP21	UCM (Madrid, Spain)
Ligilactobacillus salivarius MP22	UCM (Madrid, Spain)
Ligilactobacillus salivarius MP23	UCM (Madrid, Spain)
Ligilactobacillus salivarius MP24	UCM (Madrid, Spain)
Ligilactobacillus salivarius ZH26-12	UCM (Madrid, Spain)
Limosilactobacillus fermentum MP101	UCM (Madrid, Spain)
Limosilactobacillus fermentum MP12	UCM (Madrid, Spain)
Limosilactobacillus fermentum MP13	UCM (Madrid, Spain)
Limosilactobacillus fermentum MP14	UCM (Madrid, Spain)
Limosilactobacillus fermentum MP149	UCM (Madrid, Spain)
Limosilactobacillus fermentum MP15	UCM (Madrid, Spain)
Limosilactobacillus fermentum MP169	UCM (Madrid, Spain)
Limosilactobacillus fermentum MP170	UCM (Madrid, Spain)
Limosilactobacillus fermentum MP171	UCM (Madrid, Spain)
Limosilactobacillus fermentum MP2621	UCM (Madrid, Spain)
Limosilactobacillus fermentum MP95	UCM (Madrid, Spain)
Limosilactobacillus fermentum MP96	UCM (Madrid, Spain)
Limosilactobacillus fermentum NCFB 1750	NCFB (Reading, UK)
Loigoactobacillus coryniformis LMG 9196	LMG (Gent, Belgium)
Secundilactobacillus collinoides NCFB 2805	NCFB (Reading, UK)

DSMZ – German Collection of Microorganisms and Cell Cultures GmbH; NCFB – National Collection of Food Bacteria; UCM – Universidad Complutense de Madrid; LMG – BCCM/LMG Bacteria Collection; CTC – Meat Technology Centre Collection.

Table 2.3 Bifidobacteria, Enterococci, Lactococci and Propionibacteria strains assayed in this study and respective sources.

Strain	Source
Bifidobacteria	
Bifidobacterium animalis B94	LAFTIÒ DSM (Moorebank, NSW, Australia)
Bifidobacterium animalis Bb12	Christian Hansen (HØrsholm, Denmark)
Bifidobacterium animalis BLC	DELVO PROÒ DSM (Moorebank, NSW, Australia)
Bifidobacterium animalis BO	CSK (Ede, Netherlands)
Bifidobacterium bifidum DSM 20239	DSMZ (Braunschweig, Germany)
Bifidobacterium breve DSM 20091	DSMZ (Braunschweig, Germany)
Bifidobacterium breve MP123Te	UCM (Madrid, Spain)
Bifidobacterium breve MP302	UCM (Madrid, Spain)
Bifidobacterium pseudolongum DSM 20095	DSMZ (Braunschweig, Germany)
Enterococci	
Enterococcus 32	Cheese isolate (ESB-UCP, Portugal)
Enterococcus 37	Cheese isolate (ESB-UCP, Portugal)
Lactococci	
Lactococcus lactis ATCC 11454	ATCC (Manassas, VA, USA)
Lactococcus lactis MP238	UCM (Madrid, Spain)
Propionibacteria	
Propionibacterium freudenreichii subsp. shermanii JS	Valio Ltd. (Helsinki, Finland)
Propionibacterium freudenreichii subsp. freudenreichii DSM 20271	DSMZ (Braunschweig, Germany)

DSM – DSM Food Specialities; CSK – CSK Food Enrichment; DSMZ – German Collection of Microorganisms and Cell Cultures GmbH; UCM – Universidad Complutense de Madrid; ATCC – American Type Culture Collection.

2.1.4. Genotypic screening

To detect the presence of the putative genes encoding enzymes involved in the LA isomerization pathway (i.e., LAI, MCRA and fatty acid-hydratase – FA-HY), each strain was inoculated in fresh MRS broth (10 mL) at 2% (v/v) and incubated for 24 h at 37 ºC. Afterwards, pellets were recovered through centrifugation (1250 ×*g*, 18 ºC, 5 min) and washed twice in 0.85% (w/v) NaCl. Then, DNA was extracted from the bacterial strains as described by Alimolaei and Golchin (2016) in their P3 protocol, and subjected to PCR analysis to assess the presence of LAI, MCRA and/or FA-HY genes, using different sets of primers, depending on the strain (**Table 2.4**). More specifically, primers LISO3 and LISO4 (Gorissen et al., 2011), were used to screen the lactobacilli strains for the presence of a putative LAI gene. These primers were designed based on the partial nucleotide sequence obtained for *L. sakei* 23K and a nucleotide sequence of the LAI gene of *L. plantarum* AS1.555 (GenBank accession code DQ227322). In addition, the Entrez Gene tool (http://www.ncbi.nlm.nih.gov/gene) from the National Center for Biotechnology Information (NCBI) database was used to select the LAI sequences of lactobacilli and based on these sequences two primers were designed for their amplification: INIA-Lb1 (gaygayccrcayatsttccarac) and INIA-Lb2 (gtraagctgrcccaytcrct). In the case of the MCRA gene, primers Ev1a and Ev2a (Rosberg-Cody et al., 2011) were used to screen its presence in the bifidobacteria, propionibacteria, lactococci and enterococci strains included in this study. These degenerated primers were originally designed after

a comparison of the *L. reuteri* PYR8 LAI protein with other sequences available in databases. In addition, the Entrez Gene tool from the NCBI database was used to select the MCRA sequences of bifidobacteria and based on these sequences two primers were designed for their amplification: INIA-Bf1 (gcgyacbatgttcgcbttyg) and INIA-Bf2 (cytccatrcckgtrcgcatsga). Finally, two sets of primers based on the genome of *L. acidophilus* strains were used for the screening of two FA-HY genes (*fa-hy1* and *fa-hy2*), described as encoding enzymes with FA hydratase activity (Hirata et al., 2015), among the *L. acidophilus* strains included in this study.

For PCR amplification a Techne TC-512 Thermocycler (Keison Products, England) was used. All amplifications were carried out in a final volume of 25 µl. Each PCR mixture (*Taq* DNA polymerase 5U/µL PCR kit, Thermo Fischer Scientific) contained 2.5 µL 10× *Taq* buffer, 2 mM dNTP mix, 20 pmol of each primer, 1.5 mM $MgCl_2$, 1.25 U Taq DNA polymerase, 1.25 µL DMSO and 5 µL of the diluted DNA solution. The PCR program consisted of an initial denaturation at 95 ºC for 3 min, followed by 40 cycles of denaturation at 95 ºC for 30 s, annealing at a specific temperature for the primers used (**Table 2.4**) for 30 s, and extension at 72 ºC for 60-110s. The last step consisted of a final extension at 72 ºC for 10 min.

Table 2.4 Primers used in this study for the detection of the putative genes encoding linoleate isomerase (LAI), myosin-cross-reactive antigen (MCRA) and fatty acid-hydratase (FA-HY).

Primer set designation	Target strains (Genera/Species)	Target gene annotation	Annealing temperature (ºC)	Amplification product length (kb)	Reference
LISO3 and LISO4	Lactobacilli	Linoleate isomerase gene (*lai*)	56	≈1.0	Gorissen et al.(2011)
INIA-Lb1 and INIA-Lb2			52	≈0.6	Designed based on conserved domains
Ev1a and Ev2a	Bifidobacteria, Propionibacteria, Enterococci and Lactococci	Myosin cross-reactive-antigen gene (*mcra*)	45	≈1.0	Rosberg-Cody et al.(2011)
INIA-Bf1 and INIA-Bf2			57	≈1.0	Designed based on conserved domains
Fa-hy1-F and Fa-hy1-R	*Lactobacillus acidophilus*	Fatty acid hydratase 1 (*fa-hy1*)	51	≈1.8	Hirata et al.(2015)
Fa-hy2-F and Fa-hy2-R		Fatty acid hydratase 2 (*fa-hy2*)	51	≈1.8	

To assess the presence of PCR products, 5 µL of each PCR reaction was subjected to electrophoresis using a 1% (w/v) agarose gel in 1X Tris-acetate-EDTA (TAE) buffer (GRiSP, Porto, Portugal), stained with GreenSafe Premium (NZYTech, Lisbon, Portugal). After electrophoresis, gels were illuminated under a UV light.

2.1.5. Determination of maximum tolerance to linoleic acid

A stock solution of LA was prepared at 50 mg/mL with 2% (w/v) Tween 80 and homogenized under the conditions selected from **Section 2.1.2 Improvement of linoleic acid emulsion** (in Materials and Methods of Part I) results, i.e., using an Ultra-Turrax at 13 600 rpm during 150 s (five 30 s-intervals separated by 30 s-pauses, to not heat-up the emulsion), followed by filter-sterilization through a 0.20 µm-pore size membrane.

To find out the LA maximum tolerance of each strain, tests were conducted according to Romero-Pérez et al. (2013) with slight modifications. Briefly, after activation, cultures were plated in MRS agar (Biokar Diagnostics) containing 1, 2 or 5 mg/mL of LA and incubated for 48 h at 37 ºC in duplicate. Plates without LA were used as controls. For bifidobacteria and propionibacteria, MRS was supplemented with L-cysteine-HCl (0.05%, w/v) (cys-MRS). From LA-containing plates, colonies were assayed by spiking 96-well plates containing 250 µL of MRS (lactobacilli, lactococci and enterococci) or 200 µL of cys-MRS (bifidobacteria and propionibacteria) and LA at the same concentration present in the agar plates from which they had been isolated, in triplicate. For each strain, inoculated wells with LA-free medium were used as control. Paraffin (50 µL) was added to the top of the wells where bifidobacteria and propionibacteria were assayed. Growth was monitored in a plate reader (model FLUOSTAR optima; BMG labtech, Ortenberg, Germany) at 600 nm for 48 h at 37 ºC.

2.1.6. CLA production

To determine which of the positive strains for LAI, MCRA and/or FA-HY genes were able to produce CLA isomers, each strain was inoculated at 2% (v/v) in fresh MRS broth (10 mL) without substrate (control) or containing 0.5 mg/mL of LA (from a 15 mg/mL stock solution). Samples were incubated at 37 ºC for 48 h in duplicate. Bacterial growth was analyzed through plating on MRS agar plates of sequential decimal dilutions followed by viable cell numbers determination upon incubation at 37 ºC for 24 h. The pH was measured through a digital pH meter (Basic 20, Crison, Barcelona, Spain). The supernatants were collected after centrifugation at 1250 ×*g* and 18 ºC for 5 min for further FA analysis (**Sections 2.1.10** and **2.1.11**). Later, producing strains with high LA-tolerance were further tested for CLA production at their maximum tolerance (medium supplemented with 5 mg/mL of LA).

The LA reduction percentage was calculated as follows:

$$LA\ reduction\ (\%) = \frac{([LA_i] - [LA_f]) \times 100}{[LA_i]} \qquad \text{Equation 2.2}$$

Being LA_i the initial amount (0 h) of LA and LA_f the final amount of LA after 24 h or 48 h of incubation. The LA conversion percentage was calculated as follows:

$$LA\ conversion\ (\%) = \frac{([CLA_f] - [CLA_i]) \times 100}{[LA_i]}$$ Equation 2.3

Being CLA_f the final amount (after 24 h or 48 h) of CLA, CLA_i the initial amount (0 h) of CLA and LA_i the initial amount (0 h) of LA.

2.1.7. Influence of shaking on CLA production

To evaluate the influence of shaking on CLA production by the identified producers, each selected strain was inoculated at 1% (lactobacilli) or 2% (bifidobacteria) (v/v) in fresh MRS broth (10 mL) without substrate (control) or containing 0.5 mg/mL of LA (from a 15 mg/mL stock emulsion). Samples were incubated at 37 ºC for 24, 48 and 72 h in an incubator or in a shaking water bath (Julabo, Seelbach, Germany) at 150 rpm in triplicate. To assure anaerobiosis condition in the water bath, bifidobacteria were incubated inside sealed plastic bags with gas generating pouch systems (BD GasPak™ EZ, Wokingham, Berkshir, UK). Viable cell numbers were determined, and the pH measured. The supernatants were collected upon centrifugation according to conditions listed in **Section 2.1.6 CLA production** (in Materials and Methods of Part I) for further FA analysis (**Sections 2.1.10** and **2.1.11**). LA conversion percentage was calculated according to **Equation 2.3** (in **Section 2.1.6 CLA production** of Materials and Methods of Part I).

2.1.8. CLA production kinetics

To assess CLA production kinetics by selected producers, each strain was inoculated at 1% (lactobacilli) or 2% (bifidobacteria) (v/v) in fresh MRS broth (100 mL) without substrate (control) or containing 0.5 mg/mL of LA (from a 15 mg/mL stock emulsion). Samples were incubated at 37 ºC and aliquots taken every 2 h until 12 h and then at 24 h. Viable cell numbers were determined, and the pH measured. The supernatants were collected upon centrifugation according to conditions listed in **Section 2.1.6 CLA production** (in Materials and Methods of Part I) for further FA analysis (**Sections 2.1.10** and **2.1.11**).

2.1.9. CLNA production

Stock solution of α-LNA was prepared at 15 mg/mL with 2% (w/v) Tween 80 and homogenized as described for LA in **Section 2.1.5 Determination of maximum tolerance to linoleic acid** (in Materials and Methods of Part I) .

To test CLNA production by the identified CLA-producers, each strain was inoculated at 1% (lactobacilli) or 2% (bifidobacteria) (v/v) in fresh MRS broth (10 mL) without substrate (control) or containing 0.5 mg/mL of α-LNA. Samples were incubated at 37 ºC for 24 and 48 h in triplicate. Viable cell counts were determined, and the pH measured. The supernatants were collected upon centrifugation according to conditions listed in **Section 2.1.6 CLA production** (in Materials and Methods of Part I) for further FA analysis (**Sections 2.1.10** and **2.1.11**). The α-LNA reduction and conversion percentages were calculated as shown in **Equation 2.2** and **Equation 2.3** (in **Section**

2.1.6 CLA production of Materials and Methods of Part I), respectively, replacing LA by α-LNA and CLA by CLNA amounts.

2.1.10. Sample preparation for fatty acid and hydroxy fatty acid analyses

For the FA analysis, LA emulsions and supernatants (500 µL) were prepared according to Pimentel et al. (2015). Briefly, for quantification purposes, samples were added with 100 µL of tritridecanoin (1.5 mg/mL in hexane) and undecanoic acid (1.5 mg/mL in hexane) before derivatization. Then 2.26 mL of methanol was added, followed by 1 mL of hexane and 240 µL of sodium methoxide (5M in methanol). Samples were homogenized and incubated at 80 ºC for 10 min. After cooling in ice, 1.25 mL of dimethylformamide was added before 1.25 mL of sulphuric acid (3M in methanol). Samples were homogenized and incubated at 60 ºC for 30 min. Finally, after cooling, 1 mL of hexane was added, and samples were homogenized and centrifuged (1250 ×*g*; 18 ºC; 5 min). The upper layer containing FA methyl esters (FAME) was collected for further gas chromatographic analysis.

For the analysis of hydroxyl FAs as trimethylsilyl (TMS) derivatives in supernatant samples, the solvent was evaporated in a nitrogen stream after obtaining the FAME fraction as described above. Then, 150 µL of BSTFA and 500 µL of acetonitrile were added and the mix was incubated at 70 ºC for 30 min (Fibinger et al., 2016). Finally, acetonitrile was evaporated in a nitrogen stream and the extract was resuspended in 500 µL of hexane.

2.1.11. Gas Chromatography conditions

As previously reported by Fontes et al. (2018), FAME and TMS extracts were both analyzed in a gas chromatograph HP6890A (Hewlett-Packard, Avondale, PA, USA), equipped with a flame-ionization detector (GLC-FID) and a BPX70 capillary column (60 m x 0.32 mm x 0.25 µm; SGE Europe Ltd, Courtaboeuf, France). Analysis conditions were as follows: injector temperature – 250 ºC, split – 25:1, injection volume – 1 µL; detector (FID) temperature – 275 ºC; carrier gas – hydrogen at 20.5 psi; oven temperature program – started at 60 ºC (held 5 min), then raised at 15 ºC/min to 165 ºC (held 1 min) and, finally, at 2 ºC/min up to 225 ºC (held 2 min). Supelco 37, FAME from CRM-164 and BAME mix were used for the identification of FAs. GLC-Nestlé36 was assayed for calculation of response factors and detection and quantification limits (LOD: 0.79 ng FA/mL; LOQ: 2.64 ng FA/mL).

2.1.12. Statistical analysis

Results are reported as mean values ± standard deviation of at least duplicate samples. Data was first analyzed for normality distribution. Levene's test was applied to verify the homogeneity of the variances. Afterwards, if normality was guaranteed, T-test independent samples was applied to compare means of two groups and one-way ANOVA (equal variances) or Welch test (no equal variances) to compare three or more groups with post-hoc Bonferroni (equal variances) or Games-Howell (no equal variances). If normality was not guaranteed, Mann-Whitney test was performed to compare two groups and Kruskal-Wallis to compare three or more groups. The LA-tolerance and

genotypic screening frequencies were compared by Qui-square test. Level of significance was set in general at 0.05; for growth experiments Colony forming units (CFU) differences had to be ≥1 log cycle and pH values had to differ ≥0.5 units. Analyses were performed using the IBM SPSS Statistics 28 (SPSS Inc., IBM Corporation, NY, USA).

Part II – Study of the utilization of commercially available substrate sources to obtain a microbial CLA/CLNA-enriched fermented milk

2.2.1. Chemicals and samples

Hexane and dimethylformamide were HPLC grade (VWR Chemicals, West Chester, PA, USA), as well as methanol (Carlo Erba Reagents, Barcelona, Spain). Sulphuric acid was from Honeywell Fluka (Charlotte, NC, USA), while sodium methoxide and methyl acetate were from Acros Organics (Geel, Belgium). GLC-Nestlé36 FAME mix was obtained from Nu-Chek Prep, Inc. (Elysian, MN, USA) and butterfat CRM-164 (EU Commission; Brussels, Belgium) from Fedelco Inc. (Madrid, Spain). Undecanoic acid (98.0%) was from Alfa Aesar (Haverhill, MA, USA), while glyceryl tritridecanoate (>99.0%) was from Larodan (Solna, Sweeden). Supelco 37 FAME mix and methyl tricosanoate (≥99.0%) were purchased from Sigma-Aldrich (St. Louis, MO, USA), as well as *Candida rugosa* (CRL) type VII, Pancreatic porcine (PPL) type II and *Pseudomonas fluorescens* (PFL) lipases. Tonalin CLA was kindly given by BASF Portuguesa S.A. (Porto, Portugal) and PSO oil by Earthoil (Norfolk, United Kingdom). Pasteurized semi-skimmed cow's milk, and soybean (SBO), flaxseed (FSO) and hemp seed (HSO) oils were bought in local markets (Porto, Portugal).

2.2.2. Vegetables oils hydrolysis

For each edible vegetable oil under study, namely SBO, FSO and HSO, a 10 g reaction mixture was prepared in Erlenmeyer flasks of 100 mL according to Lu et al. (2018): water/oil ratio of 3:5 (w/w) and lipase 1% (w/w) of the vegetable oil weight. The flasks were incubated in an orbital shaker-incubator (MaxQ SHKE6000; Thermo Fisher Scientific, Waltham, MA, USA) at 35 ºC (PPL), 45 ºC (CRL) or 55 ºC (PFL) and 200 rpm for 24 h in triplicate. Then, the reaction mixture was centrifuged at 5 000 rpm and room temperature for 10 min. The vegetable oil was thereafter recovered (upper layer) and centrifuged again, at the same previous conditions, to remove any traces of water. Vegetable oil samples were taken before and after hydrolysis for further FA analysis (**Sections 2.2.5** and **2.2.6**). The hydrolysis degree was calculated based on the initial and final amount of FA in the esterified fraction as follows:

$$Hydrolysis\ degree\ (\%) = \frac{([EFA_i] - [EFA_f]) \times 100}{[EFA_i]} \qquad \text{Equation 2.4}$$

Being EFA_i the initial concentration of esterified fatty acid (before hydrolysis) and EFA_f the final concentration of esterified fatty acid (after hydrolysis).

2.2.3. Microbially CLA/CLNA-enriched milk

Stock oil emulsions were prepared at 50 mg/mL of free LA (with hydrolyzed SBO) or free α-LNA (with hydrolyzed FSO) with 2% (w/v) Tween 80 (Sigma-Aldrich) and homogenized with an Ultra-Turrax (T 25 digital; IKA Works, Inc., Wilmington, NC, USA) at 13 600 rpm for 150 s (five 30 s-intervals separated by 30 s-pauses, to not heat-up the emulsions). Then, oil emulsions were UV-sterilized in a Laminar Flow Hood (BIO-II-A; Azbil Telstar, Tokyo, Japan) for 20 min.

The selected strain (according to the results from Part I), *Bifidobacterium breve* DSM 20091 (DSMZ, Braunschweig, Germany), stored at -80 ºC in glycerol 30% (w/w) (Fisher Scientific, Hampton, NH, USA), was activated at 2% (v/v) in MRS broth (Biokar Diagnostics, Beauvais, France), supplemented with 0.01% (w/v) bacteriological meat extract (Biokar) and 0.05% (w/v) L-cysteine-HCl (Sigma-Aldrich) (cys-MRS). After overnight incubation at 37 ºC, the activated culture was then transferred to fresh cys-MRS medium at 10% (v/v) and incubated at 37 ºC for 16 h. Afterwards, the strain was inoculated at 1% (v/v) in milk (100 mL) supplemented with 0.05% (w/v) cysteine and under the following substrate conditions: *Control* – No substrate; *S0.5LA* – 0.5 mg/mL LA from hydrolyzed SBO; *S0.5LNA* – 0.5 mg/mL α-LNA from hydrolyzed FSO; *S0.25LA/LNA* – 0.25 mg/mL LA plus 0.25 mg/mL α-LNA from combined hydrolyzed SBO and FSO; *S0.25LA/LNA'* – 0.25 mg/mL LA from hydrolyzed SBO plus 0.25 mg/mL α-LNA from hydrolyzed FSO; *S0.5LA/LNA* – 0.5 mg/mL LA plus 0.5 mg/mL α-LNA from combined hydrolyzed SBO and FSO; *S0.5LA/LNA'* – 0.5 mg/mL LA from hydrolyzed SBO plus 0.5 mg/mL α-LNA from hydrolyzed FSO. Inoculated milk was then incubated at 37 ºC for 24 h in triplicate. Cultures were always grown under anaerobic conditions (Whitley DG 250; Don Withley Scientific, Yorkshire, UK; gas mixture of 80% nitrogen, 10% hydrogen and 10% carbon dioxide). Milk samples were collected before and after the incubation period for further viable cell numbers determination, pH measurement (Basic 20; Crison, Barcelona, Spain) and FA analysis (**Sections 2.2.5** and **2.2.6**). The substrate reduction percentage was calculated as follows:

$$Substrate\ reduction\ (\%) = \frac{([Subs_i] - [Subs_f]) \times 100}{[Subs_i]} \qquad \text{Equation 2.5}$$

Being $Subs_i$ the initial amount (0 h) of free substrate and $Subs_f$ the final amount (24 h) of free substrate. Linoleic acid and α-LNA conversion percentages were calculated based on the assumption that all CLA and CLNA produced derived from their former substrates only:

$$Substrate\ conversion\ (\%) = \frac{([CFA_f] - [CFA_i]) \times 100}{[Subs_i]} \qquad \text{Equation 2.6}$$

Being CFA_f the final amount (24 h) of free conjugated fatty acid, CFA_i the initial amount (0 h) of free conjugated fatty acid and $Subs_i$ the initial amount (0 h) of the corresponding free substrate (i.e., LA for CLA and α-LNA for CLNA).

2.2.4. Microbially CLNA-enriched milk

Bifidobacterium breve DSM 20091 was activated and inoculated as described in **Section 2.2.3 Microbially CLA/CLNA-enriched milk** (in Materials and Methods of Part II) using hydrolyzed FSO at the following substrate conditions: *Control* – No substrate; *S1LNA* – 1 mg/mL α-LNA; *S1.5LNA* – 1.5 mg/mL α-LNA; *S2LNA* – 2 mg/mL α-LNA; *S5LNA* – 5 mg/mL α-LNA; *S10LNA* – 10 mg/mL α-LNA. Milk samples (n=3) were collected before and after the incubation period for further viable cell numbers enumeration, pH measurement and FA analysis (**Sections 2.2.5** and **2.2.6**).

2.2.5. Sample preparation for fatty acid analysis

For the FA analysis, vegetable oil (15 mg), vegetable oil emulsion (500 µL), milk (500 mg) samples, or Tonalin CLA and PSO (5 mg) were prepared according to Pimentel et al. (2015). First, for quantification of esterified FA (EFA), samples were added with 200 µL of glyceryl tritridecanoate (1.5 mg/mL in hexane) and 100 µL of undecanoic acid (1.5 mg/mL in hexane). Then, 100 µL of methyl acetate was added, followed by 3.60 mL of hexane, 2.26 mL of methanol and 240 µL of sodium methoxide (5.4M in methanol). Samples were homogenized and incubated at 40 ºC for 10 min. After cooling on ice, 500 µL of deionized water were added (to enable phase separation), and samples were homogenized and centrifuged (1250 ×*g*, 18 ºC, 5 min). The upper layer, containing FAME, was then collected in a 15 mL centrifuge tube, containing already 200 µL of methyl tricosanoate (1.5 mg/mL in hexane), for further chromatographic analysis. To clean up, 2 mL of hexane were added to the remaining reaction mixture, and samples were homogenized and centrifuged (1250 ×*g*, 18 ºC, 5 min). The upper layer was collected into the centrifuge tube. Thereafter, for quantification of FFA, 100 µL of methyl tricosanoate (1.5 mg/mL in hexane) were added, followed by 1.25 mL of dimethylformamide, before 1.25 mL of sulphuric acid (3M in methanol). Samples were homogenized and incubated at 60 ºC for 30 min. Finally, after cooling, 700 µL of hexane were added, and samples were homogenized and centrifuged (1250 ×*g*, 18 ºC, 5 min). The upper layer, containing FAME, was collected for further chromatographic analysis.

2.2.6. Gas Chromatography conditions

The FAME extracts were analyzed in a gas chromatograph HP6890A (Hewlett-Packard, Avondale, PA, USA), equipped with a flame-ionization detector (GLC-FID) and a BPX70 capillary column (60 m x 0.32 mm x 0.25 µm; SGE Europe Ltd, Courtaboeuf, France) at conditions previously described in **Section 2.1.11 Gas Chromatography conditions** (in Materials and Methods of Part I). Supelco 37 and FAME from CRM-164 were used for the identification of FAs. Tonalin CLA was applied to identify the chromatographic region of CLA isomers. The CLA and CLNA isomers identification was based on previous works carried out with the same strain assayed herein (Coakley et al., 2009; Fontes et al., 2018). GLC-Nestlé36 was assayed for calculation of response factors and detection and quantification limits (LOD: 0.79 ng FA/mL; LOQ: 2.64 ng FA/mL).

2.2.7. UV spectroscopy

The FAME extracts of milk FFA fraction before and after fermentation with hydrolyzed FSO at 2 mg/mL α-LNA (S2LNA) and of EFA fraction from Tonalin CLA and PSO were analyzed in a UV spectrophotometer UV-1900 (Shimadzu, Kyoto, Japan). The absorption spectra were recorded between 200-400 nm, at 1 nm intervals, using diluted solutions of each sample in hexane until absorbance was below 1.50. Hexane was further used for background measurement.

2.2.8. Statistical analysis

Results are reported as mean values ± standard deviation (n=3). Data were first analyzed for normality distribution. Levene's test was applied to verify the homogeneity of the variances. When the normality assumption was not violated, one-way ANOVA was performed to verify statistical differences between groups with equal variances, otherwise, it was applied the Welch test. When normality was not guaranteed, the Kruskal-Wallis test was carried out instead. For groups with statistical differences, pairwise comparisons were made through post-hoc Bonferroni (homogeneity of variances guaranteed) or Games-Howell (homogeneity of variances not guaranteed) for normal data, otherwise, the Mann-Whitney test was applied. Regarding the analysis of FA profiles, at each substrate condition, differences from 0 h to 24 h were compared through T-test paired samples when normality was guaranteed, otherwise, the Wilcoxon test was applied. The level of significance was set, in general, at 0.05; for growth experiments, CFU differences were considered when differing ≥1 log cycle and for pH when differing ≥0.5 units. Analyses were performed using IBM SPSS Statistics 28 (SPSS Inc., IBM Corporation, NY, USA).

Part III - Evaluation of fatty acid metabolites in microbial CLA/CLNA-enrichment of milk

2.3.1. Chemicals and resources

Hexane, methanol and dimethylformamide for FFA analysis were purchased from VWR Chemicals (Radnor, PA, USA), while hexane, methanol, and dichloromethane for EFA analysis were obtained from Fisher Scientific (Loughborough, UK). Sulphuric acid and potassium hydroxide were from Honeywell (Charlotte, NC, USA), while sodium methoxide and methyl acetate were from Acros Organics (Geel, Belgium). Sodium chloride was purchased from BDH Prolabo (VWR Chemicals). GLC-Nestlé36 FAME mix was obtained from Nu-Chek Prep, Inc. (Elysian, MN, USA). Undecanoic acid (98.0%) was from Alfa Aesar (Haverhill, MA, USA). Supelco 37 FAME mix, LA, α-LNA and CRL type VII lipase were purchased from Sigma-Aldrich (St. Louis, MO, USA). Flaxseed oil (FSO) and pasteurized semi-skimmed cows' milk were bought in local markets (Porto, Portugal).

2.3.2. Substrate emulsion preparation

First, FSO was hydrolyzed with CRL and recovered as described in **Section 2.2.2 Vegetable oils hydrolysis** (in Materials and Methods of Part II). Oil emulsion was prepared at approximately

50 mg/mL of free α-LNA (from hydrolyzed FSO) with 2% (w/v) Tween 80 (Sigma-Aldrich) and homogenized and sterilized as depicted in **Section 2.2.3 Microbially CLA/CLNA-enrcihed milk** (in Materials and Methods of Part II).

Stock solutions of pure LA and α-LNA were also prepared, at 50 mg/mL with 2% (w/v) Tween 80, and further homogenized and sterilized as described in **Section 2.1.5 Determination of maximum tolerance to linoleic acid** (in Materials and Methods of Part I).

2.3.3. Milk fermentation with pure substrates or hydrolyzed oil

Bifidobacterium breve DSM 20091 (DSMZ, Braunschweig, Germany) was activated as described in **Section 2.2.3 Microbially CLACLNA-enriched milk** (in Materials and Methods of Part II). Afterwards, the strain was inoculated at 1% (v/v) in cows' milk (100 mL), supplemented with 0.05% (w/v) cysteine, and at the following precursor substrate conditions: *Control* – no substrate; *SLA* – 0.5 mg/mL LA; *SLNA* – 0.5 mg/mL α-LNA; *SLA+LNA* – 0.15 mg/mL LA + 0.5 mg/mL α-LNA; *S0.5FSO* – 0.5 mg/mL α-LNA from hydrolyzed FSO; *S2FSO* - 2 mg/mL α-LNA from hydrolyzed FSO. Inoculated milk was then incubated at 37 ºC for 24 h in triplicate. Cultures were always grown under anaerobic conditions (Whitley DG 250; Don Withley Scientific, Yorkshire, UK; gas mixture of 85% nitrogen, 5% hydrogen and 10% carbon dioxide). Milk samples were collected before and after fermentation for further viable cell numbers enumeration, pH measurement (Basic 20; Crison, Barcelona, Spain) and FA analysis (**Sections 2.3.4** and **2.3.5**).

2.3.4. Sample preparation for fatty acid analysis

For EFA analysis in milk (200 mg) samples [i.e., inoculated milk before (0 h) and after (24 h) *B. breve* DSM 20091 growth with or without substrate], lipid isolation was performed through the Folch method as described by Matyash et al. (2008) and then FAME extracts were prepared according to Reis et al. (2013). Briefly, 1.5 mL of methanol were added to the sample and homogenized for 60 s. Then, 3 mL of dichloromethane were added and homogenized for 60 s. Ultra-pure water was thereafter added to a final volume of 1.25 mL, adjusted to the natural percentage of water present in milk. The mixture was let to rest for 10 min, with occasional agitation, and then centrifuged (1000 ×*g*, room temperature, 10 min). The lower layer, containing the lipid extract, was collected in a previously weighed glass tube. About 2 mL of dichloromethane/methanol [2:1, v/v] was further added to the extraction mixture, let to rest for 10 min, with occasional agitation, and then centrifuged (1000 ×*g*, room temperature, 10 min). The lower phase was again collected. The lipid extract was then evaporated under a nitrogen stream and the glass tube was weighed to determine the lipid extract mass.

Lipid extract was resuspended in dichloromethane in a way to transfer 30 µg to a new tube, being further evaporated under a nitrogen stream. About 1 mL of hexane was added, followed by 200 µL of potassium hydroxide (2M in methanol), and the mixture was homogenized for 2 min. Then, it was added 2 mL of sodium chloride (1%, w/v in ultra-pure water), and the reaction mixture was centrifuged (2 000 rpm, room temperature, 5 min). From the upper layer, containing FAME, 600 µL

were collected to a new tube and evaporated under a nitrogen stream. The FAME extract was resuspended in 100 µL hexane for further chromatographic analysis.

For FFA analysis, FSO emulsion (500 µL) and milk (500 mg) samples were prepared according to Pimentel et al. (2015) with some modifications. The FSO emulsion samples were added with 100 µL of undecanoic acid (1.5 mg/mL in hexane) before derivatization. Then, 100 µL of methyl acetate were added, followed by 3.60 mL (FSO emulsion) or 3.70 mL (milk samples) of hexane, 2.26 mL of methanol, and 240 µL of sodium methoxide (5.4M in methanol). Samples were homogenized and incubated at 40 ºC for 10 min. After cooling in ice, 500 µL of deionized water were added, to enable phase separation, then homogenized and centrifuged (1250 ×*g*, 18 ºC, 5 min). The upper layer was recovered. To clean up, 2 mL of hexane were further added to the remaining reaction mixture, and then homogenized and centrifuged (1250 ×*g*, 18 ºC, 5 min). The upper layer was then recovered and 100 µL of methyl tricosanoate (1.5 mg/mL in hexane) were added (only to FSO emulsion samples), followed by 1.25 mL of dimethylformamide, before 1.25 mL of sulphuric acid (3M in methanol). Samples were homogenized and incubated at 60 ºC for 30 min. Finally, after cooling, 700 µL (FSO emulsion) or 800 µL (milk samples) of hexane were added, and then homogenized and centrifuged (1250 ×*g*, 18 ºC, 5 min). The upper layer, containing FAME, was collected for further chromatographic analysis.

2.3.5. Gas Chromatography conditions

2.3.5.1. Gas Chromatography – Flame Ionization Detector

For quantification of free α-LNA in hydrolyzed FSO emulsion, FAME extracts were analyzed in a gas chromatograph HP6890A (Hewlett-Packard, Avondale, PA, USA), equipped with a flame-ionization detector (GLC-FID) and a BPX70 capillary column (60 m x 0.32 mm x 0.25 µm; SGE Europe Ltd, Courtaboeuf, France) at conditions previously described in **Section 2.1.11 Gas Chromatography conditions** (in Materials and Methods of Part I). Supelco 37 was used for the identification of FAs. GLC-Nestlé36 was assayed for calculation of response factors and detection and quantification limits (LOD: 0.79 ng FA/mL; LOQ: 2.64 ng FA/ mL).

2.3.5.2. Gas Chromatography – Mass Spectrometry

For EFA and FFA profiling of milk samples, FAME extracts were analyzed in an Agilent Technologies 8860 gas chromatograph (Santa Clara, CA, USA) equipped with a DB-FFAP capillary column (30 m x 0.32 mm x 0.25 µm; J&W Scientific, Folsom, CA, USA) and coupled to an Agilent 5977B mass spectrometer (MS) detector (Agilent Technologies). The analysis conditions were as follows: injector temperature – 220 ºC, injection volume – 2 µL (splitless); detector (MS) temperature – 230 ºC; carrier gas – helium at 1.4 mL/min; oven temperature program – started at 58 ºC (held for 2 min), then raised at 25 ºC/min to 160 ºC, 2 ºC/min to 210 ºC and, finally, 20 ºC/min to 225 ºC (held for 15 min). Mass spectra were obtained at an electron impact ionization of 70 eV and a mass range of *m*/*z* 50-550 in 1-second cycles using the full scan acquisition mode. The FA compounds' identification was carried out using Agilent MassHunter Qualitative Analysis 10.0 (Agilent

Technologies) based on mass spectra NIST database and previous data [Fontes et al. (2018) and Part II results]. Results are expressed as relative abundance percentages.

2.3.6. Statistical analysis

Results are reported as mean values ± standard deviation (n=3). Data were first analyzed for normality distribution by Shapiro-Wilk test. Differences from time 0 h to the end of fermentation (24 h) were analyzed by T-student paired samples test (normality guaranteed) or Wilcoxon (normality not guaranteed). The level of significance was set, in general, at 0.05; for growth experiments, CFU differences had to be ≥1 log cycle and pH values had to differ ≥0.5 units. Analyses were performed using IBM SPSS Statistics 28 (SPSS Inc., IBM Corporation, NY, USA).

Part IV – Biochemical and organoleptic properties of microbial CLNA-enriched fermented milk

2.4.1. Chemicals and resources

Hexane, methanol and dimethylformamide were HPLC grade (VWR Chemicals, West Chester, PA, USA). Lactic acid (≥90%) was also purchased from VWR. Sulfuric acid was from Honeywell Fluka (Charlotte, NC, USA), while sodium methoxide and methyl acetate were from Acros Organics (Geel, Belgium). GLC-Nestlé36 FAME mix was obtained from Nu-Chek Prep, Inc. (Elysian, MN, USA) and butterfat CRM-164 (EU Commission; Brussels, Belgium) from Fedelco Inc. (Madrid, Spain). Undecanoic acid (98.0%) was from Alfa Aesar (Haverhill, MA, USA) while glyceryl tritridecanoate (>99.0%) was from Larodan (Solna, Sweeden). Galactose (≥98%) and acetic acid (100%) were obtained from Merck (Darmstadt, Germany). Sodium hydroxide was from LabChem (LaborSpirit Lda, Loures, Portugal) and phenolphthalein from José M. Vaz Pereira S.A. (Santarém, Portugal). Supelco 37 FAME mix, methyl tricosanoate (≥99.0%), α-lactose monohydrate (≥99%, total lactose basis), glucose (≥99.5%), CRL type VII lipase, and formic (≥95%), propionic (99%) and citric (99%) acids were purchased from Sigma-Aldrich (St. Louis, MO, USA). Pasteurized semi-skimmed cows' milk and FSO were bought in local markets (Porto, Portugal).

2.4.2. Oil emulsion preparation

First, FSO was hydrolyzed with CRL and recovered as described in **Section 2.2.2 Vegetable oils hydrolysis** (in Materials and Methods of Part II). Oil emulsion was prepared at approximately 50 mg/mL of free α-LNA (from hydrolyzed FSO) with 2% (w/v) Polysorbate 80 (food-grade; Sigma-Aldrich) and homogenized and sterilized as depicted in **Section 2.2.3 Microbially CLA/CLNA-enrcihed milk** (in Materials and Methods of Part II).

2.4.3. Optimization of incubation time

Bifidobacterium breve DSM 20091 (DSMZ, Braunschweig, Germany), stored at -80 ºC in glycerol 30% (w/w) (Fisher Scientific, Hampton, NH, USA), was activated at 2% (v/v) in MRS broth

(Biokar Diagnostics, Beauvais, France), supplemented with 0.01% (w/v) bacteriological meat extract (Biokar) and 0.05% (w/v) L-cysteine-HCl (Sigma-Aldrich), and incubated overnight at 37 ºC. The activated culture was then transferred to skim milk (Oxoid, Hampshire, UK), supplemented with 0.05% (w/v) cysteine (food-grade; Sigma-Aldrich), at 10% (v/v), and incubated at 37 ºC for 16 h. Afterwards, the strain was inoculated at 1% (v/v) in commercial pasteurized semi-skimmed milk (100 mL), supplemented with 0.05% (w/v) cysteine (food-grade) and 2 mg/mL α-LNA from hydrolyzed FSO. Inoculated milk was then incubated at 37 ºC for 16, 18, 20, 22 and 24 h in triplicate. Cultures were always grown under anaerobic conditions (Whitley DG 250; Don Withley Scientific, Yorkshire, UK; gas mixture of 85% nitrogen, 5% hydrogen and 10% carbon dioxide). Milk samples were collected at the beginning and after fermentation for further viable cell numbers determination, pH measurement (Basic 20; Crison, Barcelona, Spain) and free CLA and CLNA quantification (**Sections 2.4.10** and **2.4.11**).

2.4.4. Milk fermentation

Bifidobacterium breve DSM 20091 was activated and inoculated in milk with hydrolyzed FSO emulsion as previously described (**Section 2.4.3 Optimization of incubation time**; in Materials and Methods of Part IV). Milk devoid of α-LNA was used as control and similarly inoculated. Inoculated milk was then incubated at 37 ºC for 22 h. Cultures were always grown under anaerobic conditions using gas-generating systems in a sealed container (GasPak EZ; BD, Franklin Lakes, NJ, EUA). Fermented milk samples were stored at 4 ºC before further analysis.

2.4.5. Sugars and organic acid analysis

About 1 g of non-fermented and fermented milk samples (n=3) was dissolved in 5 mL sulfuric acid (5 mM), homogenized with Ultra-Turrax (18 000 rpm, 3 min) and centrifuged (5 000 rpm, room temperature, 10 min). The supernatant was first filtered with filter paper and then through a 0.45 µm-pore size membrane (Chromafil; Macherey-Nagel, Düren, Germany) immediately before analytical analysis. Samples were analyzed by a high-performance liquid chromatography system that consisted of a pump (K-1001 HPLC pump; Knauer, Berlin, Germany), an ion exchange Aminex HPX-87H Column (300 × 7.8 mm; Bio-Rad, Hercules, CA, USA), a column oven, and two detectors - refractive index (K-2301 RI detector; Knauer) to determine sugars, and UV spectrophotometry at 210 nm (K-2501 UV Detector; Knauer) to determine organic acids. Injection conditions were as follows: mobile phase – 5 mM sulfuric acid; flow rate – 0.6 mL/min; injection volume – 20 µL; column temperature – 65 ºC; running time – 30 min. Compounds were quantified through calibration curves of external standards: sugars (0.25-10 mg/mL) – lactose, galactose and glucose; organic acids (0.25-2.5 mg/mL) – citric acid, lactic acid, acetic acid, formic acid and propionic acid.

2.4.6. Acid titration

About 10 g of non-fermented and fermented milk samples (n=3) were diluted in 20 mL deionized water, homogenized with slight agitation, and added with 4 drops of phenolphthalein (0.5%, w/v). Samples were thereafter titrated with sodium hydroxide (0.1M) until a persistent pink

tone was attained. The volume of sodium hydroxide used was registered and the percentage of lactic acid was calculated as follows:

$$Lactic\ acid\ (\%) = \frac{V_{NaOH}(L) \times M_{NaOH}(g/mol) \times M_{C3H6O3}(g/mol)}{m_{sample}\ (g)} \times 100 \qquad \text{Equation 2.7}$$

Being, V_{NaOH} the volume of sodium hydroxide used in the titration, M_{NaOH} the molecular weight of sodium hydroxide, M_{C3H6O3} the molecular weight of lactic acid, and m_{sample} the mass of sample assayed.

Milk samples' pH was also measured (Basic 20; Crison, Barcelona, Spain).

2.4.7. Nutritional characterization

The fat and sugar contents of fermented milk (n=2) were determined according to the Portuguese norms NP 1923:1987 and NP 704:1994 (Instituto Português da Qualidade, 1988, 1996), respectively. The protein content was measured by multiplying the total nitrogen content determined through the Kjeldahl method (AOAC, 1990) by the conversion factor of 6.25. The dry residue was obtained by drying samples at 102 ºC to constant weight and ashes through incineration in muffle at 550 ºC. The total carbohydrate content was obtained by calculated difference.

2.4.8. Volatile compounds analysis

Volatiles were analyzed in a gas chromatograph (GC) 456-GC (Bruker, Billerica, MA, EUA) coupled with a mass spectrometer (MS) detector EVOQ TQ (Bruker) and a solid-phase microextraction (SPME) system. Approximately 5 g of fermented milk samples (n=2) were added to 20 mL-glass vials. Samples were first incubated at 40 ºC for 5 min and then a SPME fiber (divinylbenzene/carboxen/polydimethylsiloxane, 50/30 mm; Supelco, Bellefonte, PA, USA) was inserted to absorb volatile compounds during 30 min at 40 ºC with occasional agitation. The volatile compounds were thereafter desorbed into the injector for 15 min and separated by a CP-Wax 58 FFAP CB column (50 m x 0.25 mm x 0.20 µm; Agilent Technologies, Santa Clara, CA, USA) at the following conditions: injector temperature – 220 ºC; split – 1:30 (30 s); carrying gas – helium at 1 mL/min flow; oven temperature program – 40 ºC held for 1 min, raised at 2 ºC/min until 220 ºC and held for 30 min. Mass spectra were obtained by a mass range of *m*/*z* 45-350 in full scan mode (ion source 230 ºC). The volatile compounds identification was based on mass spectra NIST database and results were expressed as relative area percentages.

2.4.9. Sensory analysis

Before sensory analysis, additional fermented milk samples were used for the determination of total microbial and *Enterobacteriaceae* counts, through plating of sequential dilutions in Plate Count Agar (PCA; Biokar) and Violet Red Bile Glucose Agar (VRBGA; Biokar) agar plates, and incubation for 24 h at 30 ºC or 37 ºC in aerobiosis, respectively.

Once microbiological safety was guaranteed, fermented milk samples were subjected to sensory characterization by a senior trained panel (n=8). The samples were assessed in terms of appearance, aroma, texture, mouthfeel, flavor, and after-taste attributes.

2.4.10. Sample preparation for fatty acid analysis

For the FA analysis, oil emulsion (10 µL) and fermented milk (500 mg) samples were prepared as described in **Section 2.2.5 Sample preparation for fatty acid analysis** (in Materials and Methods of Part II) to obtain their FAME derivatives.

2.4.11. Gas chromatography conditions

The FAME extracts were analyzed in a gas chromatograph Agilent 8860 (Agilent Technologies), equipped with a flame-ionization detector (GLC-FID) and a BPX70 capillary column (60 m x 0.32 mm x 0.25 µm; SGE Europe Ltd, Courtaboeuf, France) at conditions previously described in **Section 2.1.11 Gas Chromatography conditions** (in Materials and Methods of Part I). Supelco 37 and FAME from CRM-164 were used for the identification of FAs. The identification of CLA and CLNA isomers was based on previous data [Fontes et al. (2018) and Part II results]. GLC-Nestlé36 was assayed for calculation of response factors and detection and quantification limits (LOD: 0.79 ng FA/mL; LOQ: 2.64 ng FA/ mL).

2.4.12. Statistical analysis

Results are reported as mean values ± standard deviation of at least duplicate samples. Data were first analyzed for normality distribution. Levene's test was applied to verify the homogeneity of the variances. Afterwards, if normality was guaranteed, one-way ANOVA (equal variances) or Welch test (no equal variances) was applied to compare more than two groups, with post-hoc Bonferroni (equal variances) or Games-Howell (no equal variances). If normality was not guaranteed, the comparison between two groups was carried out by applying the Mann-Whitney test, and to compare more than two groups the Kruskal-Wallis test was performed followed by pairwise comparison with the Mann-Whitney test. Level of significance was set in general at 0.05; in terms of CFU, differences had to be ≥1 log cycle and pH differences had to be ≥0.5 units. Analyses were performed using IBM SPSS Statistics 28 (SPSS Inc., IBM Corporation, NY, USA).

Part V – Stability assessment of developed CLNA-enriched dairy products throughout storage

2.5.1. Chemicals and resources

Hexane, methanol and dimethylformamide were HPLC grade (VWR Chemicals, West Chester, PA, USA). Sulfuric acid was from Honeywell Fluka (Charlotte, NC, USA), while sodium methoxide and methyl acetate were from Acros Organics (Geel, Belgium). GLC-Nestlé36 FAME mix was obtained from Nu-Chek Prep, Inc. (Elysian, MN, USA) and butterfat CRM-164 (EU Commission;

Brussels, Belgium) from Fedelco Inc. (Madrid, Spain). Undecanoic acid (98.0%) was from Alfa Aesar (Haverhill, MA, USA) while glyceryl tritridecanoate (>99.0%) was from Larodan (Solna, Sweeden). Supelco 37 FAME mix, methyl tricosanoate (≥99.0%) and CRL type VII lipase were purchased from Sigma-Aldrich (St. Louis, MO, USA). Pasteurized semi-skimmed cows' milk and FSO oil were bought in local markets (Porto, Portugal).

2.5.2. Oil emulsion preparation

First, FSO was hydrolyzed with CRL and recovered as described in **Section 2.2.2 Vegetable oils hydrolysis** (in Materials and Methods of Part II). Oil emulsion was prepared at approximately 50 mg/mL of free α-LNA (from hydrolyzed FSO) with 2% (w/v) Polysorbate 80 (food-grade; Sigma-Aldrich) and homogenized and sterilized as depicted in **Section 2.2.3 Microbially CLA/CLNA-enriched milk** (in Materials and Methods of Part II).

2.5.3. Milk fermentation

Bifidobacterium breve DSM 20091 (DSMZ, Braunschweig, Germany) was activated and inoculated as stated in **Section 2.4.4 Milk fermentation** (in Materials and Methods of Part IV). Cultures were always grown under anaerobic conditions (Whitley DG 250; Don Withley Scientific, Yorkshire, UK; gas mixture of 85% nitrogen, 5% hydrogen and 10% carbon dioxide).

2.5.4. Stability of CLNA-enriched fermented milk

Fermented milk samples were stored at 4 ºC for 28 days, being air- and light-protected with parafilm and aluminum foil. Independent flasks (n=3) were collected at time 0 and every 7 days until the end of the storage period for further analyses: *B. breve* viable cell numbers determination through plating on cys-MRS agar plates of sequential decimal dilutions, total microbial counts determination through plating on PCA (Biokar) plates of sequential decimal dilutions, pH measurement (Basic 20; Crison, Barcelona, Spain) and FA analysis (**Sections 2.5.6** and **2.5.7**).

2.5.5. Stability of CLNA-enriched lyophilized milk

Fermented milk was frozen at -20 ºC and then lyophilized in a freeze dryer (SB4 model, Armfield, Ringwood, UK). Lyophilized milk was stored in a desiccator at room temperature (20-21 ºC) for 12 weeks, being air- and light-protected with parafilm and aluminum foil. Independent flasks (n=3) were collected at time 0 and every 4 weeks until the end of the established storage period for further analyses: *B. breve* viable cell numbers, total microbial counts determination and FA analysis (**Sections 2.5.6** and **2.5.7**).

2.5.6. Sample preparation for fatty acid analysis

For the FA analysis, oil emulsion (10 µL), fermented milk (500 mg) and lyophilized milk (100 mg) samples were prepared as described in **Section 2.2.5 Sample preparation for fatty acid analysis** (in Materials and Methods of Part II) to obtain their FAME derivatives.

2.5.7. Gas chromatography conditions

The FAME extracts were analyzed in a gas chromatograph Agilent 8860 (Agilent Technologies), equipped with a flame-ionization detector (GLC-FID) and a BPX70 capillary column (60 m x 0.32 mm x 0.25 µm; SGE Europe Ltd, Courtaboeuf, France) at conditions previously described in **Section 2.1.11 Gas Chromatography conditions** (in Materials and Methods of Part I). Supelco 37 and FAME from CRM-164 were used for the identification of FAs. The identification of CLA and CLNA isomers was based on previous data [Fontes et al. (2018) and Part II results]. GLC-Nestlé36 was assayed for calculation of response factors and detection and quantification limits (LOD: 0.79 ng FA/mL; LOQ: 2.64 ng FA/ mL).

2.5.8. Statistical analysis

Results are reported as mean values ± standard (n=3). Data were first analyzed for normality distribution. Levene's test was applied to verify the homogeneity of the variances. Afterward, if normality was guaranteed, one-way ANOVA (equal variances) or Welch test (no equal variances) was applied to compare groups, with post-hoc Bonferroni (equal variances) or Games-Howell (no equal variances). If normality was not guaranteed, the Kruskal-Wallis test was performed followed by pairwise comparison with the Mann-Whitney test. Level of significance was set in general at 0.05; in terms of CFU, differences had to be ≥1 log cycle and pH differences had to be ≥0.5 units. Analyses were performed using IBM SPSS Statistics 28 (SPSS Inc., IBM Corporation, NY, USA).

CHAPTER 3

Results and Discussion

CHAPTER 3 – RESULTS AND DISCUSSION

Part I - Identification of CLA/CLNA-producing strains

The aim of Part I was to select probiotic strains capable of producing CLA and CLNA isomers from their precursor substrates (i.e., LA and α-LNA, respectively). To do so, it was first necessary to improve the homogenization of substrate emulsion before further application. Then, to select potential CLA-producers, a molecular detection of genes encoding enzymes involved in LA isomerization was performed and the maximum tolerance to LA was further determined. Thereafter, the genotypically-positive strains were assayed for CLA production quantification. The influence of shaking on CLA production and CLA kinetics were further studied with the identified CLA-producing strains. Finally, these strains were tested for CLNA production.

3.1.1. Improvement of linoleic acid emulsion

Being LA a FA, Tween 80 was applied as a surfactant to allow its dispersion in bacteria culture media. However, to guarantee sterility and not compromise LA stability, the only viable way to sterilize LA emulsion was through filtration; however, if the emulsion was not well homogenized, part of the LA could be retained in the filter. Indeed, when it was filtered through 0.45 µm, 17.09% of the initial LA amount was lost (**Table 3.1**). The lowest LA losses were achieved with a previous treatment using the Ultra-Turrax (7.17-7.40%) (**Table 3.1**). Although the Ultrasonic treatment enabled smaller and more uniform droplets in the oil-in-water emulsions than by the Ultra-Turrax (Liu & Wang, 2021; Urbánková et al., 2019), it was too aggressive for LA integrity.

Table 3.1 Linoleic acid loss after treatment with Ultra-Turrax, Ultrasonic Liquid Processor and/or Filtration at 0.45 µm.

	F45	UT90+F45	UT150+F45	US90+F45
LA loss (%)	17.09 ± 1.47^{a}	7.17 ± 2.87^{c}	7.40 ± 2.82^{c}	13.01 ± 1.34^{b}

Average values ± standard deviation (n=2). Different superscript letters indicate significant differences ($p<0.05$). LA – Linoleic acid; F45 – Filtration at 0.45 µm; UT90 – Ultra-Turrax during 90 s; UT150 – Ultra-Turrax during 150 s; US90 – Ultrasonic Liquid Processor during 90 s.

Even though several studies assaying CLA/CLNA microbial production filter the substrate emulsion through a 0.45 µm-pore size membrane (Hennessy et al., 2012; Renes et al., 2017a), to guarantee microbiological sterility, it would be more appropriate to filter the emulsion through a smaller pore-size membrane, namely, 0.20 µm. Thus, it was further tested if a previous treatment using the Ultra-Turrax for 150 s would still assure a good homogenization for filtration through 0.20 µm. Results showed that LA was lost by 10.07% after filtration (**Table 3.2**), but with the Ultra-Turrax treatment LA loss was reduced to just 3.71%. Therefore, this procedure was selected for further preparations of stock LA solutions.

Table 3.2 Linoleic acid loss after treatment with the Ultra-Turrax and/or Filtration at 0.20 µm.

	F20	UT150+F20
LA loss (%)	10.07 ± 0.05[a]	3.71 ± 1.28[b]

Average values ± standard deviation (n=2). Different superscript letters indicate significant differences ($p<0.05$). LA – Linoleic acid; F20 – Filtration at 0.20 µm; UT150 – Ultra-Turrax during 150 s.

3.1.2. Genotypic screening

Some studies have detected the presence of LAI gene in lactobacilli strains with associated CLA production capacity (Gorissen et al., 2011; Tyagi et al., 2020). Among the 70 strains of lactobacilli assayed, the putative LAI gene was detected with the designed primers in 32 of them (**Table 3.3** and **Table 3.4**). In contrast, with the primers described by Gorissen et al. (2011) the LAI gene was only detected in 14 of them (**Table 3.3** and **Table 3.4**). Different LAI gene sequences corresponding to different species are available in GenBank (Fontes et al., 2017), but the designed primers, which were constructed targeting conserved motifs of the lactobacilli LAI protein, showed to be more efficient in identifying potential CLA-producing strains than the primers described by Gorissen et al. (2011), which were constructed based on the LAI gene sequence of only one strain of *L. plantarum* and one strain of *L. sakei*.

Table 3.3 Outcome from molecular detection of *lai* among lactobacilli.

Strain	Gorissen et al. (2011) primers	Designed primers
Lacticaseibacillus casei 01	-	+
Lacticaseibacillus casei 431	-	+
Lacticaseibacillus casei MP10	-	+
Lacticaseibacillus casei MP11	-	-
Lacticaseibacillus casei MP8	-	+
Lacticaseibacillus casei MP9	-	+
Lacticaseibacillus casei NCTC 6375	-	-
Lacticaseibacillus paracasei L26	-	+
Lacticaseibacillus paracasei subsp. paracasei NCFB 151	-	+
Lacticaseibacillus rhamnosus MP18	+	+
Lacticaseibacillus rhamnosus R11	-	+
Lactiplantibacillus plantarum 229v	+	+
Lactiplantibacillus plantarum CECT 7482	+	+
Lactiplantibacillus plantarum D36	+	+
Lactiplantibacillus plantarum DSM 12028	+	+
Lactiplantibacillus plantarum DSM 20174	+	+
Lactiplantibacillus plantarum DSM 20205	+	+
Lactiplantibacillus plantarum MP16	+	+
Lactiplantibacillus plantarum MP17	+	+
Lactiplantibacillus plantarum NCFB 1752	+	+
Lactobacillus acidophilus ATCC 4356	-	+
Lactobacillus acidophilus Ki	-	+
Lactobacillus acidophilus L10	-	-

(-) gene was not detected, (+) gene was detected.

Table 3.4 Outcome from molecular detection of *lai* gene among lactobacilli (*continued*).

Strain	Gorissen et al. (2011) primers	Designed primers
Lactobacillus acidophilus LA-5	-	-
Lactobacillus acidophilus MP169	-	+
Lactobacillus acidophilus MP198	-	-
Lactobacillus acidophilus MP289	-	+
Lactobacillus acidophilus MP345	-	-
Lactobacillus acidophilus MP4	-	-
Lactobacillus acidophilus MP5	-	-
Lactobacillus acidophilus MP6	-	-
Lactobacillus acidophilus MP7	-	-
Lactobacillus delbruecki subsp. bulgaricus DSM 20081	+	+
Lactobacillus delbrueckii subsp. bulgaricus NCFB 1489	-	-
Lactobacillus gasseri MP199	-	-
Lactobacillus gasseri MP289	-	-
Lactobacillus gasseri MP321	-	-
Lactobacillus gasseri MP551	-	+
Latilactobacillus curvatus LMG 9198	-	-
Latilactobacillus sakei CTC 372	-	+
Latilactobacillus sakei DSM 20017	+	+
Latilactobacillus sakei Lb 706	-	-
Lentilactobacillus buchneri NCFB 110	-	-
Lentilactobacillus hilgardii NCFB 264	-	+
Levilactobacillus brevis D24	+	+
Ligilactobacillus salivarius DSM 20555	-	+
Ligilactobacillus salivarius MP143	-	-
Ligilactobacillus salivarius MP160	-	-
Ligilactobacillus salivarius MP19	-	-
Ligilactobacillus salivarius MP20	-	-
Ligilactobacillus salivarius MP21	-	-
Ligilactobacillus salivarius MP22	-	-
Ligilactobacillus salivarius MP23	-	-
Ligilactobacillus salivarius MP24	-	-
Ligilactobacillus salivarius ZH26-12	-	-
Limosilactobacillus fermentum MP101	-	-
Limosilactobacillus fermentum MP12	-	-
Limosilactobacillus fermentum MP13	-	-
Limosilactobacillus fermentum MP14	-	-
Limosilactobacillus fermentum MP149	-	-
Limosilactobacillus fermentum MP15	-	-
Limosilactobacillus fermentum MP169	-	-
Limosilactobacillus fermentum MP170	-	-
Limosilactobacillus fermentum MP171	-	-
Limosilactobacillus fermentum MP2621	-	-
Limosilactobacillus fermentum MP95	-	-
Limosilactobacillus fermentum MP96	-	-
Limosilactobacillus fermentum NCFB 1750	-	+
Loigolactobacillus coryniformis LMG 9196	+	+
Secundilactobacillus collinoides NCFB 2805	-	+

(-) gene was not detected, (+) gene was detected.

As previously mentioned in **Section 1.4.3 Linoleate isomerase and other involved enzymes** (in Introduction), LAI activity may consist of a multienzymatic system, and it has been suggested that the hydration/dehydration steps are catalyzed by the MCRA protein (Yang, Qi, et al., 2017). Despite the significant homology existing between LAI and MCRA (Farmani et al., 2010), hydratase activity has been associated only with MCRA, with hydroxy FAs production from LA (Rosberg-Cody et al., 2011). The MCRA role in LA isomerization is not clear, but its high homology with LAI suggests that detection of the MCRA gene may be associated with CLA production. Within the 15 strains belonging to the *Bifidobacterium*, *Enterococcus*, *Lactococcus* and *Propionibacterium* genera, an MCRA-like gene was detected only in 6 bifidobacteria strains (**Table 3.5**). The designed primers were less efficient in detecting this gene (3 out of the 6 bifidobacteria; **Table 3.5**), even though both were constructed based on conserved motifs. In fact, the MCRA sequence is highly conserved among different bacterial species (Volkov et al., 2010).

Table 3.5 Outcome from molecular detection of *mcra* gene among bifidobacteria, enterococci, lactococci and propionibacteria.

Strain	Rosberg-Cody et al. (2011) primers	Designed primers
Bifidobacteria		
Bifidobacterium animalis B94	-	-
Bifidobacterium animalis Bb12	+	-
Bifidobacterium animalis BLC	+	-
Bifidobacterium animalis BO	+	-
Bifidobacterium bifidum DSM 20239	+	+
Bifidobacterium breve DSM 20091	+	+
Bifidobacterium breve MP123Te	-	-
Bifidobacterium breve MP302	-	-
Bifidobacterium pseudolongum DSM 20095	+	+
Enterococci		
Enterococcus 32	-	-
Enterococcus 37	-	-
Lactococci		
Lactococcus lactis ATCC 11454	-	-
Lactococcus lactis MP238	-	-
Propionibacteria		
Propionibacterium freudenreichii subsp. shermanii JS	-	-
Propionibacterium freudenreichii subsp. freudenreichii DSM 20271	-	-

(-) gene was not detected, (+) gene was detected.

A LA hydratase (CLA-HY) that catalyzes the hydration and dehydration steps of the CLA production pathway has been further described (Zhao et al., 2020). Consequently, the detection of the corresponding structural gene could allow the identification of potential CLA-producers. Hirata et al. (2015) identified two proteins in the genome of *L. acidophilus* ATCC4796 that shared 32% amino acid identity with CLA-HY and were able to clone the respective structural genes (*fa-hy1* and *fa-hy2*). But since both sets of primers were designed based on a single *L. acidophilus* genome, only the *L.*

acidophilus strains were screened with them. However, only one strain (out of 12) was positive and contained both hydratase genes (**Table 3.6**).

Table 3.6 Outcome from molecular detection of *fa-hy* gene among *Lactobacillus acidophilus*.

Strain	Hirata et al. (2015) primers
Lactobacillus acidophilus Ki	-
Lactobacillus acidophilus L10	-
Lactobacillus acidophilus LA-5	+
Lactobacillus acidophilus ATCC 4356	-
Lactobacillus acidophilus MP169	-
Lactobacillus acidophilus MP198	-
Lactobacillus acidophilus MP289	-
Lactobacillus acidophilus MP4	-
Lactobacillus acidophilus MP5	-
Lactobacillus acidophilus MP345	-
Lactobacillus acidophilus MP6	-
Lactobacillus acidophilus MP7	-

(-) gene was not detected, (+) gene was detected.

Accordingly, based on the full results listed in **Table 3.3** to **Table 3.6** a total of 39 strains were identified as potential CLA-producers.

3.1.3. Determination of maximum tolerance to LA

Approximately 33% (28/85) of the strains tested in this study were not able to grow at the lowest LA concentration assayed (i.e., 1 mg/mL) (**Table 3.7** and **Table 3.8**). Among the remaining strains, about 18% (15/85) tolerated 5 mg/mL of LA (**Table 3.7** and **Table 3.8**), 27% (23/85) tolerated 2 mg/mL, and 22% (19/85) tolerated 1 mg/mL. Considering the genotypically-positive strains, i.e., the potential CLA-producers, a large fraction (~38%; 15/39) revealed LA tolerance <1 mg/mL (**Table 3.7** and **Table 3.8**). The number of strains that tolerated 1 and 5 mg/mL was similar (10/39 and 9/39, respectively) (**Table 3.7** and **Table 3.8**), while ~13% (5/39) tolerated 2 mg/mL. According to Qui-square test, LA-tolerance level is independent of genotypical-positivity/negativity [$\chi 2$ (3, N = 85) = 7.62; p>0.05] (**Table 3.9**), even when frequencies of levels <1 and 1 mg/mL were grouped as “low tolerance” and of 2 and 5 mg/mL as “high tolerance” [$\chi 2$ (1, N = 85) = 2.26; p>0.05] (**Table 3.10**).

The LA conversion to CLA isomers may consist of a detoxification mechanism since the presence of LA impairs microbial growth (Mei et al., 2021). Xu et al. (2008) observed that, among different probiotic strains, the best CLA producer could tolerate up to 3 mg LA/mL after 42 h of incubation. Therefore, it was expected that the genotypically-positive strains, i.e., the potential CLA-producers, would reveal a high LA-tolerance (≥2 mg/mL), and the negative ones a low LA-tolerance (<2 mg/mL). However, results showed that strain tolerance level to LA has no relation to their CLA production potential. This might happen because i) the LA bioconversion into CLA is not in fact a detoxifying mechanism, and only some strains genetically possess that potential while others do not, or ii) it is a detoxifying mechanism but the degree of tolerance to LA is particular to each potential

CLA producing-strain, which would merely reflect on different production capacities, i.e., the high-tolerant strains would yield more CLA than the low-tolerant ones.

Table 3.7 Maximum tolerance to linoleic acid of the assayed strains.

Strain	LA tolerance (mg/mL)
Bifidobacteria	
Bifidobacterium animalis B94	<1
Bifidobacterium animalis Bb12*	<1
Bifidobacterium animalis BLC*	2
Bifidobacterium animalis BO*	<1
Bifidobacterium bifidum DSM 20239*	<1
Bifidobacterium breve DSM 20091*	1
Bifidobacterium breve MP123Te	5
Bifidobacterium breve MP302	5
Bifidobacterium pseudolongum DSM 20095*	<1
Enterococci	
Enterococcus 32	2
Enterococcus 37	2
Lactobacilli	
Lacticaseibacillus casei 01*	<1
Lacticaseibacillus casei 431*	<1
Lacticaseibacillus casei MP10*	<1
Lacticaseibacillus casei MP11	2
Lacticaseibacillus casei MP8*	1
Lacticaseibacillus casei MP9*	1
Lacticaseibacillus casei NCTC 6375	5
Lacticaseibacillus paracasei L26*	<1
Lacticaseibacillus paracasei subsp. paracasei NCFB 151*	1
Lacticaseibacillus rhamnosus MP18*	2
Lacticaseibacillus rhamnosus R11*	<1
Lactiplantibacillus plantarum 229v*	1
Lactiplantibacillus plantarum CECT 7482*	5
Lactiplantibacillus plantarum D36*	5
Lactiplantibacillus plantarum DSM 12028*	1
Lactiplantibacillus plantarum DSM 20174*	<1
Lactiplantibacillus plantarum DSM 20205*	<1
Lactiplantibacillus plantarum MP16*	2
Lactiplantibacillus plantarum MP17*	2
Lactiplantibacillus plantarum NCFB 1752*	5
Lactobacillus acidophilus ATCC 4356*	1
Lactobacillus acidophilus Ki*	<1
Lactobacillus acidophilus L10	<1
Lactobacillus acidophilus LA-5*	2
Lactobacillus acidophilus MP169*	<1
Lactobacillus acidophilus MP198	<1
Lactobacillus acidophilus MP289*	<1
Lactobacillus acidophilus MP345	<1
Lactobacillus acidophilus MP4	<1
Lactobacillus acidophilus MP5	<1
Lactobacillus acidophilus MP6	2

LA – Linoleic acid.

Genotypically-positive strains are marked with an asterisk (*).

Table 3.8 Maximum tolerance to linoleic acid of the assayed strains (*continued*).

Strain	LA tolerance (mg/mL)
Lactobacilli	
Lactobacillus acidophilus MP7	<1
Lactobacillus delbruecki subsp. bulgaricus DSM 20081*	5
Lactobacillus delbrueckii subsp. bulgaricus NCFB 1489	<1
Lactobacillus gasseri MP199	<1
Lactobacillus gasseri MP289	<1
Lactobacillus gasseri MP321	<1
Lactobacillus gasseri MP551*	5
Latilactobacillus curvatus LMG 9198	1
Latilactobacillus sakei CTC 372*	1
Latilactobacillus sakei DSM 20017*	5
Latilactobacillus sakei Lb 706	5
Lentilactobacillus buchneri NCFB 110	5
Lentilactobacillus hilgardii NCFB 264*	<1
Levilactobacillus brevis D24*	5
Ligilactobacillus salivarius DSM 20555*	1
Ligilactobacillus salivarius MP143	1
Ligilactobacillus salivarius MP160	2
Ligilactobacillus salivarius MP19	2
Ligilactobacillus salivarius MP20	2
Ligilactobacillus salivarius MP21	2
Ligilactobacillus salivarius MP22	2
Ligilactobacillus salivarius MP23	2
Ligilactobacillus salivarius MP24	1
Ligilactobacillus salivarius ZH26-12	2
Limosilactobacillus fermentum MP101	2
Limosilactobacillus fermentum MP12	2
Limosilactobacillus fermentum MP13	2
Limosilactobacillus fermentum MP14	<1
Limosilactobacillus fermentum MP149	<1
Limosilactobacillus fermentum MP15	1
Limosilactobacillus fermentum MP169	2
Limosilactobacillus fermentum MP170	1
Limosilactobacillus fermentum MP171	1
Limosilactobacillus fermentum MP2621	2
Limosilactobacillus fermentum MP95	2
Limosilactobacillus fermentum MP96	1
Limosilactobacillus fermentum NCFB 1750*	5
Loigolactobacillus coryniformis LMG 9196*	1
Secundilactobacillus collinoides NCFB 2805*	5
Lactococci	
Lactococcus lactis ATCC 11454	1
Lactococcus lactis MP238	2
Propionibacteria	
Propionibacterium freudenreichii subsp. shermanii JS	1
Propionibacterium freudenreichii subsp. freudenreichii DSM 20271	5

LA – Linoleic acid.

Genotypically-positive strains are marked with an asterisk (*).

Table 3.9 Qui-square test results for conjugated linoleic acid production potential *vs*. linoleic acid tolerance (4 levels: <1, 1, 2 and 5 mg/mL).

	Value	Df	Asymp. Sig. (2-sided)
Pearson Chi-Square	7.619[a]	3	0.055
Likelihood Ratio	8.022	3	0.046
Linear-by-Linear Association	0.463	1	0.496
N of Valid Cases	85		

[a] 0 cell (0.0%) has an expected count of less than 5. The minimum expected count is 6.88.

Table 3.10 Qui-square test results for conjugated linoleic acid production potential *vs*. linoleic acid tolerance (2 levels: Low and High).

	Value	Df	Asymp. Sig. (2-sided)
Pearson Chi-Square	2.262[a]	1	0.133
Continuity Correction[b]	1.651	1	0.199
Likelihood Ratio	2.277	1	0.131
Linear-by-Linear Association	2.235	1	0.135
N of Valid Cases	85		

[a] 0 cell (0.0%) has an expected count of less than 5. The minimum expected count is 17.44.

[b] Computed only for a 2x2 table.

3.1.4. CLA production

From the 39 microorganisms containing CLA-related genes, only 4 strains showed the capacity to transform LA into CLA: *B. breve* DSM 20091, *L. gasseri* MP551, *L. plantarum* DSM 20205 and *L. sakei* DSM 20017.

A 1000 bp amplification product was detected from *B. breve* DSM 20091 DNA with both sets of MCRA gene primers (**Figure 3.1**). This strain was able to grow on MRS agar supplemented with 2 mg/mL of LA (**Figure 3.2**); however, when re-exposed to 2 mg/mL in MRS broth, its growth dropped considerably, while at 1 mg/mL it was only slightly affected (**Figure 3.3**).

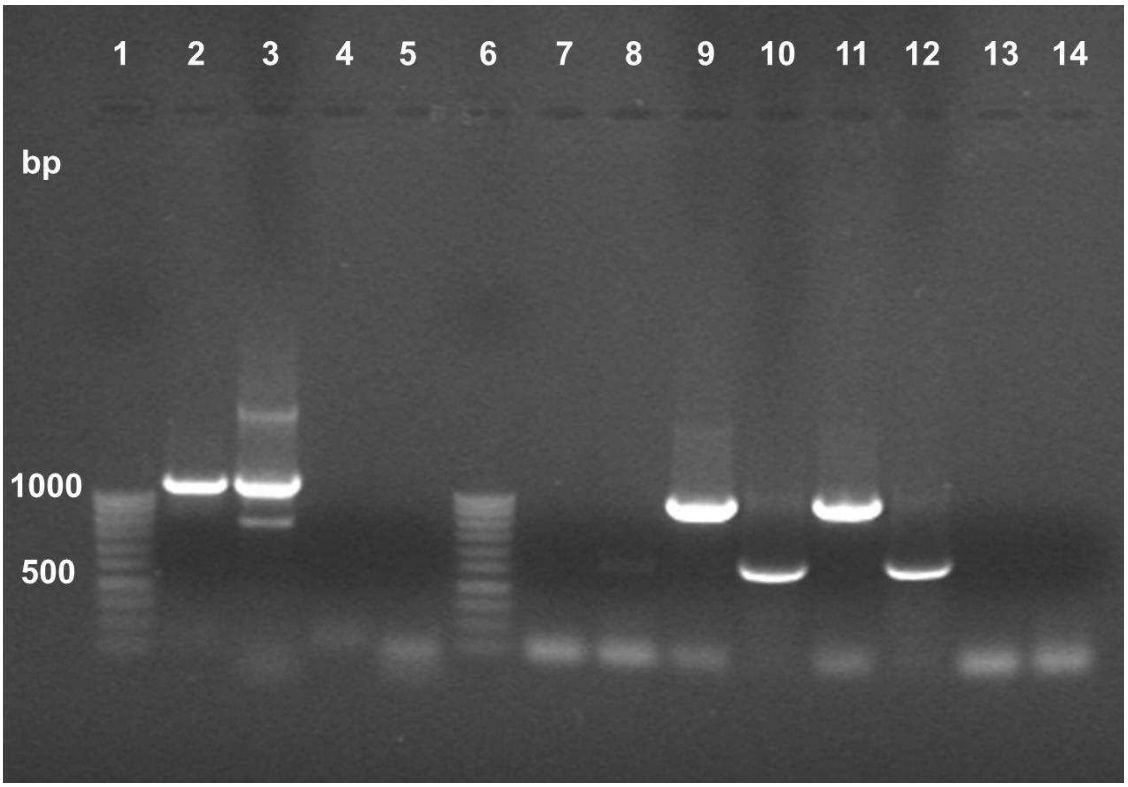


Figure 3.1 Detection of the putative genes encoding linoleate isomerase (LAI) or myosin-cross-reactive antigen (MCRA) in the identified producing strains. Each lane corresponds to the following samples: 1 and 6 – SmartLadder small fragments (Eurogent); 2 – *Bifidobacterium breve* DSM 20091 MCRA gene amplification using Ev1a and Ev2a primers; 3 – *Bifidobacterium breve* DSM 20091 MCRA gene amplification using INIA-Bf1 and INIA-Bf2 primers; 4 – Primers Ev1a and Ev2a negative control; 5 – Primers INIA-Bf1 and INIA-Bf2 negative control; 7 – *Lactobacillus gasseri* MP551 LAI gene amplification using primers LISO3 and LISO4; 8 – *Lactobacillus gasseri* MP551 LAI gene amplification using primers INIA-Lb1 and INIA-Lb2; 9 – *Lactiplantibacillus plantarum* DSM 20205 LAI gene amplification using primers LISO3 and LISO4; 10 – *Lactiplantibacillus plantarum* DSM 20205 LAI gene amplification using primers INIA-Lb1 and INIA-Lb2; 11 – *Latilactobacillus sakei* DSM 20017 LAI gene amplification using primers LISO3 and LISO4; 12 – *Latilactobacillus sakei* DSM 20017 LAI gene amplification using INIA-Lb1 and INIA-Lb2; 13 – Primers LISO3 and LISO4 negative control; 14 – Primers INIA-Lb1 and INIA-Lb2 negative control.

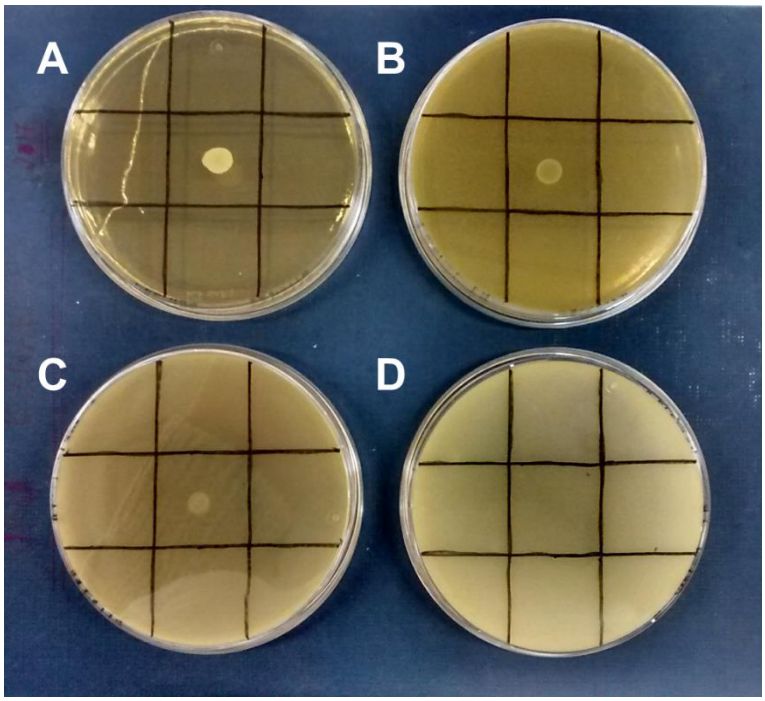


Figure 3.2 Growth of *Bifidobacterium breve* DSM 20091 in cys-MRS agar plates containing 0 (A), 1 (B), 2 (C) or 5 mg/mL (D) of linoleic acid.

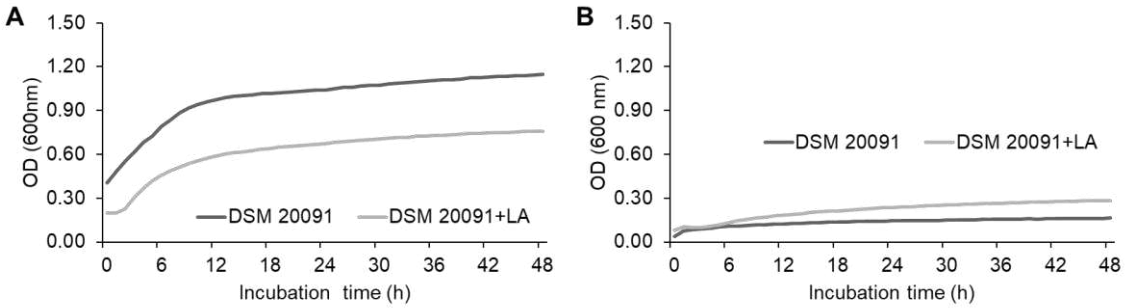


Figure 3.3 Growth curves of *Bifidobacterium breve* in the presence of 1 (A) or 2 mg/mL (B) of linoleic acid (LA). Optical density recorded at 600 nm.

Regarding CLA production, *B. breve* DSM 20091 converted 51% and 47% of LA after 24 and 48 h of incubation, yielding 312 and 287 µg CLA/mL, respectively (**Table 3.11**). The CLA isomers produced were essentially C18:2 c9t11 and an all *trans* CLA (**Figure 3.4**). The above conversion percentages are in accordance with those reported for other *B. breve* strains (>40%) at equal temperature and LA concentration conditions (Choi et al., 2018; Raimondi et al., 2016). However, it has been widely verified that CLA production capacity is not only species-specific, but also strain-dependent (Dahiya & Puniya, 2017; Gao et al., 2020), existing *B. breve* strains that can achieve up to 90% of LA conversion at similar culture conditions (Gao et al., 2020; Mei et al., 2021).

Table 3.11 Linoleic acid reduction and conversion percentages and conjugated linoleic acid yield by the identified producing strains.

Strain	LA reduction (%)		CLA production (µg/mL)		LA conversion (%)	
	24 h	48 h	24 h	48 h	24 h	48 h
Bifidobacterium breve DSM 20091	85.23 ± 0.48	86.49 ± 0.16	312.39 ± 3.42[a]	287.30 ± 5.81[b]	50.81 ± 0.55[a]	46.73 ± 0.94[b]
Lactobacillus gasseri MP551	39.08 ± 3.91	34.19 ± 0.52	ND	5.49 ± 0.76	ND	1.08 ± 0.15
Lactiplantibacillus plantarum DSM 20205	61.19 ± 0.45[b]	67.24 ± 1.61[a]	6.12 ± 3.42[a]	4.94 ± 1.48	1.23 ± 0.05	0.99 ± 0.30
Latilactobacillus sakei DSM 20017	41.65 ± 1.00[b]	58.69 ± 1.83[a]	6.85 ± 1.52	5.1 ± 0.77	1.22 ± 0.27	0.91 ± 0.14

Average value ± standard deviation (n=2). Different superscript letters for significant differences between 24 and 48 h ($p<0.05$).

LA – Linoleic acid; CLA – Conjugated linoleic acid; ND – not detected.

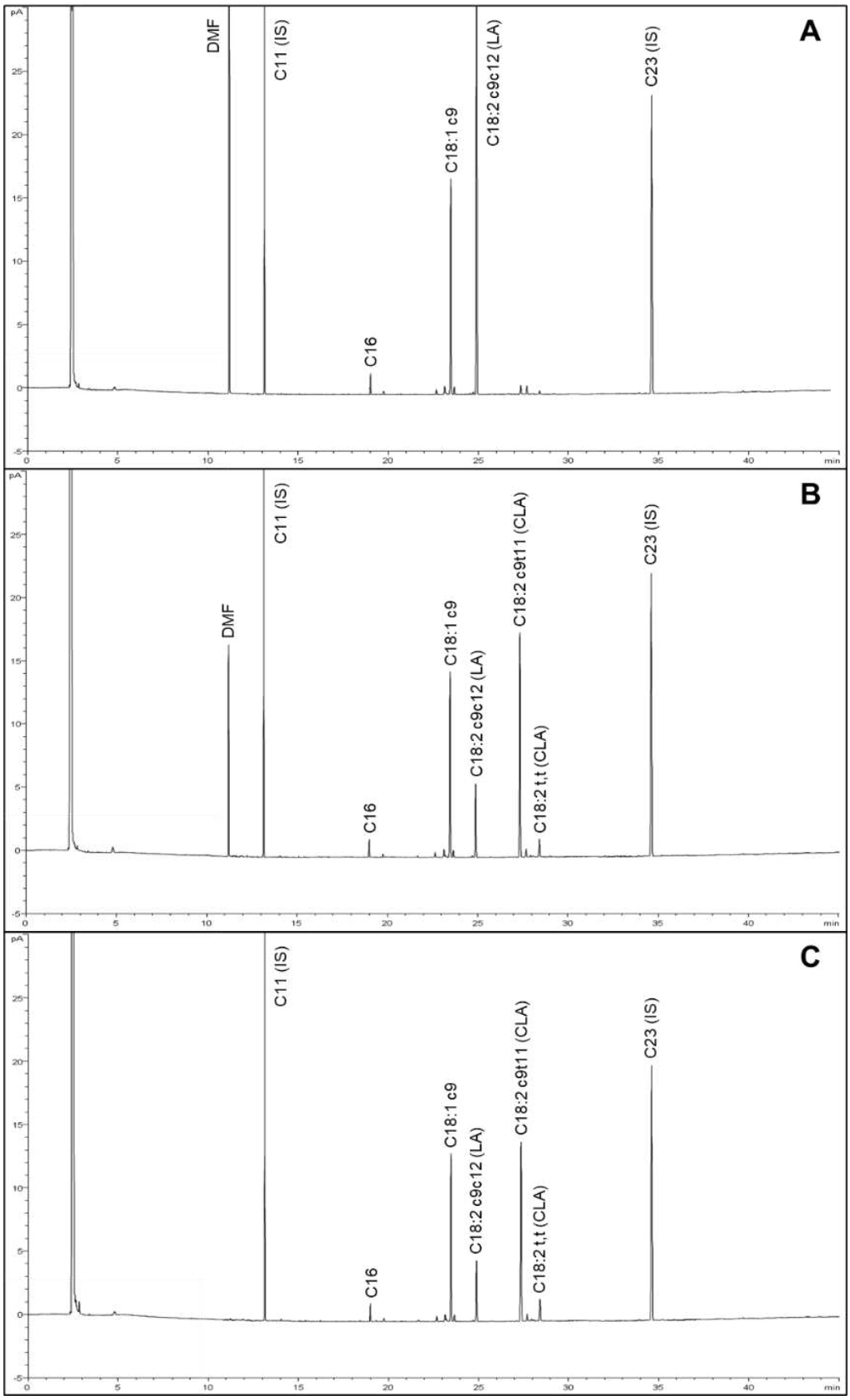


Figure 3.4 Chromatogram profile by GC-FID of *Bifidobacterium breve* DSM 20091 culture supernatant at 0 h (A), 24 h (B) and 48 h (C) of incubation with 0.5 mg/mL of linoleic acid (LA). DMF – dimethylformamide, IS – Internal standard, LA – Linoleic acid, CLA – Conjugated linoleic acid.

The application of LAI gene-targeting primers designed in this study led to the amplification of a product of 500 bp from DNA of *L. gasseri* MP551, *L. plantarum* DSM 20205 and *L. sakei* DSM 20017 (**Figure 3.1**). However, an amplification product of 1000 bp was also detected from *L. plantarum* DSM 20205 and *L. sakei* DSM 20017 DNA by using the LISO3 and LISO4 primers described by Gorissen et al. (2011) (**Figure 3.1**). All these three lactobacilli strains were able to grow in the presence of 5 mg/mL of LA in the agar plates (**Figure 3.5**) but the growth of *L. plantarum* DSM 20205 was inhibited when re-exposed to LA, even at 1 mg/mL (**Figure 3.6**). On the contrary, *L. gasseri* MP551 and *L. sakei* DSM 20017 tolerated up to 5 mg/mL of LA when re-exposure was performed (**Figure 3.7** and **Figure 3.8**).

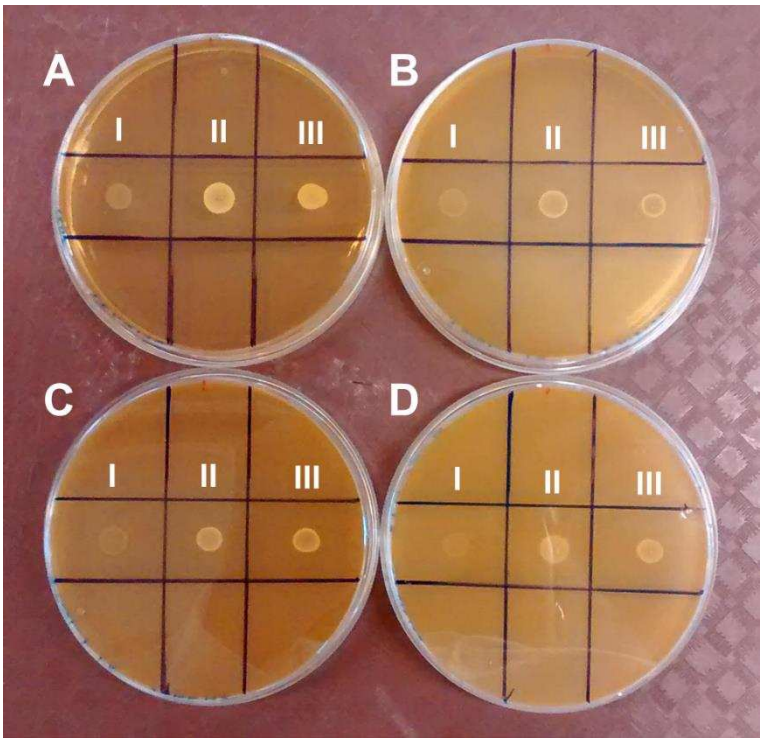


Figure 3.5 Growth of *Lactobacillus gasseri* MP551 (I), *Latilactobacillus sakei* DSM 20017 (II) and *Lactiplantibacillus plantarum* DSM 20205 (III) in MRS agar plates containing 0 (A), 1 (B), 2 (C) or 5 mg/mL (D) of linoleic acid.

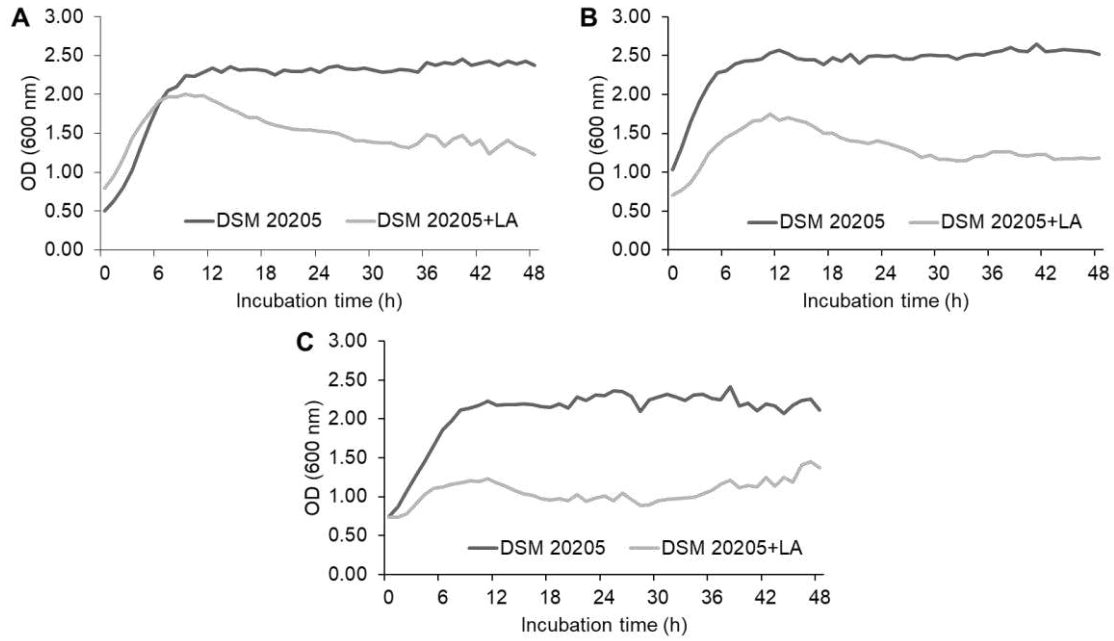


Figure 3.6 Growth curves of *Lactiplantibacillus plantarum* DSM 20205 in the presence of 1 (A), 2 (B) or 5 mg/mL (C) of linoleic acid (LA). Optical density (OD) recorded at 600 nm.

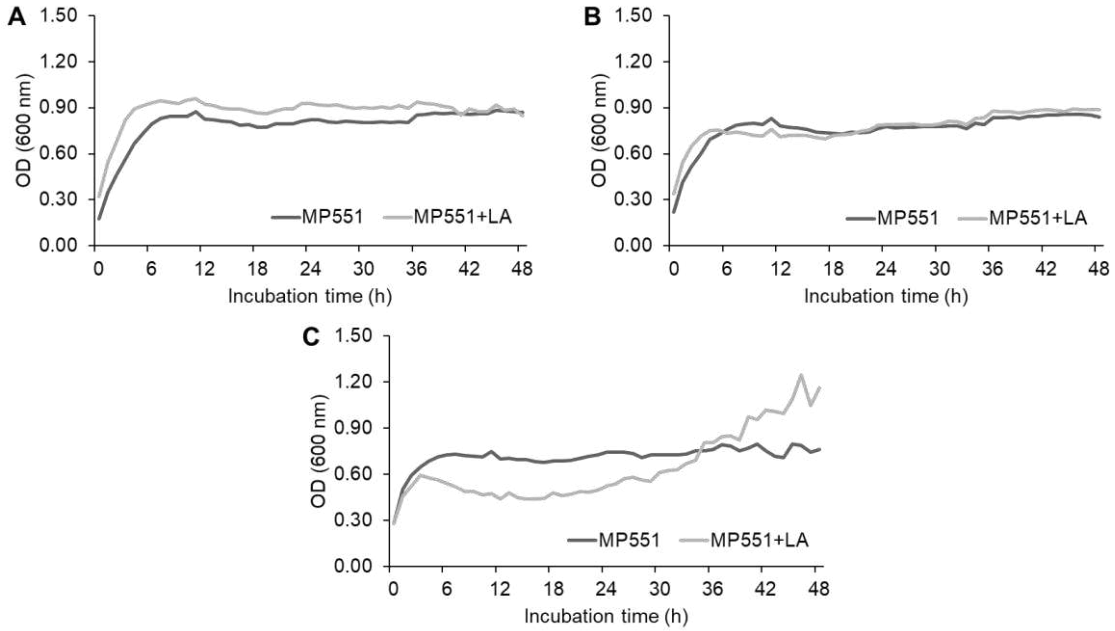


Figure 3.7 Growth curves of *Lactobacillus gasseri* MP551 in the presence of 1 (A), 2 (B) or 5 mg/mL (C) of linoleic acid (LA). Optical density (OD) recorded at 600 nm.

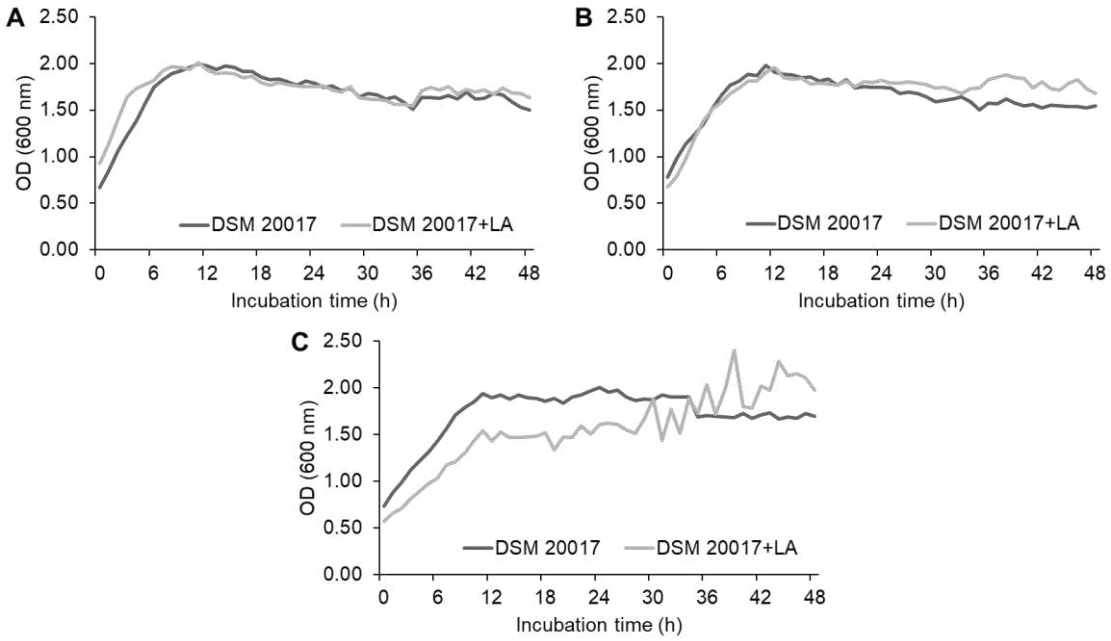


Figure 3.8 Growth curves of *Latilactobacillus sakei* DSM 20017 in the presence of 1 (A), 2 (B) or 5 mg/mL (C) of linoleic acid (LA). Optical density (OD) recorded at 600 nm.

In what concern CLA production, *L. gasseri* MP551 was able to convert 1.08% of LA, only after 48 h, resulting in 5 µg CLA/mL (**Table 3.11**). A higher conversion percentage (~11%) has been reported for *L. gasseri* CCFM5115 after 48 h under similar assay conditions (Yang et al., 2014). Furthermore, *L. gasseri* strains isolated from human infant feces showed higher CLA amounts as well, approximately 11-29 µg/mL after 48 h (Dahiya & Puniya, 2017). *Lactiplantibacillus plantarum* DSM 20205 and *L. sakei* DSM 20017 converted just over 1% LA after 24 h of incubation, which marginally decreased after 48 h, releasing about 5 to 7 µg CLA/mL (**Table 3.11**). Slightly higher conversion percentages (about 3% to 5%) have been obtained for *L. sakei* strains when incubated at a lower temperature (30 ºC) (Terán et al., 2015). On the other hand, *L. plantarum* DSM 20205 values are within the range reported by this latter study for other *L. plantarum* strains grown at 37 ºC (ca. 0.4-3.5%), but other research works have detected higher yields of CLA (approximately 10 to 55 µg/mL), whether at equal or lower incubation temperature (30 ºC) than that employed in our work (Dahiya & Puniya, 2017; Renes et al., 2017b). The above-mentioned lactobacilli strains produced the same CLA isomers as *B. breve* DSM 20091, plus C18:2 t10c12, with exception of *L. sakei* DSM 20017 (data not shown).

Since *L. gasseri* MP551 and *L. sakei* DSM 20017 showed a high tolerance to LA (5 mg/mL), and considering the detoxification mechanism previously stated, it was hypothesized that these strains were not stressed enough at 0.5 mg/mL LA to produce higher CLA amounts. However, when these strains were further tested at 5 mg/mL LA, no CLA production was detected at all (data not shown). Therefore, the data presented here do not corroborate the idea of CLA production as a detoxifying strategy.

Two further factors can exert an influence on CLA production: (a) the antimicrobial activity of LA; and (b) the pH. Kishino et al. (2002) observed that when bacterial growth of *L. plantarum* AKU

1009a was inhibited by LA, CLA production decreased. In the current study, viable cell counts were lower with LA for *L. gasseri* MP551 (48 h; 8.19 log CFU/mL *vs*. 9.17 log CFU/mL) and *B. breve* DSM 20091 (24 h; 8.10 log CFU/mL *vs*. 9.53 log CFU/mL), compared to control (**Figure 3.9**). Thus, LA affected the bacterial growth of *L. gasseri* MP551 only when this strain showed CLA production, and interfered with *B. breve* DSM 20091, which was, in fact, the best producer (**Table 3.11**). Therefore, growth inhibition does not seem an influencing factor in CLA production. Concerning pH, it decreased from around pH 6.0 to pH 3.8-4.6 (24/48 h) independently of LA presence/absence (**Figure 3.10**). Suteebut et al. (2016) observed that CLA production by *L. plantarum* GSI 303 was higher when the initial pH of the culture medium was set at pH 6.5. Moreover, according to other studies, when *B. breve* LMC 520 was grown under buffered pH, a maximal level of CLA production was achieved between pH 5.5 and 6.0 (Park et al., 2009). Thus, this may suggest that pH could have critically influenced CLA production in our study, but further assays would be needed to confirm that assumption.

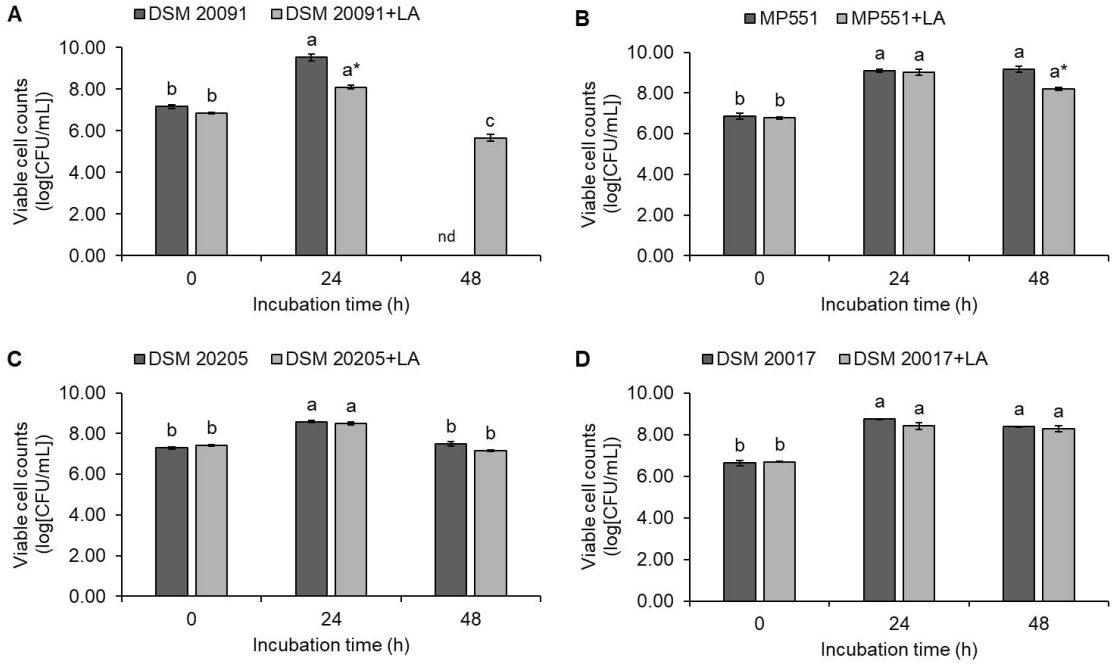


Figure 3.9 Viable cell counts of *Bifidobacterium breve* DSM 20091 (A), *Lactobacillus gasseri* MP551 (B), *Lactiplantibacillus plantarum* DSM 20205 (C) and *Latilactobacillus sakei* DSM 20017 (D) grown with or without 0.5 mg/mL of linoleic acid (LA). Different superscript letters indicate ≥1 log cycle difference between incubation times. *Indicates ≥1 log cycle difference between LA presence/absence within incubation time. nd = bacterial growth below the countable range (<5.00×10^5 CFU/mL).

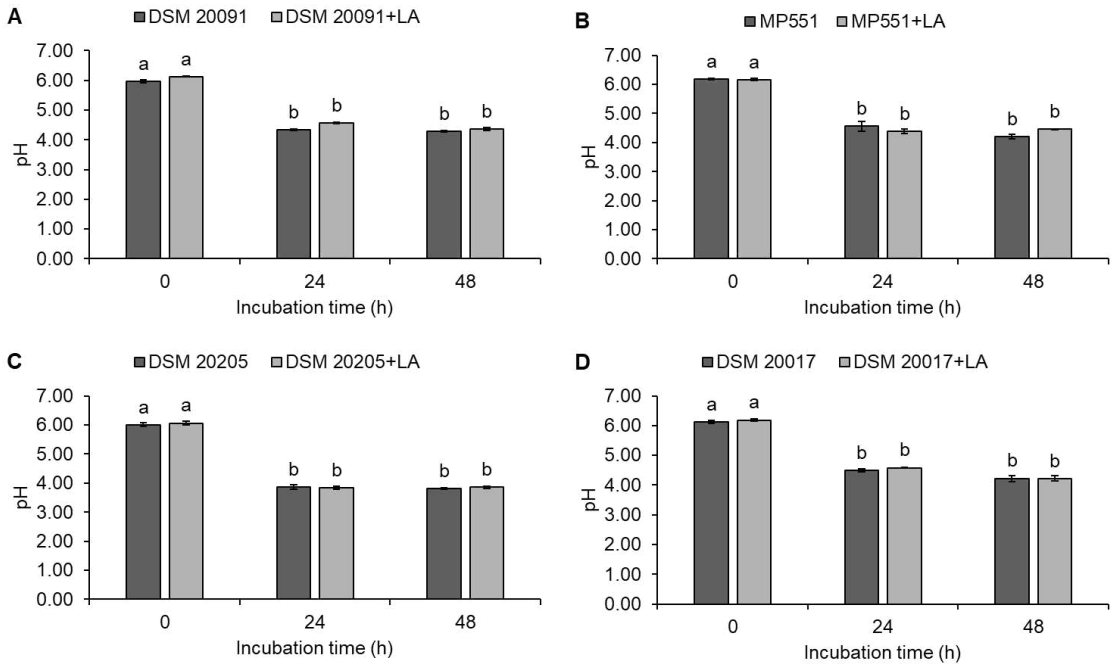


Figure 3.10 pH of *Bifidobacterium breve* DSM 20091 (A), *Lactobacillus gasseri* MP551 (B), *Lactiplantibacillus plantarum* DSM 20205 (C) and *Latilactobacillus sakei* DSM 20017 (D) cultures with or without 0.5 mg/mL of linoleic acid (LA). Different superscript letters indicate ≥0.5 units difference between incubation times.

The LA conversion into other compounds may be an additional reason explaining low CLA production levels by the identified producers, because LA reduction percentages reached considerable values (up to 86%; **Table 3.11**). As mentioned in **Section 1.4.3 Linoleate isomerase and other involved enzymes** (in Introduction), CLA production may include multiple reactions with hydroxy and oxo FAs intermediates of LA conversion into CLA isomers (Salsinha et al., 2018), therefore an hydroxy FA analysis was performed, but no other compounds were detected at levels that could justify LA reduction amounts. However, Aziz et al. (2020) have identified different metabolites produced from LA by *L. plantarum* YW11, such as *trans*/*trans*-9,12-octadecadienoic acid propyl ester, *trans*-9-octadecenoic acid ethyl ester and linoelaidic acid, among others.

Contrary to what was expected, few positive strains identified in the genotypic screening could produce CLA isomers. Another previous study also reported that some strains of lactobacilli did not convert LA despite the presence of the LAI gene (Renes et al., 2017a). This suggests that genotypic screening is still not a reliable technique for the identification of CLA-producing strains. On the other hand, among the non-CLA-producers, LA reduction percentages were considerably high (up to ~70%; data not shown), suggesting that other compounds might have been produced instead, as stated above.

Based on the results achieved, there are, at least, four hypotheses that could explain the lack of LA conversion into CLA isomers by the genotypically-positive strains: i) low or no expression of the genes encoding required enzymes; ii) inhibition of expressed enzymes; iii) production of other

compounds from LA; and iv) non-optimal growth conditions for CLA production. However, these hypotheses were not all pursued within this research work.

3.1.5. Influence of shaking on CLA production

A factor that might have influenced CLA production by the identified producing strains is that culturing conditions were not optimal, specifically, lack of homogenization during incubation, thus not enabling a uniform contact between bacterial cells and substrate. Therefore, it was hypothesized that culturing these strains with shaking would improve CLA yields. Consequently, to prove this, the identified CLA-producing strains (**Section 3.1.4 CLA production;** in Results and Discussion of Part I) were cultured with LA under continuous agitation and this was compared to steady conditions.

Viable cell numbers of *B. breve* DSM 20091 in control conditions were maintained stable from 24 h to 48 h with shaking (8.82-9.04 log CFU/mL) (**Figure 3.11**), while without shaking *B. breve* lost viability after 48 h. Moreover, at 48 h, viable cell numbers were higher with shaking for the LA-condition (8.14 log CFU/mL *vs*. 5.66 log CFU/mL) (**Figure 3.11**). After 72 h, growth within the countable range (≥5.00×10^5 CFU/mL) was only detected for bacteria cultured with LA and shaking (**Figure 3.11**). *Lactobacillus gasseri* MP551 growth was similar whether with shaking or not, however, at 72 h, viable cell numbers were lower with shaking for the LA-condition (5.39 log CFU/mL *vs*. 7.00 log CFU/mL) (**Figure 3.11**). As for *L. plantarum* DSM 20205, viable cell counts were similar until 48 h, however, at this point, viable cell numbers were lower with LA for the shaking condition (6.48 log CFU/mL *vs*. 7.65 log CFU/mL) (**Figure 3.11**). After 72 h, with shaking, cell numbers were 1 to 2-log cycle lower (**Figure 3.11**), and no considerable differences were observed between control and LA presence. In the case of *L. sakei* DSM 20017, it was not able to grow in the presence of LA with shaking (**Figure 3.11**), while under control conditions growth was similar with or without shaking. Taken all together, while shaking improved *B. breve* DSM 20091 growth with LA when incubated for a prolonged time (72 h), it negatively affected the remaining strains.

Concerning the influence of shaking on pH values, differences were only observed for *B. breve* DSM 20091 and at 24 and 48 h, where pH values were higher with shaking for LA-conditions (pH 4.92-5.74 *vs*. pH 4.37-4.56) (**Figure 3.12**). Moreover, for shaking condition, pH values were lower in control (i.e., no LA; pH 4.29-4.50) (**Figure 3.12**).

Regarding CLA production, in general, it was observed that shaking led to a LA conversion increment throughout the assayed times (**Table 3.12**). However, substrate conversion degrees were significantly higher ($p<0.05$) without shaking for all strains, except for *L. gasseri* MP551 (**Table 3.12**). In a previous work, carried out with a *L. acidophillus* strain, CLA yields increased proportionally to shaking level (0-180 rpm) (Wei et al., 2014). However, according to the present results, shaking was not a critical factor for CLA production transversal to all species. Hence, the maximum LA conversion achieved by *B. breve* DSM 20091, *L. plantarum* DSM 20205 and *L. sakei* DSM 20017 was after 24 h of incubation with no shaking (52.17%, 0.97% and 0.82%, respectively) (**Table 3.12**), and *L. gasseri* MP551 after 72 h with shaking (1.58%).

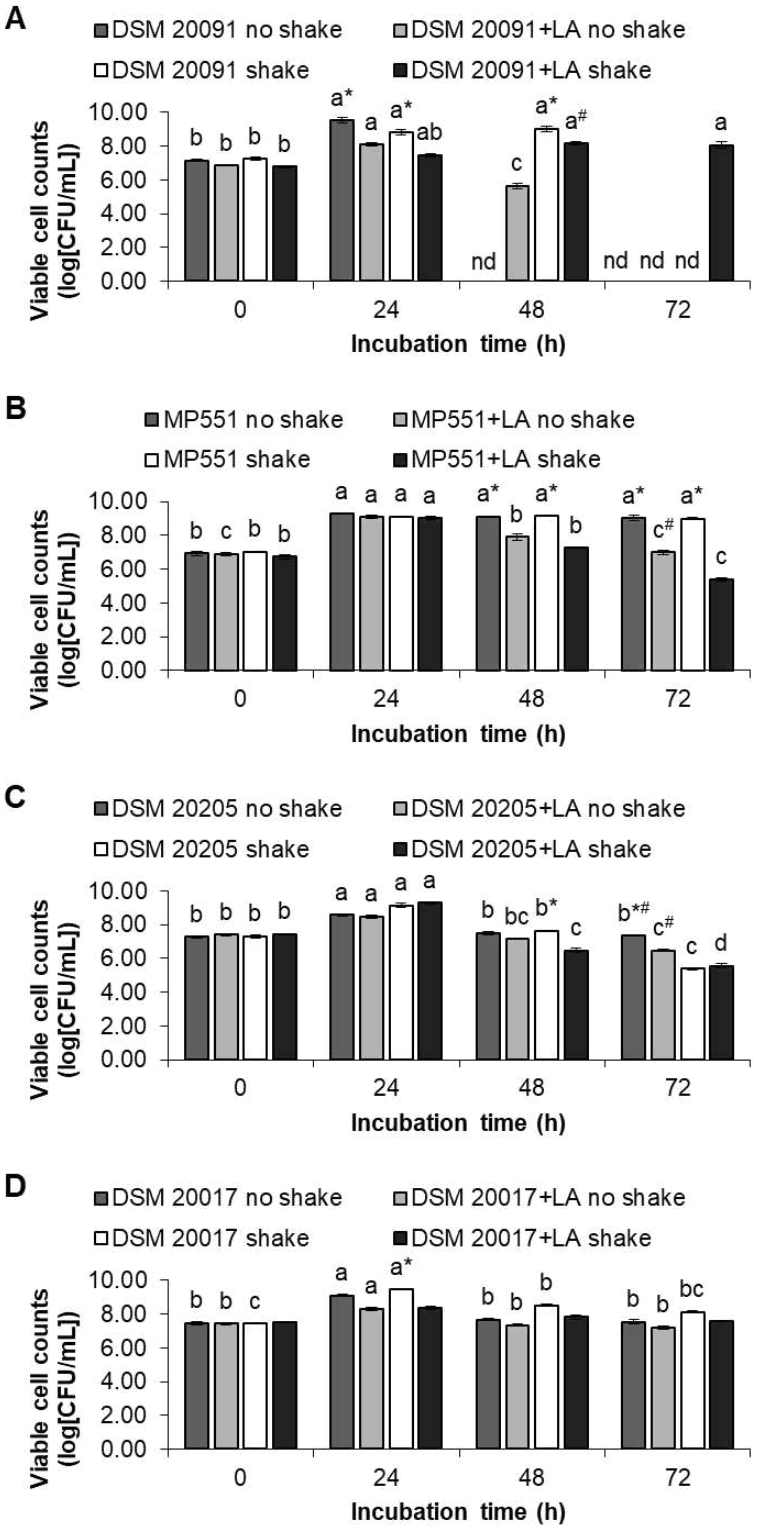


Figure 3.11 Viable cell numbers of *Bifidobacterium breve* DSM 20091 (A), *Lactobacillus gasseri* MP551 (B), *Lactiplantibacillus plantarum* DSM 20205 (C) and *Latilactobacillus sakei* DSM 20017 (D) cultured with or without linoleic acid (LA) and with or without shaking. Different superscript letters indicate ≥1 log cycle difference between incubation times. *Indicates ≥1 log cycle difference between control and LA-condition within incubation time. #Indicates ≥1 log cycle difference between no shaking and shaking within incubation time. nd = bacterial growth below the countable range (<5.00×10^5 CFU/mL).

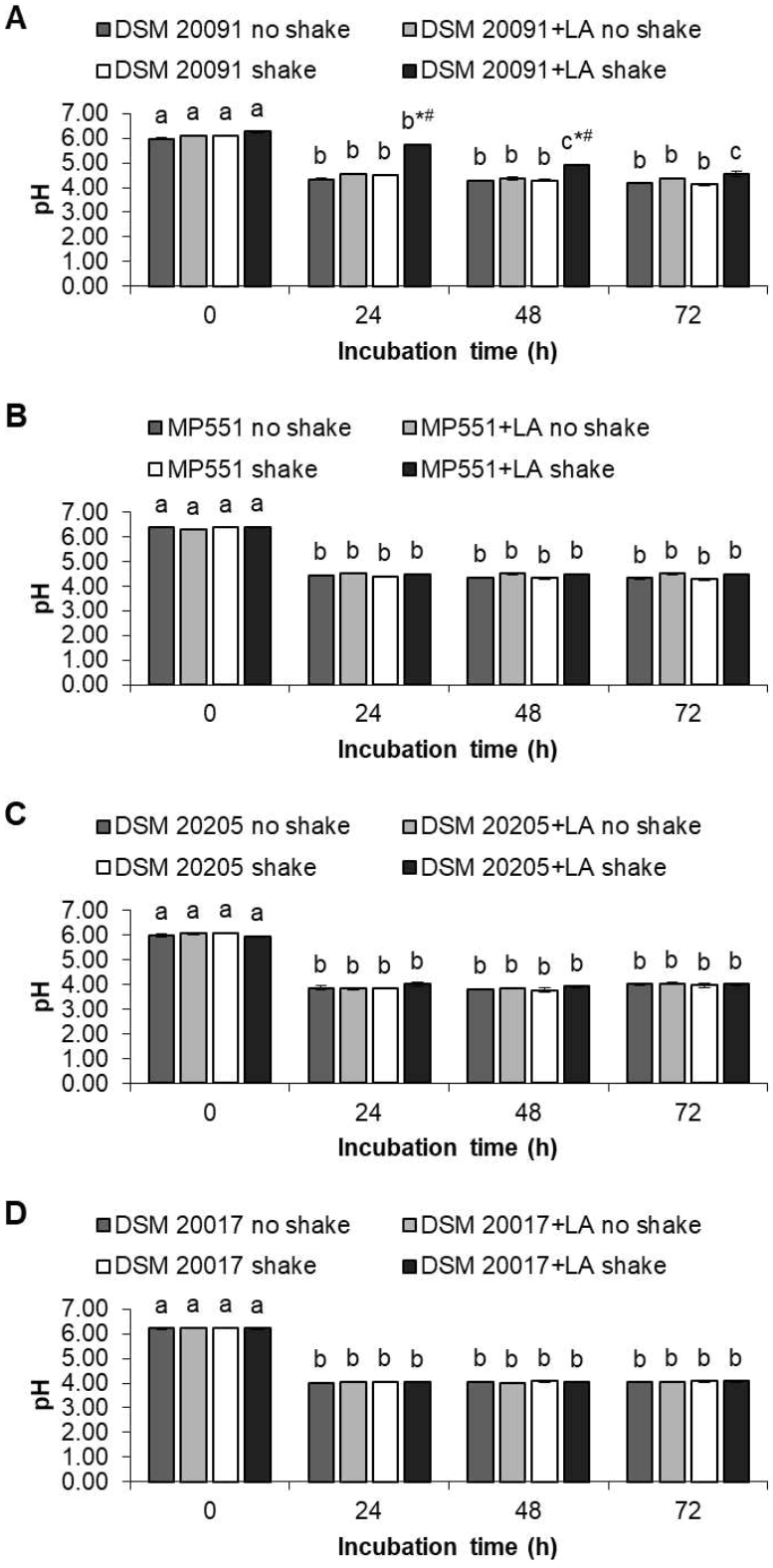


Figure 3.12 pH values of *Bifidobacterium breve* DSM 20091 (A), *Lactobacillus gasseri* MP551 (B), *Lactiplantibacillus plantarum* DSM 20205 (C) and *Latilactobacillus sakei* DSM 20017 (D) cultures grown with or without linoleic acid (LA) and with or without shaking. Different superscript letters indicate ≥0.5 units difference between incubation times. *Indicates ≥0.5 units difference between control and LA-condition within incubation time. #Indicates ≥0.5 units difference between no shaking and shaking within incubation time.

Table 3.12 Linoleic acid conversion degree (%) by the identified conjugated linoleic acid-producing strains cultured with or without shaking.

Strain	Condition	24 h	48 h	72 h
Bifidobacterium breve DSM 20091	no shake	52.17 ± 8.35^{a*}	43.81 ± 1.25^{a*}	33.30 ± 1.23^{b*}
	shake	5.34 ± 0.12^{b}	8.93 ± 0.85^{a}	11.37 ± 1.96^{a}
Lactobacillus gasseri MP551	no shake	ND	0.08 ± 0.01	0.11 ± 0.01
	shake	0.14 ± 0.01^{c}	0.50 ± 0.10^{b*}	1.58 ± 0.16^{a*}
Lactiplantibacillus plantarum DSM 20205	no shake	0.97 ± 0.27*	0.65 ± 0.04*	0.84 ± 0.25*
	shake	0.26 ± 0.06^{b}	0.25 ± 0.06^{b}	0.42 ± 0.07^{a}
Latilactobacillus sakei DSM 20017	no shake	0.82 ± 0.36^{a*}	0.50 ± 0.09^{a*}	0.24 ± 0.02^{b}
	shake	0.09 ± 0.03^{b}	0.10 ± 0.01^{b}	0.23 ± 0.03^{a}

Average value ± standard deviation (n=3). Different superscript letters indicate significant differences between 24, 48 and 72 h ($p<0.05$). *Significant differences between no shaking and shaking within incubation time ($p<0.05$).

ND – not detected.

3.1.6. CLA production kinetics

To understand strains behavior in terms of CLA production, a kinetic study was carried out with the identified CLA-producers for 24 h, since no increment was verified in CLA yield for longer incubation times (**Table 3.12**). *Lactobacillus gasseri* MP551 was excluded from this assay since it was the only one requiring specific conditions (72 h of incubation with shaking; **Table 3.12**) to show a better CLA production capacity. Accordingly, the selected producing strains were inoculated in culture medium containing LA and viable cell counts, pH values and LA and CLA contents were monitored throughout 24 h of incubation.

Regarding the pH evolution, for *B. breve* DSM 20091, pH values started to decrease more considerably in the middle of the exponential phase (at 8 h) (**Figure 3.13**), and in the case of *L. sakei* DSM 200017 at 6 h upon inoculation (**Figure 3.13**). On the other hand, for *L. plantarum* DSM 20205, pH values started to decrease from the beginning of the exponential phase (at 4 h) (**Figure 3.13**).

In what concerns LA and CLA levels, in *B. breve* DSM 20091 culture, LA content started to decrease more substantially in the middle of the exponential phase (at 6 h), and at the same time, CLA content began to increase, reaching its maximum at 24 h, during the stationary phase (**Figure 3.14**). This is in line with what was observed for other *B. breve* strains, where most CLA was produced during the exponential growth phase (Mei et al., 2021; Raimondi et al., 2016). As for *L. plantarum* DSM 20205, LA content started to reduce more abruptly by the end of the exponential phase at 10 h, and CLA began to be released at 12 h, already during the stationary phase (**Figure 3.14**). Regarding *L. sakei* DSM 20017, LA content dropped during the stationary phase (at 12 h) and CLA was detected at 24 h, during the death phase, at very low concentrations (**Figure 3.14**). However, since no sampling was performed between 12 and 24 h, there is the possibility that CLA production started earlier. Our results agree with those observed for other lactobacilli strains, where most CLA was released during the stationary phase (Li et al., 2013; Liu et al., 2011).

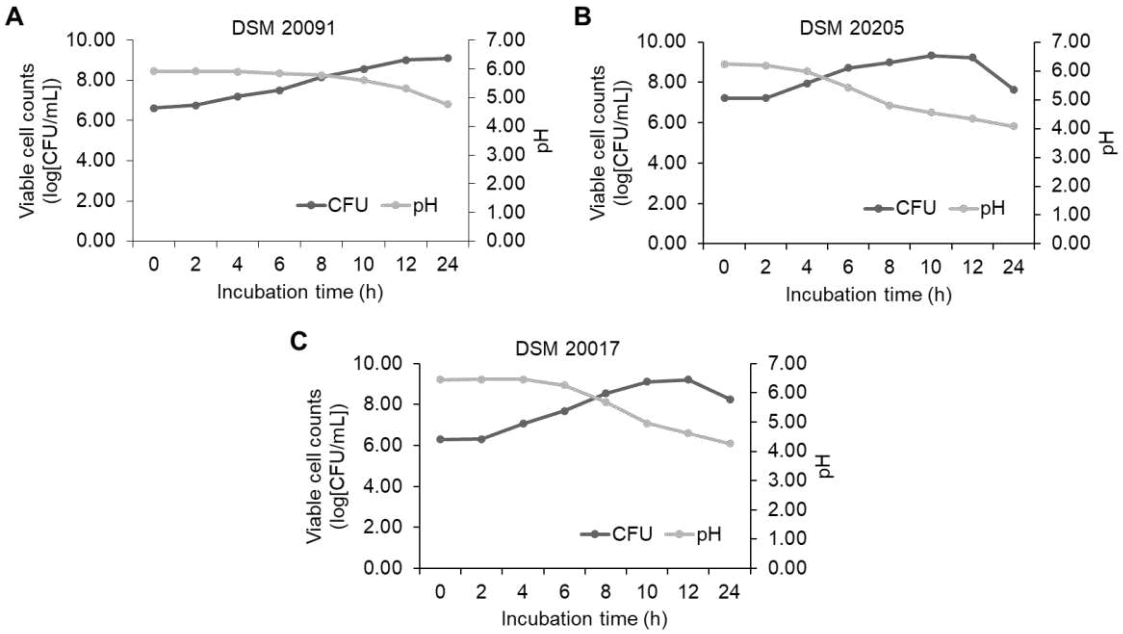


Figure 3.13 Viable cell numbers (log[CFU/mL]) and pH variation throughout *Bifidobacterium breve* DSM 20091 (A), *Lactiplantibacillus plantarum* DSM 20205 (B) and *Latilactobacillus sakei* (C) growth in MRS medium containing 0.5 mg/mL of linoleic acid.

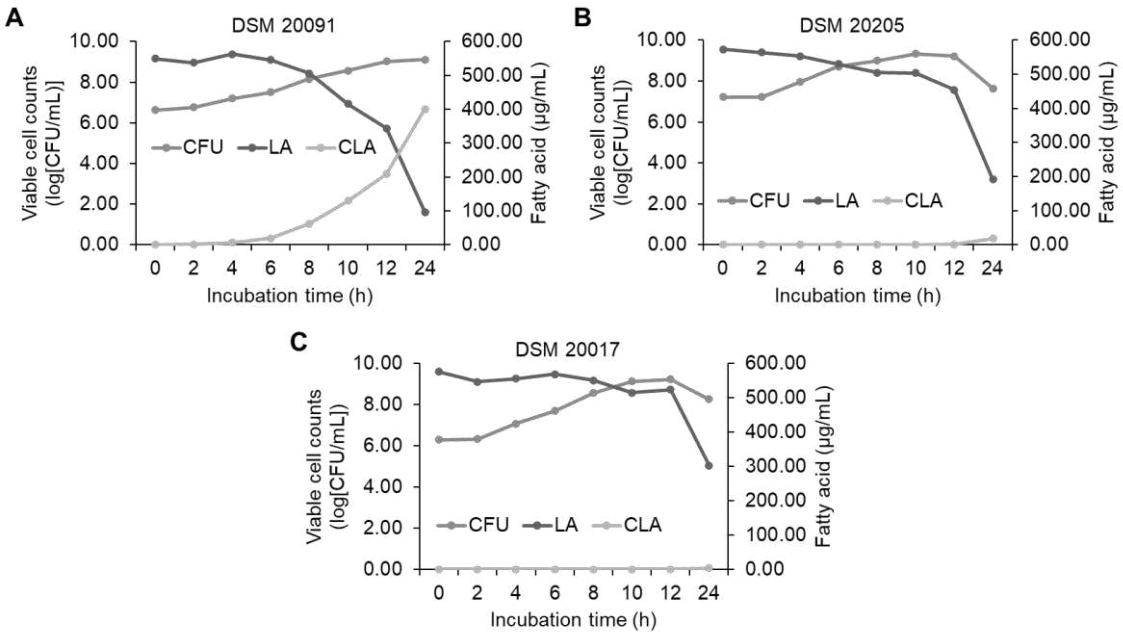


Figure 3.14 Evolution of viable cell numbers (log[CFU/mL]), linoleic acid (LA) and conjugated linoleic acid (CLA) contents (µg/mL) throughout *Bifidobacterium breve* DSM 20091 (A), *Lactiplantibacillus plantarum* DSM 20205 (B) and *Latilactobacillus sakei* (C) growth in MRS medium containing 0.5 mg/mL of linoleic acid.

3.1.7. CLNA production

Other studies have shown that probiotic strains with CLA-production capacity were also capable of converting α-LNA into CLNA isomers (Hennessy et al., 2012; Villar-Tajadura et al., 2014). Being this compound of interest for this project as well, the identified CLA-producers were further tested with α-LNA.

Regarding bacterial growth in the presence of α-LNA, all strains tested increased their viable cell numbers 1 to 2-log cycles after 24 h (**Figure 3.15**). After 48 h viable cell numbers generally decreased (1 to 2-log cycles) for all strains, except for *L. sakei* DSM 20017 (**Figure 3.15**). When comparing with the control, viable cell numbers were lower with α-LNA for *L. gasseri* MP551 at 48 h (7.23 lof CFU/mL *vs*. 9.17 log CFU/mL) (**Figure 3.15**), but higher for *L. plantarum* DSM 20205 at 24 h (9.26 log CFU/mL *vs*. 8.12 log CFU/mL). Interestingly, similarly to what was observed with LA (**Figure 3.9**), at 48 h, *B. breve* DSM 20091 cells were kept viable only with the presence of α-LNA (**Figure 3.15**). An inhibitory effect of α-LNA on bacterial growth has been reported for different probiotic strains (Gorissen et al., 2010; Kankaanpää et al., 2001), however, in the present work, that was observed for just one strain, and only at 48 h. Moreover, in some cases, growth was even prompted.

Regarding the pH variation, it decreased between 1-2 units after 24 h and remained constant until 48 h of incubation for all strains (pH 3.54-4.48; **Figure 3.16**). In comparison to the control, differences were only observed for *B. breve* DSM 20091 at 24 h, where pH values were higher with α-LNA (pH 4.57 *vs*. pH 4.11) (**Figure 3.16**).

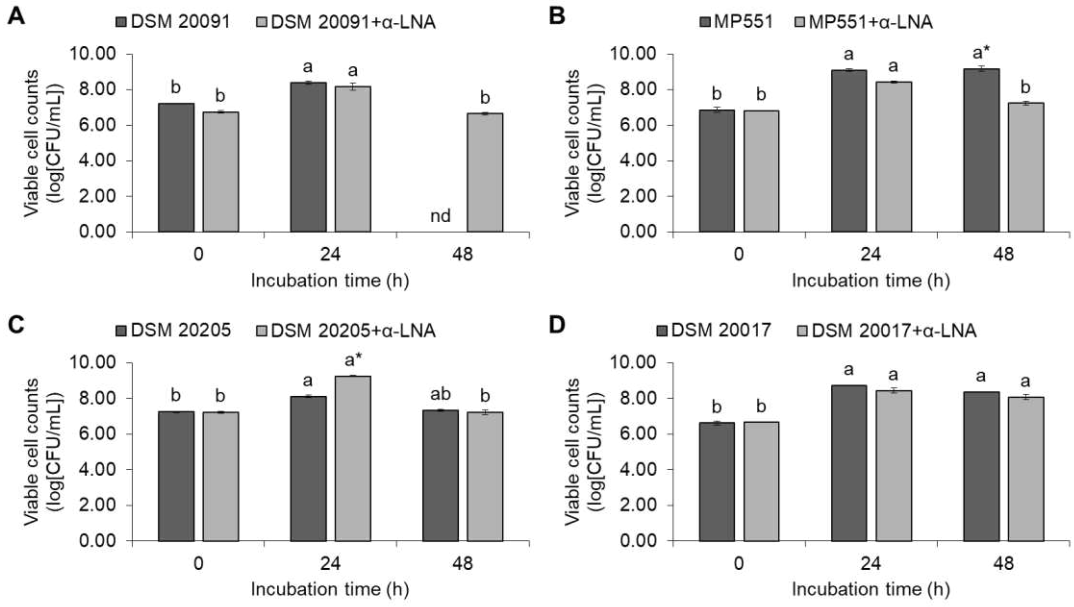


Figure 3.15 Viable cell numbers of *Bifidobacterium breve* DSM 20091 (A), *Lactobacillus gasseri* MP551 (B), *Lactiplantibacillus plantarum* DSM 20205 (C) and *Latilactobacillus sakei* DSM 20017 (D) grown in MRS medium with α-linolenic acid (α-LNA). Different superscript letters indicate ≥1 log cycle difference between incubation times. *Indicates ≥1 log cycle difference between control and α-LNA-condition within incubation time. nd = bacterial growth below the countable range (<5.00×10^5 CFU/mL).

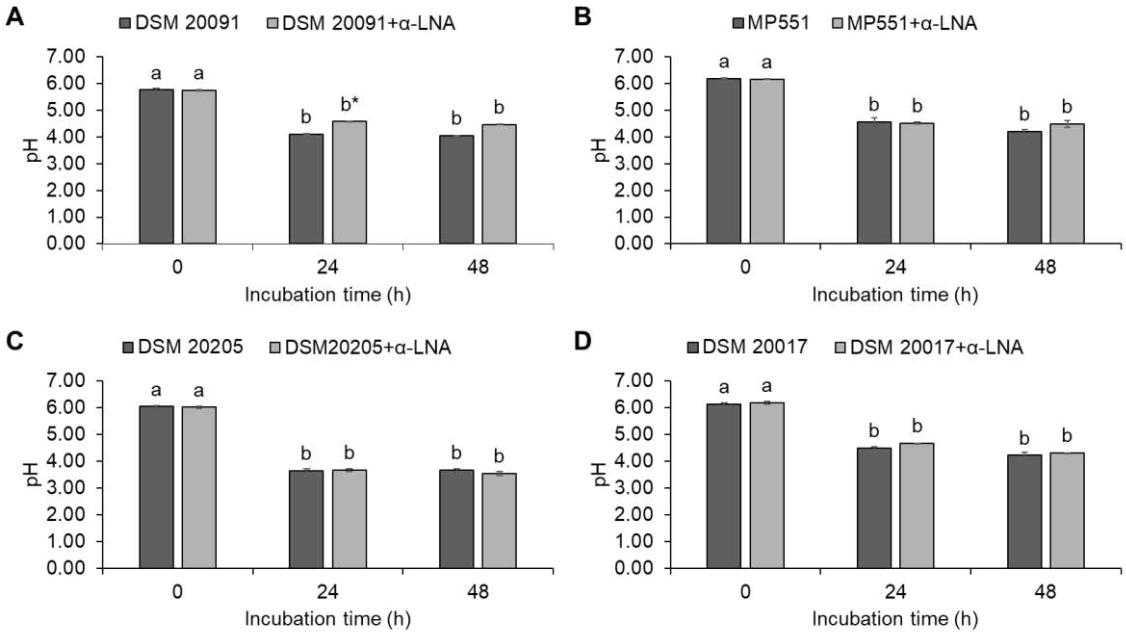


Figure 3.16 pH of *Bifidobacterium breve* DSM 20091 (A), *Lactobacillus gasseri* MP551 (B), *Lactiplantibacillus plantarum* DSM 20205 (C) and *Latilactobacillus sakei* DSM 20017 (D) cultures grown with α-linolenic acid (α-LNA). Different superscript letters indicate ≥0.5 units difference between incubation times. *Indicates ≥0.5 units difference between control and α-LNA-condition within incubation time.

As for CLNA production, *B. breve* DSM 20091 was able to produce 319.35 µg/mL after 24 h and 250.16 µg/mL after 48 h, corresponding to an α-LNA conversion of 58.44% and 45.78%, respectively (**Table 3.13**). Moreover, α-LNA was reduced by 93.40%. Other *B. breve* strains were described with a 49.74% to 82.97% conversion capacity, when assayed at 0.45 mg/mL α-LNA for 72 h (Hennessy et al., 2012). However, similarly to CLA, the microbial production of CLNA was also shown to be strain-dependent, since, at similar growth conditions to those employed in the current work, *B. breve* strains isolated form human breast milk were able to attain α-LNA conversion percentages of 95.70-98.8% (Villar-Tajadura et al., 2014).

Regarding *L. gasseri* MP551, it was not able to produce CLNA isomers at detectable levels, even though α-LNA was reduced by 50.65% (24 h) to 59.63% (48 h) (**Table 3.13**). To the best of our knowledge no other work has evaluated CLNA production by *L. gasseri* strains.

Lactiplantibacillus plantarum DSM 20205 converted 3.54% (24 h) to 3.60% (48 h) of the substrate into 19.35 and 19.66 µg/mL of CLNA, respectively (**Table 3.13**). However, α-LNA reduction achieved values of 70.39% (24 h) and 77.06% (48 h). Téran et al. (2015) observed low α-LNA conversion percentages as well (0.54-2.08%) with *L. plantarum* strains at similar growth conditions. In another study, substrate conversions varied from 3.4% to 7.4%, with only *L. plantarum* ATCC 8014 achieving 26.8% (Gorissen et al., 2011). However, in this last research work, α-LNA was added to culture medium only 7 h after incubation.

Latilactobacillus sakei DSM 20017 was able to produce 85.19 µg CLA/mL (24 h) and 74.87 µg CLA/mL (48 h), with substrate (i.e., α-LNA) conversions of 15.59% and 13.70%, respectively (**Table**

3.13). On the other hand, 53.66% (24 h) to 69.27% (48 h) of the α-LNA was reduced. Lower substrate conversion percentages have been reported for other *L. sakei* strains (Terán et al., 2015), with values ranging from 2.45% to 2.78%, but assaying at lower incubation temperature (i.e., 30 ºC). On the other hand, at 30 ºC, but adding the substrate only 7 h after incubation, *L. sakei* CG1 and *L. sakei* LMG 13558 revealed conversion percentages of 28.3% and 60.1%, respectively (Gorissen et al., 2011). Thus, the above-mentioned discrepancies could be attributed to growth conditions differences, but, in the meantime, strain influence cannot be discharged.

As observed for LA (**Table 3.11**), α-LNA reduction degree was much higher than its conversion into CLNA isomers for any of the strains tested. As discussed in **Section 1.4.3 Linoleate isomerase and other involved enzymes** (in Introduction), the α-LNA bioconversion pathway might also involve hydroxy and oxo intermediates. Therefore, hydroxy fatty analysis was performed, but no other compounds were detected at levels that could justify such substrate reduction (data not shown).

Comparing to CLA production (**Table 3.11**), *B. breve* DSM 20091 converted similar α-LNA amounts, while *L. gasseri* MP551 was not able to produce CLNA. On the other hand, the quantity of α-LNA converted by *L. plantarum* DSM 20205 and *L. sakei* DSM 20017 was 2.88-3.64 times and 12.78-15.05 times higher than that of LA conversion, respectively (**Table 3.11** and **Table 3.13**). Some studies (Gorissen et al., 2010; Villar-Tajadura et al., 2014) have reported similar findings for other probiotic strains, suggesting, through the detoxification mechanism hypothesis, that α-LNA would be more toxic than LA. On the other hand, the discrepancy observed between CLA and CLNA yield by *L. plantarum* DSM 20205 and *L. sakei* DSM 20017 might be attributed to LAI efficiency. Elsewhere, it has been described higher K_m values for α-LNA enzymatic isomerization than LA, thus showing a lower affinity for α-LNA. Nevertheless, V_{max} was also higher for α-LNA, which suggested a higher efficiency for α-LNA isomerization (Meynadier et al., 2018; Peng et al., 2007).

Since *B. breve* DSM 20091 revealed the best yields of both CFA, it was selected for the following experiments regarding the development of a dairy product enriched with microbial CLA/CLNA isomers.

Table 3.13 Alpha-linolenic acid reduction and conversion degrees and conjugated linolenic acid yield by the selected strains.

Strain	α-LNA reduction (%)		CLNA production (μg/mL)		α-LNA conversion (%)	
	24 h	48 h	24 h	48 h	24 h	48 h
Bifidobacterium breve DSM 20091	93.42 ± 0.60	93.40 ± 0.08	319.35 ± 3.02[a]	250.16 ± 9.52[b]	58.44 ± 0.55[a]	45.78 ± 1.74[b]
Lactobacillus gasseri MP551	50.65 ± 0.69[b]	59.63 ± 2.33[a]	ND	ND	ND	ND
Lactiplantibacillus plantarum DSM 20205	70.39 ± 1.12[b]	77.06 ± 0.64[a]	19.35 ± 0.63	19.66 ± 0.95	3.54 ± 0.12	3.60 ± 0.17
Latilactobacillus sakei DSM 20017	53.66 ± 2.63[b]	69.27 ± 2.24[a]	85.19 ± 3.89	74.87 ± 4.89	15.59 ± 0.71	13.70 ± 0.89

Average value ± standard deviation (n=3). Different superscript letters indicate significant differences between 24 and 48 h ($p<0.05$).

α-LNA – Linolenic acid; CLNA – Conjugated linolenic acid; ND – not detected.

Part II – Study of the utilization of commercially available substrate sources to obtain a microbial CLA/CLNA-enriched fermented milk

According to results from Part I, *B. breve* DSM 20091 revealed the best yields of CLA and CLNA isomers, and therefore was selected for the development of a CLA/CLNA-enriched fermented milk. The aim of Part II was to study the production of CLA/CLNA isomers in milk, by the selected strain, using commercial vegetable oils as substrate sources. To increase LA and α-LNA bioavailability, vegetable oils were first hydrolyzed, for which different lipases were tested. Thereafter, a vegetable oil rich in LA (SBO) and another one rich in α-LNA (FSO), previously hydrolyzed with the lipase showing the best yields (CRL lipase), were further assayed in milk at different proportions for the microbial enrichment with CLA and CLNA isomers by *B. breve* DSM 20091. Not being possible to simultaneously enrich milk with CLA and CLNA by the selected strain, higher substrate concentrations were further tested for the development of a CLNA-enriched fermented milk with hydrolyzed FSO. Furthermore, it was performed a UV spectroscopy analysis to confirm the structure configuration of the conjugated isomers present in the developed fermented milk.

3.2.1. Vegetable oils hydrolysis

A preliminary analysis of the edible vegetable oils' composition revealed that HSO and SBO oils had a high amount of LA (49.57 and 43.71 g/100 g, respectively) (**Table 3.14**), while FSO was rich in α-LNA (41.32 g/100 g). A smaller proportion of α-LNA was also detected in HSO (12.83 g/100 g) and SBO (6.07 g/100g), and of LA in FSO (15.06 g/100 g) (**Table 3.14**). However, only a small amount of these FA was present as free form (<1.5 g/100 g; data not shown), thus, remaining FAs should be esterified in other lipids found in oils' composition, like triacylglycerides.

Table 3.14 Hemp seed, flaxseed and soybean oils' total fatty acid composition.

FA (g/100 g oil)	HSO	FSO	SBO
C14:0	0.05 ± <0.01	0.07 ± <0.01	0.10 ± <0.01
C16:0	6.70 ± 0.41	5.99 ± 0.22	10.66 ± 0.35
C16:1 c9	0.12 ± 0.01	0.09 ± <0.01	0.11 ± 0.01
C18:0	5.20 ± 0.74	6.96 ± 0.56	6.46 ± 0.19
C18:1 c9	14.62 ± 1.07	21.35 ± 0.96	23.59 ± 0.74
C18:2 c9c12 (LA)	49.57 ± 4.60	15.06 ± 0.76	43.71 ± 1.52
C18:3 c6c9c12 (γ-LNA)	0.47 ± 0.04	ND	ND
C18:3 c9c12c15 (α-LNA)	12.83 ± 1.22	41.32 ± 2.04	6.07 ± 0.23
C20:0	0.80 ± 0.05	0.23 ± 0.02	0.40 ± 0.03
C20:1 c11	0.35 ± 0.03	0.15 ± 0.01	0.22 ± 0.01
C20:3 c11c14c17	0.31 ± 0.03	0.20 ± 0.01	0.41 ± 0.02

Average values ± standard deviation (n=3).

FA – Fatty acid; HSO – Hemp seed oil; FSO – Flaxseed oil; SBO – Soybean oil; *c* – *cis* double bond; LA – Linoleic acid; γ- LNA – gamma-Linolenic acid; α-LNA – alpha-Linolenic acid; ND – not detected.

After hydrolysis, it was observed that the CRL lipase produced the best outcome, with hydrolysis percentages of at least 91.7% for LA and α-LNA in all the edible vegetable oils tested (**Table 3.15**). Based on FFA release, hydrolysis degrees ranging from 79.6% to 97.2% have also

been reported for other vegetable oils treated with this lipase, including FSO (Rupani et al., 2012), coconut (Nguyen et al., 2018) and palm oils (Serri et al., 2008).

Pancreatic porcine lipase revealed the worst hydrolytic efficiency (0.2-17.5%) (**Table 3.15**). In other studies, PPL exhibited a 23% hydrolysis degree with SBO (Freitas et al., 2007), but, with safflower oil, it reached 91.6% (Aziz et al., 2015). The PPL hydrolytic efficiency seems oil-dependent, but differences in reaction conditions could have also influenced it.

In what concerns PFL, it was able to hydrolyze 67.8-70.3% of LA or α-LNA in all the assayed oils (**Table 3.15**). A similar hydrolysis degree (~65%) has been reported for blackcurrant oil by Vacek et al. (2000).

Given CRL's high hydrolytic efficiency, this lipase led to the highest concentration of free LA in HSO (63.63 g/100 g) (**Table 3.16**), followed by SBO (53.85 g/100 g), and of α-LNA in FSO (48.94 g/100 g). Therefore, the CRL lipase was selected and tested in the following assays. For cost-effective reasons, HSO was not included in the following experiments, since it is more expensive than SBO, and this latter oil can provide a good source of LA as well.

Table 3.15 Hydrolysis degree (%) of esterified linoleic, α-linolenic and total fatty acids.

	HSO			**FSO**			**SBO**		
	CRL	PFL	PPL	CRL	PFL	PPL	CRL	PFL	PPL
LA	95.0 ± 0.1^{a}	68.7 ± 1.6^{b}	13.4 ± 0.1^{c}	94.8 ± <0.1^{a}	67.8 ± 1.4^{b}	4.3 ± 1.5^{c}	92.6 ± 3.6^{a}	70.3 ± 1.8^{b}	0.2 ± <0.1^{c}
α-**LNA**	94.3 ± 0.1^{a}	69.2 ± 1.5^{b}	17.5 ± 0.2^{c}	94.6 ± 0.1^{a}	68.5 ± 1.2^{b}	5.6 ± 1.5^{c}	91.7 ± 2.9^{a}	68.5 ± 2.3^{b}	0.5 ± 0.1^{c}
Σ FA	94.4 ± 0.1^{a}	67.2 ± 1.6^{b}	14.9 ± 0.1^{c}	94.5 ± 0.1^{a}	66.2 ± 1.4^{b}	5.4 ± 2.1^{c}	90.7 ± 5.7^{a}	68.2 ± 1.9^{b}	0.5 ± 0.1^{c}

Average values ± standard deviation (n=3). Different superscript letters for significant differences ($p<0.05$) within rows of the same oil.

HSO – Hempseed oil; FSO – Flaxseed oil; SBO – Soybean oil; CRL – *Candida rugosa* lipase; PFL – *Pseudomonas fluorescens* lipase; PPL – Pancreatic porcine lipase; LA – Linoleic acid; α-LNA – alpha-Linolenic acid; Σ FA – Total fatty acids.

Table 3.16 Free linoleic, α-linolenic and total fatty acids (g/100 g) after hydrolysis.

	HSO			**FSO**			**SBO**		
	CRL	PFL	PPL	CRL	PFL	PPL	CRL	PFL	PPL
LA	63.63 ± 1.42^{a}	46.47 ± 0.86^{b}	13.81 ± 0.98^{c}	19.32 ± 0.77^{a}	14.26 ± 0.40^{b}	0.95 ± 0.03^{c}	53.85 ± 2.23^{a}	39.78 ± 0.92^{b}	1.00 ± 0.01^{c}
α-**LNA**	15.13 ± 0.36^{a}	11.27 ± 0.20^{b}	3.94 ± 0.29^{c}	48.94 ± 2.05^{a}	35.27 ± 1.00^{b}	3.01 ± 0.08^{c}	6.84 ± 0.22^{a}	5.03 ± 0.12^{a}	0.18 ± 0.01^{b}
Σ FA	114.15 ± 2.98^{a}	81.43 ± 1.43^{b}	28.24 ± 2.40^{c}	112.00 ± 4.65^{a}	79.95 ± 1.42^{b}	6.89 ± 0.14^{c}	108.70 ± 5.43^{a}	78.66 ± 2.23^{b}	2.38 ± 0.03^{c}

Average values ± standard deviation (n=3). Different superscript letters for significant differences ($p<0.05$) within rows of the same oil.

HSO – Hempseed oil; FSO – Flaxseed oil; SBO – Soybean oil; CRL – *Candida rugosa* lipase; PFL – *Pseudomonas fluorescens* lipase; PPL – Pancreatic porcine lipase; LA – Linoleic acid; α-LNA – alpha-Linolenic acid; Σ FA – Total fatty acids.

3.2.2. Microbially CLA/CLNA-enriched milk

Bifidobacterium breve DSM 20091 was able to grow well at the different substrate concentrations assayed, corroborated by the considerable increase in viable cell numbers from 7.10-7.22 to 9.44-9.61 log CFU/mL after 24 h of incubation (**Figure 3.17**), followed by a decrease in pH values from pH 6.23-6.50 to pH 4.23-4.42 (**Figure 3.18**), with no differences from control (i.e., strain without precursor substrate). No differences were found between substrate conditions at 24 h as well (**Figure 3.17** and **Figure 3.18**). In a previous work (Fontes et al., 2018) this strain could also grow with pure substrates as well as without them in semi-skimmed milk. Changing substrate source to vegetable oils revealed that these do not negatively affect *B. breve* DSM 20091. Besides, dairy matrices exhibit a protective effect on probiotic bacteria viability (de Almeida et al., 2018).

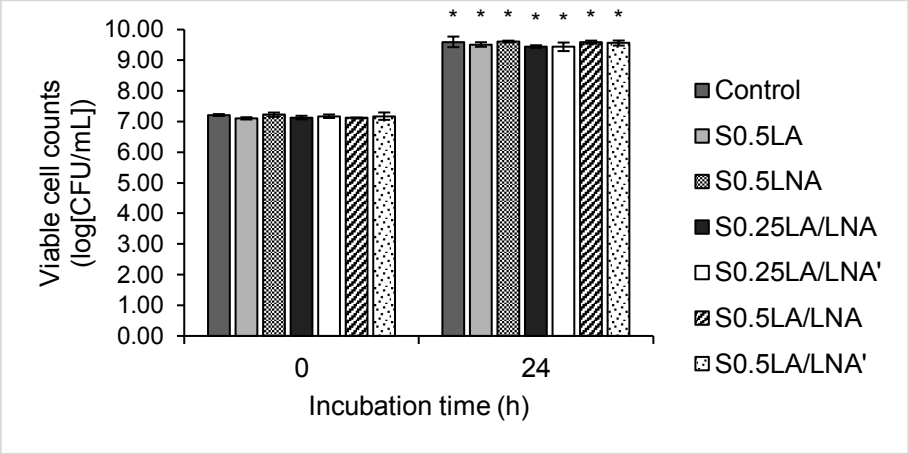


Figure 3.17 Evolution of viable cell numbers (log [CFU/mL]) of *Bifidobacterium breve* DSM 20091 between 0 and 24 h of incubation in semi-skimmed milk with different substrate concentrations: Control – No substrate; S0.5LA – 0.5 mg/mL linoleic acid (LA) from hydrolyzed soybean oil (SBO); S0.5LNA – 0.5 mg/mL α-linolenic acid (α-LNA) from hydrolyzed flaxseed oil (FSO); S0.25LA/LNA – 0.25 mg/mL LA plus 0.25 mg/mL α-LNA from combined hydrolyzed SBO and FSO; S0.25LA/LNA' – 0.25 mg/mL LA from hydrolyzed SBO plus 0.25 mg/mL α-LNA from hydrolyzed FSO; S0.5LA/LNA – 0.5 mg/mL LA plus 0.5 mg/mL α-LNA from combined hydrolyzed SBO and FSO; S0.5LA/LNA' – 0.5 mg/mL LA from hydrolyzed SBO plus 0.5 mg/mL α-LNA from hydrolyzed FSO. *Indicates ≥1 log cycle difference from 0 h.

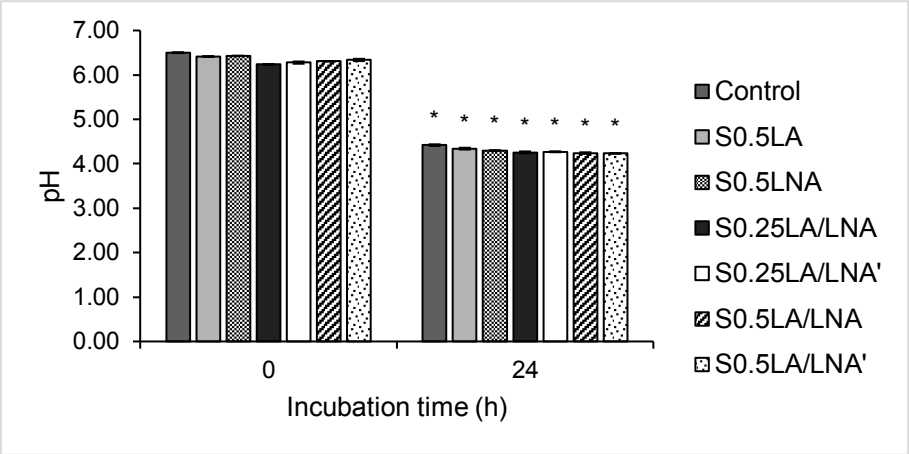


Figure 3.18 Evolution of pH of semi-skimmed milk between 0 and 24 h of fermentation with *Bifidobacterium breve* DSM 20091 and different substrate concentrations: Control – No substrate; S0.5LA – 0.5 mg/mL linoleic acid (LA) from hydrolyzed soybean oil (SBO); S0.5LNA – 0.5 mg/mL α-linolenic acid (α-LNA) from hydrolyzed flaxseed oil (FSO); S0.25LA/LNA – 0.25 mg/mL LA plus 0.25 mg/mL α-LNA from combined hydrolyzed SBO and FSO; S0.25LA/LNA' – 0.25 mg/mL LA from hydrolyzed SBO plus 0.25 mg/mL α-LNA from hydrolyzed FSO; S0.5LA/LNA – 0.5 mg/mL LA plus 0.5 mg/mL α-LNA from combined hydrolyzed SBO and FSO; S0.5LA/LNA' – 0.5 mg/mL LA from hydrolyzed SBO plus 0.5 mg/mL α-LNA from hydrolyzed FSO. *Indicates ≥0.5 units difference from 0 h.

Regarding the CFA production, in control conditions, although milk has a certain amount of LA and α-LNA, the majority is in the esterified form (0.352 mg/g and 0.061 mg/g, respectively; **Section 3.2.3 Effect on the fatty acid profile**, in Results and Discussion of Part II), and therefore not bioavailable for the microorganism. Substrate reduction was minimal – 4.51% for LA and 11.45% for α-LNA (**Table 3.17**). Furthermore, the CLA initially present in the esterified fraction of control samples (0.049 mg/g) did not increase (**Section 3.2.3 Effect on the fatty acid profile**, in Results and Discussion of Part II). All this supports the fact that microbial production of CLA and CLNA can only occur from free substrates. Regarding CFA production in the presence of the assayed vegetable oils, the amount of the CLA released remained low at any of the tested substrate conditions (0.04-0.08 mg/g), being detected C18:2 c9t11 and C18:2 CLA t,t (**Table 3.17**). With both vegetable oils added (i.e., S0.25LA/LNA, S0.25LA/LNA', S0.5LA/LNA and S0.5LA/LNA'), CLNA production was higher than that of CLA (**Table 3.17**). The highest CFA production was achieved for S0.5LNA (0.37 mg/g), corresponding mainly to CLNA isomers (0.34 mg/g) – C18:3 c9t11c15 and C18:3 t9t11c15 (**Table 3.17**). Accordingly, α-LNA conversion degrees were higher than those of LA for all tested conditions, ranging from 43.31-61.69 mg/g for α-LNA and from 12.93-23.58 mg/g for LA (**Table 3.17**). Taken all together, it seems that *B. breve* DSM 20091 has a preference to convert α-LNA instead of LA. In a previous research work (Fontes et al., 2018), the authors reported similar behavior by this same strain when grown in culture medium or semi-skimmed milk supplemented with pure precursor substrates, either alone or in combination. In this latter case, two possible reasons were suggested for such findings: i) α-LNA is more toxic than LA, since CLA and CLNA production by bacteria may

be part of a detoxification mechanism (Sosa-Castañeda et al., 2015); ii) LAI, described as the enzyme responsible for LA and α-LNA isomerization (Salsinha et al., 2018), has a higher specificity for α-LNA. Comparison of the conversion degrees, reveal that the percentages of the precursor substrate reduction were higher, especially for LA, with a nearly 2.4 to 3.3-fold difference (**Table 3.17**). Previous results (Fontes et al., 2018) suggested that both FA enter the bacteria membrane, but α-LNA bioconversion to CLNA is faster.

The results of this second stage of the research work showed that it is not possible to simultaneously enrich milk with CLA and CLNA by *B. breve* DSM 20091 using vegetable oils as precursor substrate sources, and the best option would be to use just FSO and obtain a food product enriched in CLNA. Based on this rationale, the third stage was performed with only FSO.

Few studies have been carried out on dairy microbial CLA/CLNA-enrichment with vegetable oils so far. When different bifidobacteria strains were tested with rapeseed and sunflower oils as LA/α-LNA sources, no production was detected in milk (Gorissen et al., 2012). However, in this previous study, the vegetable oils were not hydrolyzed, thus, the substrate was not bioavailable for further conversion. On the other hand, a *Lc. lactis* strain was able to produce up to 11 mg CLA/g fat from sunflower oil in milk (Kim & Liu, 2002), which could be associated with the fact that LAB are described as possessing weak lipolytic activity (García-Cano et al., 2019). With hydrolyzed SBO, Xu et al. (2004) were able to enrich milk with 1.04 to 2.21 mg CLA/g lipid by different probiotic strains after 24 h, but none was a *B. breve* strain. Therefore, CLA production capacity from SBO could also be a strain-dependent feature. In a further study, with previously selected strains, Xu et al. (2005) could no longer detect CLA production in milk with hydrolyzed SBO, but that could be related to the high LA concentration tested (5 mg/mL). To the best of our knowledge, the study presented herein is the first research work to show microbial CLNA-enrichment of a dairy matrix using an edible substrate source.

Table 3.17 Reduction and conversion percentages of linoleic and α-linolenic acids and amounts of conjugated linoleic and linolenic acid isomers produced by *Bifidobacterium breve* DSM 20091 cultured in semi-skimmed milk supplemented with different substrate concentrations (based on free fatty acid profile).

	Control	S0.5LA	S0.5LNA	S0.25LA/LNA	S0.25LA/LNA'	S0.5LA/LNA	S0.5LA/LNA'
LA reduction (%)	4.51 ± 0.45^{c}	41.56 ± 4.74^{ab}	32.31 ± 4.57^{b}	49.23 ± 4.56^{a}	50.25 ± 2.33^{a}	36.67 ± 3.86^{b}	38.86 ± 2.74^{b}
α-LNA reduction (%)	11.45 ± 2.52^{e}	59.85 ± 5.09^{d}	84.48 ± 0.47^{a}	79.60 ± 3.69^{ab}	79.08 ± 1.72^{ab}	72.75 ± 5.16^{bc}	69.95 ± 2.36^{c}
CLA (mg/g)	ND	0.06 ± 0.01^{b}	0.04 ± <0.01^{c}	0.06 ± 0.01^{b}	0.06 ± <0.01^{b}	0.08 ± 0.01^{a}	0.07 ± <0.01^{ab}
CLNA (mg/g)	ND	0.03 ± <0.01^{d}	0.34 ± 0.04^{a}	0.11 ± <0.01^{c}	0.11 ± 0.01^{c}	0.20 ± 0.01^{b}	0.17 ± 0.01^{b}
CFA (mg/g)	ND	0.09 ± 0.01^{d}	0.37 ± 0.04^{a}	0.17 ± 0.01^{c}	0.17 ± 0.01^{c}	0.28 ± 0.02^{b}	0.24 ± 0.01^{b}
LA conversion (%)	ND	14.69 ± 1.37^{bc}	19.21 ± 2.55^{ab}	23.58 ± 2.86^{a}	18.81 ± 0.73^{ab}	16.79 ± 1.83^{bc}	12.93 ± 0.49^{c}
α-LNA conversion (%)	ND	48.52 ± 4.57^{bc}	61.69 ± 5.90^{a}	56.46 ± 2.97^{ab}	49.36 ± 1.83^{b}	51.41 ± 3.47^{ab}	43.31 ± 2.06^{c}

Average values ± standard deviation (n=3). Different superscript letters indicate significant differences ($p<0.05$) within rows.

Control – No substrate; S0.5LA – 0.5 mg/mL linoleic acid (LA) from hydrolyzed soybean oil (SBO); S0.5LNA – 0.5 mg/mL α-linolenic acid (α-LNA) from hydrolyzed flaxseed oil (FSO); S0.25LA/LNA – 0.25 mg/mL LA plus 0.25 mg/mL α-LNA from combined hydrolyzed SBO and FSO; S0.25LA/LNA' – 0.25 mg/mL LA from hydrolyzed SBO plus 0.25 mg/mL α-LNA from hydrolyzed FSO; S0.5LA/LNA – 0.5 mg/mL LA plus 0.5 mg/mL α-LNA from combined hydrolyzed SBO and FSO; S0.5LA/LNA' – 0.5 mg/mL LA from hydrolyzed SBO plus 0.5 mg/mL α-LNA from hydrolyzed FSO; CLA – Conjugated linoleic acid (C18:2 c9t11 + C18:2 CLA t,t); CLNA – Conjugated linolenic acid (C18:3 c9t11c15 + C18:3 t9t11c15); CFA – Conjugated fatty acids (CLA + CLNA); ND – not detected.

3.2.3. Effect on the fatty acid profile

When comparing the FFA composition at the beginning of the experiment (at 0 h) versus fermented samples (at 24 h) (**Table 3.18** and **Table 3.19**), a significant decrease ($p<0.05$) in butyric (C4:0; from 0.018-0.021 mg/g to 0.013-0.015 mg/g), oleic (C18:1 c9; from 0.314-0.503 mg/g to 0.184-0.335 mg/g), total monounsaturated FA (MUFA; from 0.342-0.540 mg/g to 0.201-0.355 mg/g) and total FA levels (from 1.524-2.454 mg/g to 0.931-1.605 mg/g) was observed in most of the milk samples at the substrate conditions tested. Total PUFA was also changed significantly ($p<0.05$; from 0.450-0.952 mg/g to 0.340-0.704 mg/g), given the reduction in LA and α-LNA (**Table 3.19**). As for EFA, few significant changes were observed, although there was a general reduction in FAs for all tested conditions (**Table 3.20** and **Table 3.21**), which may be due to their use during microbial growth (Fontes et al., 2018). Analyses of the FA composition of sucuk (Özer et al., 2016) and cow's milk (Vieira et al., 2017), fermented with LAB, have also revealed a decrease in oleic acid and MUFA when compared with control (i.e. without LAB). Vieira et al. (2017) reported that a decrease in MUFA, attributed to the consumption of oleic acid, occurs as a way to control bacterial membrane fluidity under stress conditions, such as fermentation. Furthermore, this former study observed an increase in stearic acid (C18:0), as did Özer and Kılıç (2021) in beef fermented with *L. plantarum* strains and hydrolyzed safflower oil. It has been stated that it could be associated with the detoxification mechanism (Özer & Kılıç, 2021) since stearic acid is the final product of the LA biohydrogenation pathway (Fontes et al., 2017). Yet, a significant increment in stearic acid was never observed in the current work.

Table 3.18 Free fatty acids profile of semi-skimmed milk cultured with *Bifidobacterium breve* DSM 20091 and different substrate concentrations at 0 h and 24 h of incubation.

FA (mg/g)	Control		S0.5LA		S0.5LNA		S0.25LA/LNA		S0.25LA/LNA'		S0.5LA/LNA		S0.5LA/LNA'	
	0 h	24 h	0 h	24 h	0 h	24 h	0 h	24 h	0 h	24 h	0 h	24 h	0 h	24 h
C4:0	0.015± 0.001	0.013± 0.001	0.018± 0.003	0.013± 0.002*	0.024± 0.002	0.023± 0.001	0.020± 0.002	0.013± 0.001*	0.021± 0.001	0.014± 0.003*	0.021± 0.002	0.015± 0.002*	0.019± 0.001	0.015± 0.001*
C6:0	0.012± 0.002	0.010± 0.001	0.015± 0.003	0.010± 0.002*	0.025± 0.003	0.021± 0.001	0.017± 0.002	0.010± <0.001*	0.019± 0.002	0.011± 0.002*	0.017± 0.004	0.011± 0.001	0.018± 0.001	0.012± 0.001*
C8:0	0.010± 0.002	0.009± 0.001	0.013± 0.004	0.008± 0.001	0.016± 0.001	0.015± 0.002	0.014± 0.001	0.008± 0.002*	0.018± 0.003	0.009± 0.001*	0.014± 0.004	0.009± 0.001	0.015± 0.001	0.010± 0.002
C10:0	0.027± 0.003	0.023± 0.002	0.029± 0.007	0.018± 0.004	0.039± 0.002	0.034± 0.005	0.036± 0.006	0.016± 0.001*	0.041± 0.005	0.020± 0.003*	0.031± 0.009	0.019± 0.001	0.038± 0.003	0.021± 0.002*
C12:0	0.035± 0.004	0.028± 0.002*	0.036± 0.009	0.020± 0.005	0.048± 0.003	0.039± 0.007	0.045± 0.008	0.018± 0.001*	0.050± 0.007	0.021± 0.003*	0.037± 0.012	0.020± 0.001	0.046± 0.004	0.023± 0.003*
C14:0	0.098± 0.017	0.088± 0.006	0.101± 0.026	0.053± 0.012	0.132± 0.008	0.105± 0.018	0.131± 0.026	0.049± 0.003*	0.145± 0.022	0.058± 0.008*	0.106± 0.033	0.055± 0.003	0.132± 0.011	0.062± 0.007*
C14:1 c9	0.010± 0.002	0.008± 0.001	0.010± 0.003	0.007± 0.001	0.014± 0.001	0.012± 0.002	0.013± 0.002	0.006± <0.001*	0.015± 0.002	0.007± 0.001*	0.011± 0.003	0.007± <0.001	0.014± 0.001	0.007± 0.001*
C15:0	0.009± 0.001	0.008± <0.001	0.009± 0.002	0.006± 0.001	0.013± 0.001	0.011± 0.001	0.012± 0.002	0.005± <0.001*	0.014± 0.002	0.007± 0.001*	0.010± 0.003	0.007± 0.001	0.014± 0.002	0.007± <0.001*
C16:0	0.303± 0.052	0.301± 0.019	0.390± 0.082	0.229± 0.047*	0.473± 0.027	0.388± 0.059	0.464± 0.093	0.206± 0.018	0.513± 0.080	0.236± 0.025*	0.440± 0.102	0.270± 0.016	0.527± 0.042	0.293± 0.019*
C16:1 c9	0.017± 0.002	0.015± 0.001	0.018± 0.004	0.012± 0.003	0.023± 0.001	0.020± 0.003	0.022± 0.005	0.011± 0.001	0.024± 0.004	0.012± 0.001*	0.019± 0.005	0.012± 0.001	0.023± 0.002	0.013± 0.001*
C17:0	0.004± 0.001	0.004± <0.001	0.005± 0.001	0.005± 0.002	0.006± 0.001	0.006± 0.001	0.006± 0.002	0.004± 0.001	0.007± 0.001	0.004± <0.001*	0.006± 0.001	0.004± <0.001	0.007± <0.001	0.005± <0.001*
C18:0	0.054± 0.012	0.059± 0.006	0.097± 0.019	0.063± 0.011	0.133± 0.007	0.115± 0.011	0.110± 0.020	0.062± 0.005	0.122± 0.019	0.068± 0.006*	0.139± 0.022	0.097± 0.005	0.146± 0.011	0.099± 0.002*
C18:1 c9	0.132± 0.025	0.127± 0.011	0.314± 0.043	0.211± 0.032*	0.387± 0.013	0.311± 0.017*	0.323± 0.045	0.184± 0.008*	0.369± 0.040	0.207± 0.017*	0.442± 0.041	0.322± 0.017*	0.503± 0.023	0.335± 0.001*

Average values ± standard deviation (n=3). * Significant differences ($p<0.05$) from 0 h to 24 h.

FA – Fatty acid; Control – No substrate; S0.5LA – 0.5 mg/mL linoleic acid (LA) from hydrolyzed soybean oil (SBO); S0.5LNA – 0.5 mg/mL α-linolenic acid (α-LNA) from hydrolyzed flaxseed oil (FSO); S0.25LA/LNA – 0.25 mg/mL LA plus 0.25 mg/mL α-LNA from combined hydrolyzed SBO and FSO; S0.25LA/LNA' – 0.25 mg/mL LA from hydrolyzed SBO plus 0.25 mg/mL α-LNA from hydrolyzed FSO; S0.5LA/LNA – 0.5 mg/mL LA plus 0.5 mg/mL α-LNA from combined hydrolyzed SBO and FSO; S0.5LA/LNA' – 0.5 mg/mL LA from hydrolyzed SBO plus 0.5 mg/mL α-LNA from hydrolyzed FSO; *c* – *cis* double bond.

Table 3.19 Free fatty acids profile of semi-skimmed milk cultured with *Bifidobacterium breve* DSM 20091 and different substrate concentrations at 0 h and 24 h of incubation (*continued*).

FA (mg/g)	Control		S0.5LA		S0.5LNA		S0.25LA/LNA		S0.25LA/LNA'		S0.5LA/LNA		S0.5LA/LNA'	
	0 h	24 h	0 h	24 h	0 h	24 h	0 h	24 h	0 h	24 h	0 h	24 h	0 h	24 h
C18:2 c9c12 (LA)	0.025± 0.004	0.024± 0.001	0.403± 0.019	0.236± 0.030*	0.189± 0.007	0.128± 0.008*	0.254± 0.011	0.129± 0.010*	0.310± 0.009	0.154± 0.010*	0.466± 0.004	0.295± 0.020*	0.543± 0.019	0.331± 0.004*
C18:3 c9c12c15 (α-LNA)	0.005± 0.001	0.004± <0.001	0.060± 0.003	0.024± 0.004*	0.546± 0.021	0.085± 0.005*	0.190± 0.005	0.039± 0.007*	0.219± 0.005	0.046± 0.004*	0.384± 0.004	0.104± 0.019*	0.402± 0.012	0.121± 0.006*
C18:2 c9t11 (CLA)	0.003± <0.001	0.003± <0.001	0.004± 0.001	0.044± 0.006*	0.008± <0.001	0.033± 0.004*	0.005± <0.001	0.046± 0.006*	0.005± 0.001	0.046± 0.001*	0.006± 0.001	0.061± 0.008*	0.007± <0.001	0.056± 0.002*
C18:2 CLA t,t	ND	ND	ND	0.020± 0.003	ND	0.011± 0.001	ND	0.019± 0.001	ND	0.018± 0.001	ND	0.023± 0.002	ND	0.021± 0.001
C18:3 c9t11c15 (CLNA)	ND	ND	ND	0.021± 0.003	ND	0.268± 0.028	ND	0.080± 0.006	ND	0.083± 0.002	ND	0.150± 0.013	ND	0.135± 0.006
C18:3 t9t11c15 (CLNA)	ND	ND	ND	0.008± 0.001	ND	0.069± 0.008	ND	0.027± 0.002	ND	0.025± 0.003	ND	0.048± 0.003	ND	0.039± 0.002
SFA	0.567± 0.095	0.543± 0.037	0.714± 0.155	0.425± 0.085	0.909± 0.051	0.756± 0.105	0.856± 0.162	0.390± 0.023*	0.951± 0.140	0.448± 0.051*	0.822± 0.192	0.508± 0.028	0.962± 0.077	0.546± 0.034*
MUFA	0.159± 0.029	0.150± 0.012	0.342± 0.050	0.229± 0.036*	0.424± 0.015	0.343± 0.021	0.358± 0.052	0.201± 0.009*	0.408± 0.046	0.226± 0.018*	0.472± 0.050	0.341± 0.018*	0.540± 0.026	0.355± 0.003*
PUFA	0.032± 0.004	0.030± 0.001	0.468± 0.022	0.353± 0.039*	0.742± 0.027	0.593± 0.049*	0.450± 0.016	0.340± 0.009*	0.534± 0.014	0.372± 0.022*	0.856± 0.005	0.681± 0.036*	0.952± 0.030	0.704± 0.010*
Σ FA	0.757± 0.127	0.724± 0.050	1.524± 0.223	1.007± 0.159*	2.075± 0.076	1.692± 0.102	1.664± 0.229	0.931± 0.034*	1.893± 0.200	1.046± 0.083*	2.150± 0.243	1.530± 0.080*	2.454± 0.116	1.605± 0.027*

Average values ± standard deviation (n=3). * Significant differences ($p<0.05$) from 0 h to 24 h.

FA – Fatty acid; Control – No substrate; S0.5LA – 0.5 mg/mL linoleic acid (LA) from hydrolyzed soybean oil (SBO); S0.5LNA – 0.5 mg/mL α-linolenic acid (α-LNA) from hydrolyzed flaxseed oil (FSO); S0.25LA/LNA – 0.25 mg/mL LA plus 0.25 mg/mL α-LNA from combined hydrolyzed SBO and FSO; S0.25LA/LNA' – 0.25 mg/mL LA from hydrolyzed SBO plus 0.25 mg/mL α-LNA from hydrolyzed FSO; S0.5LA/LNA – 0.5 mg/mL LA plus 0.5 mg/mL α-LNA from combined hydrolyzed SBO and FSO; S0.5LA/LNA' – 0.5 mg/mL LA from hydrolyzed SBO plus 0.5 mg/mL α-LNA from hydrolyzed FSO; *c* – *cis* double bond; *t* – *trans* double bond; CLA – Conjugated linoleic acid; CLNA – Conjugated linolenic acid; SFA – Saturated fatty acids; MUFA – Monounsaturated fatty acids; PUFA – Polyunsaturated fatty acids; Σ FA – Total fatty acids; ND – not detected.

Table 3.20 Esterified fatty acids profile of semi-skimmed milk cultured with *Bifidobacterium breve* DSM 20091 and different substrate concentrations at 0 h and 24 h of incubation.

FA (mg/g)	Control		S0.5LA		S0.5LNA		S0.25LA/LNA		S0.25LA/LNA'		S0.5LA/LNA		S0.5LA/LNA'	
	0 h	24 h	0 h	24 h	0 h	24 h	0 h	24 h	0 h	24 h	0 h	24 h	0 h	24 h
C4:0	0.247± 0.012	0.251± 0.059	0.372± 0.057	0.310± 0.089	0.180± 0.011	0.153± 0.019	0.329± 0.030	0.275± 0.024*	0.315± 0.029	0.258± 0.052	0.257± 0.021	0.266± 0.014	0.320± 0.023	0.278± 0.010
C6:0	0.208± 0.003	0.202± 0.036	0.326± 0.009	0.296± 0.096	0.203± 0.023	0.140± 0.015*	0.288± 0.035	0.237± 0.020*	0.291± 0.030	0.235± 0.036	0.234± 0.023	0.235± 0.021	0.291± 0.027	0.251± 0.018
C8:0	0.133± 0.011	0.124± 0.027	0.207± 0.033	0.159± 0.040	0.105± 0.011	0.095± 0.009	0.193± 0.008	0.150± 0.017*	0.173± 0.010	0.136± 0.023	0.157± 0.017	0.162± 0.016	0.185± 0.007	0.152± 0.012
C10:0	0.322± 0.028	0.295± 0.063	0.362± 0.031	0.281± 0.069	0.189± 0.016	0.153± 0.008	0.344± 0.037	0.254± 0.019*	0.348± 0.020	0.252± 0.032	0.301± 0.024	0.260± 0.007	0.355± 0.022	0.281± 0.017
C12:0	0.413± 0.040	0.380± 0.085	0.444± 0.036	0.348± 0.086	0.231± 0.018	0.187± 0.009	0.416± 0.039	0.305± 0.024*	0.428± 0.027	0.306± 0.039	0.364± 0.026	0.317± 0.008	0.430± 0.029	0.345± 0.017
C14:0	1.317± 0.098	1.212± 0.280	1.406± 0.112	1.081± 0.263	0.718± 0.059	0.579± 0.028	1.312± 0.121	0.953± 0.074*	1.359± 0.080	0.973± 0.128	1.147± 0.085	0.989± 0.025	1.366± 0.088	1.084± 0.053
C14:1 c9	0.125± 0.010	0.116± 0.028	0.148± 0.018	0.119± 0.031	0.074± 0.007	0.060± 0.005	0.136± 0.017	0.099± 0.006*	0.137± 0.011	0.097± 0.011	0.115± 0.010	0.101± 0.001	0.140± 0.011	0.112± 0.006
C15:0	0.122± 0.013	0.113± 0.031	0.156± 0.031	0.129± 0.030	0.073± 0.008	0.060± 0.005	0.137± 0.010	0.100± 0.005*	0.138± 0.025	0.093± 0.012	0.113± 0.011	0.100± 0.004	0.143± 0.012	0.110± 0.009
C16:0	4.700± 0.374	4.221± 0.998	5.032± 0.426	3.899± 0.966	2.621± 0.219	2.107± 0.119	4.688± 0.413	3.387± 0.241*	4.873± 0.301	3.486± 0.464	4.085± 0.302	3.534± 0.094	4.896± 0.323	3.882± 0.233
C16:1 c9	0.238± 0.021	0.215± 0.051	0.240± 0.024	0.188± 0.044*	0.125± 0.011	0.102± 0.006	0.223± 0.022	0.160± 0.013*	0.231± 0.014	0.161± 0.022	0.196± 0.015	0.166± 0.005	0.230± 0.015	0.186± 0.011
C17:0	0.061± 0.006	0.053± 0.009	0.071± 0.010	0.058± 0.013*	0.034± 0.004	0.027± 0.002	0.070± 0.010	0.050± 0.007*	0.070± 0.006	0.051± 0.009	0.054± 0.004	0.051± 0.004	0.069± 0.002	0.061± 0.004
C18:0	0.957± 0.054	0.815± 0.169	1.090± 0.095	0.825± 0.197*	0.532± 0.050	0.424± 0.023	1.019± 0.089	0.715± 0.048*	1.120± 0.078	0.745± 0.102	0.899± 0.061	0.757± 0.019	1.071± 0.074	0.831± 0.047
C18:1 c9	2.175± 0.148	1.877± 0.394	2.278± 0.199	1.709± 0.415*	1.124± 0.097	0.893± 0.045	2.104± 0.174	1.492± 0.102*	2.155 ±0.140	1.502± 0.190	1.863± 0.126	1.574± 0.036	2.260± 0.153	1.752± 0.109

Average values ± standard deviation (n=3). * Significant differences ($p<0.05$) from 0 h to 24 h.

FA – Fatty acid; Control – No substrate; S0.5LA – 0.5 mg/mL linoleic acid (LA) from hydrolyzed soybean oil (SBO); S0.5LNA – 0.5 mg/mL α-linolenic acid (α-LNA) from hydrolyzed flaxseed oil (FSO); S0.25LA/LNA – 0.25 mg/mL LA plus 0.25 mg/mL α-LNA from combined hydrolyzed SBO and FSO; S0.25LA/LNA' – 0.25 mg/mL LA from hydrolyzed SBO plus 0.25 mg/mL α-LNA from hydrolyzed FSO; S0.5LA/LNA – 0.5 mg/mL LA plus 0.5 mg/mL α-LNA from combined hydrolyzed SBO and FSO; S0.5LA/LNA' – 0.5 mg/mL LA from hydrolyzed SBO plus 0.5 mg/mL α-LNA from hydrolyzed FSO; *c* – *cis* double bond.

Table 3.21 Esterified fatty acids profile of semi-skimmed milk cultured with *Bifidobacterium breve* DSM 20091 and different substrate concentrations at 0 h and 24 h of incubation (*continued*).

FA (mg/g)	Control		S0.5LA		S0.5LNA		S0.25LA/LNA		S0.25LA/LNA'		S0.5LA/LNA		S0.5LA/LNA'	
	0 h	24 h	0 h	24 h	0 h	24 h	0 h	24 h	0 h	24 h	0 h	24 h	0 h	24 h
C18:2 c9c12 (LA)	0.352± 0.029	0.312± 0.072	0.361± 0.034	0.285± 0.073	0.174± 0.016	0.137± 0.009	0.323± 0.026	0.237± 0.025*	0.321± 0.025	0.234± 0.037	0.286± 0.022	0.251± 0.013	0.359± 0.029	0.281± 0.025
C18:3 c9c12c15 (α-LNA)	0.061± 0.015	0.055± 0.023	0.080± 0.016	0.074± 0.027	0.068± 0.005	0.049± 0.008	0.082± 0.014	0.071± 0.009	0.078± 0.008	0.070± 0.016	0.068± 0.009	0.074± 0.005	0.099± 0.010	0.082± 0.012
C18:2 c9t11 (CLA)	0.049± 0.005	0.038± 0.009*	0.059± 0.009	0.046± 0.014	0.031± 0.003	0.024± 0.002	0.058± 0.011	0.044± 0.004	0.053± 0.008	0.037± 0.004	0.042± 0.007	0.043± 0.005	0.052± 0.001	0.048± 0.005
SFA	8.481± 0.631	7.666± 1.754	9.466± 0.823	7.388± 1.845	4.885± 0.396	3.925± 0.234	8.794± 0.785	6.427± 0.474*	9.115± 0.589	6.535± 0.888	7.611± 0.564	6.671± 0.200	9.126± 0.604	7.275± 0.415
MUFA	2.538± 0.180	2.209± 0.473	2.666± 0.240	2.016± 0.489*	1.323± 0.114	1.055± 0.055	2.463± 0.213	1.751± 0.121*	2.522± 0.165	1.760± 0.223	2.174± 0.152	1.841± 0.043	2.630± 0.178	2.050± 0.126
PUFA	0.461± 0.049	0.405± 0.102	0.500± 0.058	0.405± 0.112	0.273± 0.023	0.211± 0.019	0.463± 0.048	0.352± 0.036*	0.451± 0.041	0.341± 0.056	0.396± 0.037	0.368± 0.020	0.509± 0.040	0.411± 0.039
Σ FA	11.481± 0.858	10.279± 2.329	12.632± 1.116	9.808± 2.445	6.481± 0.532	5.190± 0.308	11.720± 1.045	8.530± 0.630*	12.088± 0.769	8.636± 1.166	10.181± 0.751	8.881± 0.261	12.265± 0.815	9.736± 0.578

Average values ± standard deviation (n=3). * Significant differences ($p<0.05$) from 0 h to 24 h.

FA – Fatty acid; Control – No substrate; S0.5LA – 0.5 mg/mL linoleic acid (LA) from hydrolyzed soybean oil (SBO); S0.5LNA – 0.5 mg/mL α-linolenic acid (α-LNA) from hydrolyzed flaxseed oil (FSO); S0.25LA/LNA – 0.25 mg/mL LA plus 0.25 mg/mL α-LNA from combined hydrolyzed SBO and FSO; S0.25LA/LNA' – 0.25 mg/mL LA from hydrolyzed SBO plus 0.25 mg/mL α-LNA from hydrolyzed FSO; S0.5LA/LNA – 0.5 mg/mL LA plus 0.5 mg/mL α-LNA from combined hydrolyzed SBO and FSO; S0.5LA/LNA' – 0.5 mg/mL LA from hydrolyzed SBO plus 0.5 mg/mL α-LNA from hydrolyzed FSO; *c* – *cis* double bond; *t* – *trans* double bond; CLA – Conjugated linoleic acid; SFA – Saturated fatty acids; MUFA – Monounsaturated fatty acids; PUFA – Polyunsaturated fatty acids; Σ FA – Total fatty acids.

3.2.4. Microbially CLNA-enriched milk

At all tested α-LNA concentrations (with hydrolyzed FSO), *B. breve* DSM 20091 was able to grow well, even at 5 (S5LNA) or 10 (S10LNA) mg/mL, as seen by the considerable increase in viable cell numbers from 7.10-7.26 to 8.97-9.64 log CFU/mL after 24 h of incubation (**Figure 3.19**), with no differences from the control (strain without precursor substrate), neither between tested α-LNA concentrations at 24 h (**Figure 3.19**). At the beginning of incubation, the milk pH of S5LNA was more acidic (pH 6.02) than the control (pH 6.51), and S10LNA was more acidic (pH 5.82) than all conditions, except for S5LNA (**Figure 3.20**); this is related to the higher amount of FA provided. However, after 24 h of incubation, the pH values decreased considerably for any condition tested to pH 4.02-4.43, and no differences were found from the control or between tested α-LNA concentrations at 24 h (**Figure 3.20**), which is in accordance with the bacterial growth observed. This behavior is an upgrade to previous results where the assessed strain could only tolerate up to 1 mg/mL of pure LA (**Section 3.1.3 Determination of maximum tolerance to linoleic acid**; in Results and Discussion of Part I). According to free LA:α-LNA proportion in hydrolyzed FSO (1:2.53; **Table 3.16**), 10 mg/mL of α-LNA added from FSO would also provide nearly 4 mg/mL of LA. Within this framework of results, it seems that the use of edible vegetable oils, as substrate vehicles, in particular FSO, confers somehow a protective system to *B. breve* DSM 20091 against associated inhibitory effects at high substrate concentrations. Additionally, it could be related to the milk protective effect (de Almeida et al., 2018).

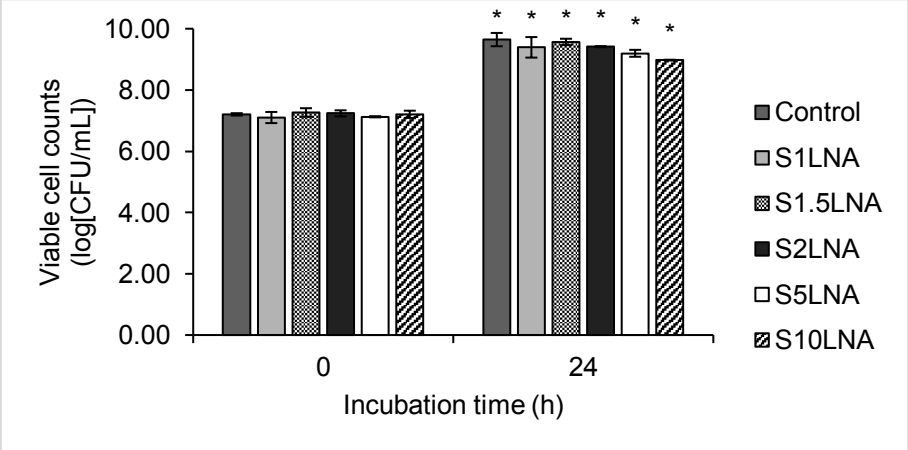


Figure 3.19 Evolution of viable cell numbers (log[CFU/mL]) of *Bifidobacterium breve* DSM 20091 between 0 and after 24 h of incubation in semi-skimmed milk with different α-linolenic acid (α-LNA) concentrations from hydrolyzed flaxseed oil: Control – No substrate; S1LNA – 1 mg/mL α-LNA; S1.5LNA – 1.5 mg/mL α-LNA; S2LNA – 2 mg/mL α-LNA; S5LNA – 5 mg/mL α-LNA; S10LNA – 10 mg/mL α-LNA. *Indicates ≥1 log cycle difference from 0 h.

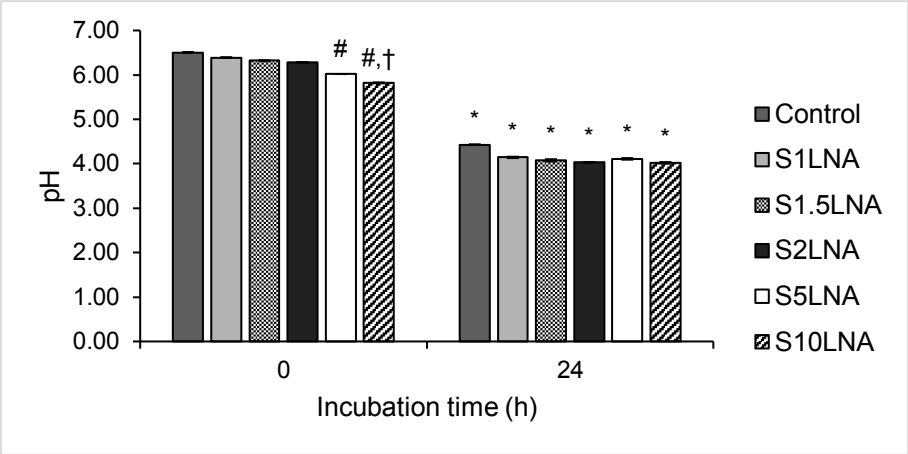


Figure 3.20 Evolution of pH of semi-skimmed milk between 0 and 24 h of fermentation with *Bifidobacterium breve* DSM 20091 and different α-linolenic acid (α-LNA) concentrations from hydrolyzed flaxseed oil: Control – No substrate; S1LNA – 1 mg/mL α-LNA; S1.5LNA – 1.5 mg/mL α-LNA; S2LNA – 2 mg/mL α-LNA; S5LNA – 5 mg/mL α-LNA; S10LNA – 10 mg/mL α-LNA. #Indicates ≥0.5 units difference from Control. †Indicates ≥0.5 units difference from S1LNA, S1.5LNA and S2LNA. *Indicates ≥0.5 units difference from 0 h.

At the conditions assayed, it was possible to enrich milk with a maximum of 0.95 mg/g of CFA (S2LNA), being mainly CLNA isomers (0.88 mg/g) – C18:3 c9t11c15 and C18:3 t9t11c15 (**Table 3.22**; **Figure 3.21**). The 2 mg/mL content was shown to be the limiting substrate concentration for *B. breve* DSM 20091 LA/LNA conversion ability since no significant differences ($p>0.05$) were observed on CFA production at 5 (S5LNA) or 10 (S10LNA) mg/mL α-LNA (**Table 3.22**). Indeed, this strain maintained its substrate conversion rates at the same level of capacity until 2 mg/mL α-LNA (50.46-55.81%) (**Table 3.22**), and then dropped these considerably to 15.50-17.03%. Moreover, from 2 mg/mL, inclusively, substrate reduction degree decreased in a concentration dependent-manner (**Table 3.22**). This strongly suggests that from 2 mg/mL onward LAI becomes saturated with substrate and therefore has no further capacity to produce more CFA. To the best of our knowledge, no other studies have reported such values in CLNA-enrichment of dairy products. Indeed, this is the first study to employ α-LNA concentrations above 0.5 mg/mL for dairy fermentation. Only Chung et al. (2008) had achieved comparable levels, but in CLA isomers, with a ~1.06 mg/mL production in skim milk fermented with a *B. breve* strain, as well, and 0.5% (v/v) of monolinolein.

Table 3.22 Reduction and conversion percentages of linoleic and α-linolenic acids and conjugated linoleic and linolenic acid isomers contents produced by *Bifidobacterium breve* DSM 20091 cultured in semi-skimmed milk supplemented with different α-linolenic acid concentrations from hydrolyzed flaxseed oil (based on the free fatty acid profiles).

	Control	S1LNA	S1.5LNA	S2LNA	S5LNA	S10LNA
LA reduction (%)	6.77 ± 0.68^{c}	37.85 ± 8.62^{a}	26.30 ± 5.23^{a}	14.19 ± 1.42^{b}	6.85 ± 0.68^{c}	0.26 ± 0.03^{d}
α-LNA reduction (%)	12.78 ± 1.41^{e}	82.78 ± 8.67^{ab}	72.03 ± 8.21^{bc}	65.95 ± 6.59^{c}	20.89 ± 3.53^{d}	7.06 ± 0.71^{f}
CLA (mg/g)	ND	0.05 ± 0.02^{ab}	0.05 ± 0.01^{ab}	0.07 ± 0.01^{a}	0.04 ± <0.01^{b}	0.04 ± <0.01^{b}
CLNA (mg/g)	ND	0.44 ± 0.05^{cd}	0.61 ± 0.07^{bc}	0.88 ± 0.09^{a}	0.70 ± 0.05^{ab}	0.77 ± <0.01^{a}
CFA (mg/g)	ND	0.49 ± 0.07^{cd}	0.67 ± 0.08^{bc}	0.95 ± 0.09^{a}	0.74 ± 0.06^{ab}	0.81 ± 0.01^{a}
LA conversion (%)	ND	19.67 ± 2.00^{a}	14.54 ± 2.47^{a}	14.77 ± 1.48^{a}	2.58 ± 0.16^{b}	2.70 ± 0.20^{b}
α-LNA conversion (%)	ND	54.57 ± 8.36^{ab}	50.46 ± 5.52^{b}	55.81 ± 5.58^{ab}	15.50 ± 1.22^{c}	17.03 ± 0.07^{c}

Average values ± standard deviation (n=3). Different superscript letters indicate significant differences ($p<0.05$) within rows.

Control – No substrate; S1LNA – 1 mg/mL α-linolenic acid (α-LNA); S1.5LNA – 1.5 mg/mL α-LNA; S2LNA – 2 mg/mL α-LNA; S5LNA – 5 mg/mL α-LNA; S10LNA – 10 mg/mL α-LNA; LA – Linoleic acid; CLA – Conjugated linoleic acid (C18:2 c9t11 + C18:2 CLA t,t); CLNA – Conjugated linolenic acid (C18:3 c9t11c15 + C18:3 t9t11c15); CFA – Conjugated fatty acids (CLA + CLNA); ND – not detected.

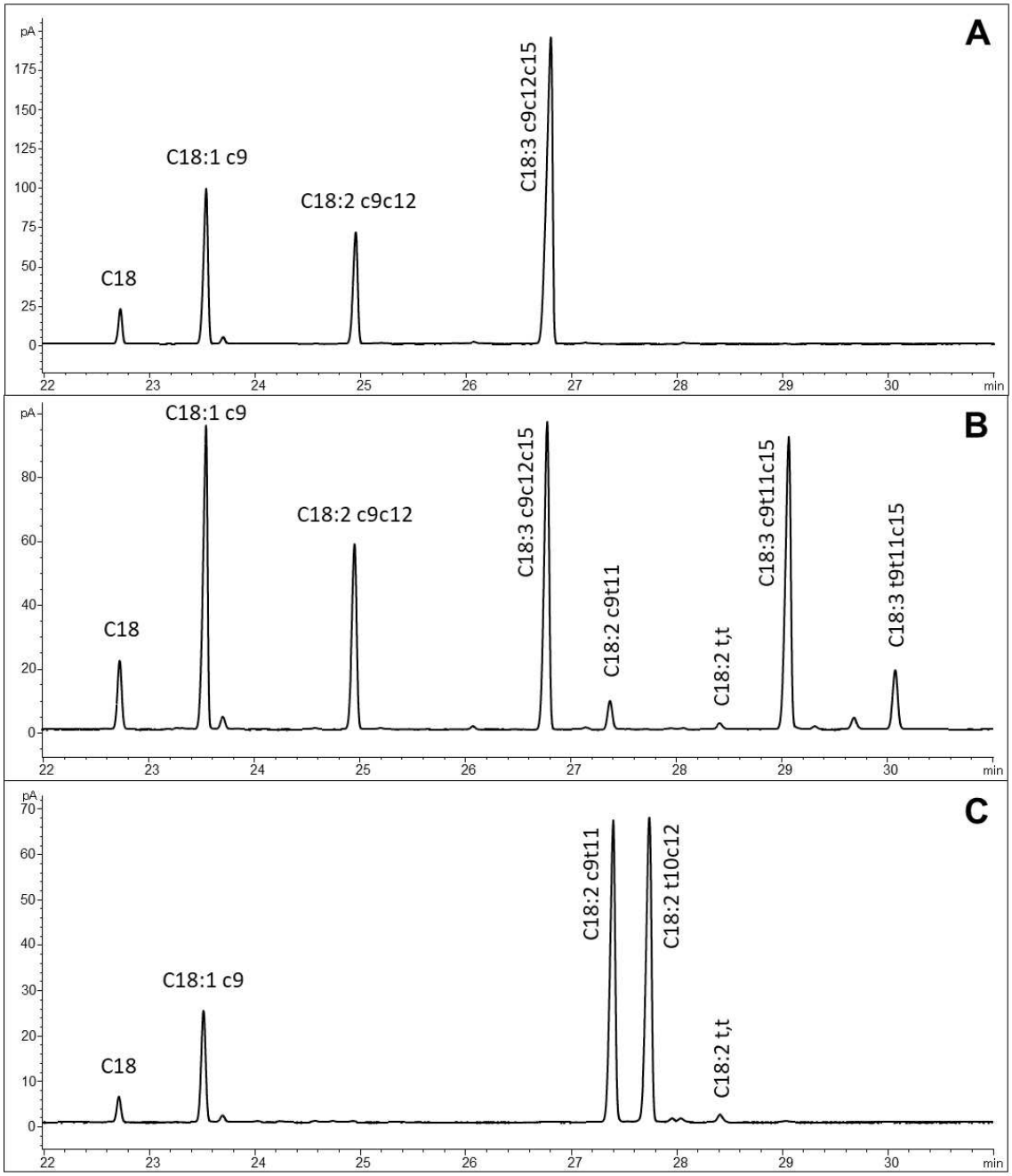


Figure 3.21 Chromatogram profile by GC-FID of milk free fatty acids at 0 h (A) and 24 h (B) of fermentation with *Bifidobacterium breve* DSM 20091 and hydrolyzed flaxseed oil at 2 mg/mL α-linolenic acid (S2LNA), plus esterified profile of Tonalin CLA (C). Tonalin CLA isomers identification was based on results reported by Rodríguez-Alcalá and Fontecha (2007).

Regarding milk FFA composition, it was observed that PUFA levels changed significantly ($p<0.05$) from 0 h (1.065-2.045 mg/g) to 24 h (0.792-1.889 mg/g) of fermentation only until 2 mg/mL α-LNA (S2LNA) (**Table 3.23** and **Table 3.24**), which is explained by the low LA/α-LNA reduction and conversion rates verified at 5 (S5LNA) or 10 (S10LNA) mg/mL α-LNA (**Table 3.22**). As for EFA, although not always significant, there was a general decrease in FAs for all tested conditions (**Table 3.25** and **Table 3.26**).

Analysis of milk composition revealed the presence of 0.05 mg/g of CLA only (0.004 mg/g free CLA + 0.048 mg/g esterified CLA; **Table 3.27**). By culturing milk with *B. breve* DSM 20091 at 2 mg/mL α-LNA (with hydrolyzed FSO), it was possible to increase CFA contents (mostly CLNA isomers) to a total of 1.01 mg/g (0.060 mg/g free C18:2 c9t11 + 0.017 mg/g free C18:2 CLA t,t + 0.722 mg/g free C18:3 c9t11c15 + 0.154 mg/g free C18:3 t9t11c15 + 0.054 mg/g esterified C18:2 c9t11; **Table 3.24** and **Table 3.26**). According to the European Commission Regulation (EC) 983/2009, amended by Regulation (EC) 376/2010, α-LNA is recognized as a functional ingredient. Totalizing CFAs and α-LNA, the CLNA-enriched fermented milk obtained at 2 mg/mL α-LNA (with hydrolyzed FSO) could account for 1.69 mg/g of bioactive compounds (0.530 mg/g free C18:3 c9c12c15 + 0.060 mg/g free C18:2 c9t11 + 0.017 mg/g free C18:2 CLA t,t + 0.722 mg/g free C18:3 c9t11c15 + 0.154 mg/g free C18:3 t9t11c15 + 0.155 mg/g esterified C18:3 c9c12c15 + 0.054 mg/g esterified C18:2 c9t11; **Table 3.24** and **Table 3.26**). Although at 10 mg/mL α-LNA (with hydrolyzed FSO) the CLNA-enriched fermented milk reached 9.47 mg/g in bioactive compounds (7.822 mg/g free C18:3 c9c12c15 + 0.041 mg/g free C18:2 c9t11 + 0.015 free C18:2 CLA t,t + 0.0638 mg/g free C18:3 c9t11c15 + 0.130 mg/g free C18:3 t9t11c15 + 0.689 mg/g esterified C18:3 c9c12c15 + 0.092 mg/g esterified C18:2 c9t11 + 0.040 mg/g esterified C18:2 CLA t,t; **Table 3.24** and **Table 3.26**), most part corresponded to the α-LNA that was not used by the strain (low conversion rate), therefore, the 2 mg/mL α-LNA condition revealed to be the most cost-effective process regarding the development of a functional dairy product through this *in situ* microbial production strategy.

Table 3.23 Free fatty acids profile of semi-skimmed milk cultured with *Bifidobacterium breve* DSM 20091 and different α-linolenic acid concentrations from hydrolyzed flaxseed oil at 0 h and 24 h of incubation.

FA (mg/g)	Control		S1LNA		S1.5LNA		S2LNA		S5LNA		S10LNA	
	0 h	24 h	0 h	24 h	0 h	24 h	0 h	24 h	0 h	24 h	0 h	24 h
C4:0	0.015± 0.001	0.013± 0.001	0.017± 0.001	0.012± 0.002*	0.017± 0.002	0.011± 0.002*	0.016± 0.002	0.014± 0.001*	0.013± 0.001	0.012± 0.001	0.012± <0.001	0.011± 0.001
C6:0	0.013± 0.002	0.011± 0.001	0.016± 0.001	0.010± 0.003*	0.016± 0.002	0.010± 0.001*	0.014± 0.001	0.012± 0.001	0.011± 0.001	0.010± 0.001	0.011± <0.001	0.009± 0.002
C8:0	0.010± 0.002	0.009± 0.001	0.013± 0.001	0.008± 0.002*	0.013± 0.001	0.007± 0.001*	0.011± 0.002	0.010± 0.001	0.009± 0.001	0.008± <0.001	0.010± 0.001	0.009± 0.001
C10:0	0.028± 0.004	0.024± 0.001	0.031± 0.004	0.019± 0.007	0.031± 0.003	0.017± 0.002*	0.026± 0.005	0.022± 0.004	0.020± 0.001	0.018± 0.002*	0.021± 0.002	0.018± 0.002
C12:0	0.036± 0.004	0.029± 0.001	0.039± 0.005	0.022± 0.009	0.038± 0.004	0.019± 0.001*	0.032± 0.006	0.025± 0.005	0.025± 0.002	0.021± 0.003*	0.025± 0.002	0.021± 0.003
C14:0	0.103± 0.021	0.091± 0.006	0.107± 0.014	0.060± 0.030	0.104± 0.011	0.051± 0.004*	0.089± 0.019	0.066± 0.013	0.075± 0.004	0.061± 0.007*	0.076± 0.007	0.064± 0.008
C14:1 c9	0.010± 0.002	0.008± 0.001	0.011± 0.001	0.007± 0.003	0.011± 0.001	0.006± <0.001*	0.009± 0.002	0.007± 0.001	0.008± 0.001	0.007± 0.001*	0.008± 0.001	0.007± 0.001
C15:0	0.009± 0.001	0.008± <0.001	0.011± 0.001	0.007± 0.003	0.010± 0.001	0.006± <0.001*	0.009± 0.002	0.008± 0.001	0.009± <0.001	0.008± 0.001	0.011± 0.001	0.010± 0.001
C16:0	0.320± 0.060	0.308± 0.020	0.411± 0.046	0.270± 0.127	0.436± 0.024	0.261± 0.019*	0.414± 0.063	0.351± 0.044	0.748± 0.011	0.677± 0.040	1.204± 0.021	1.185± 0.041
C16:1 c9	0.017± 0.003	0.016± 0.001	0.019± 0.002	0.012± 0.005	0.019± 0.002	0.011± 0.001*	0.017± 0.003	0.015± 0.002	0.021± 0.001	0.020± 0.001	0.028± 0.001	0.028± 0.001
C17:0	0.004± 0.001	0.004± <0.001	0.006± 0.001	0.004± 0.002	0.006± <0.001	0.004± <0.001*	0.006± 0.001	0.006± 0.002	0.009± <0.001	0.008± 0.001	0.014± <0.001	0.013± <0.001
C18:0	0.058± 0.014	0.061± 0.007	0.135± 0.009	0.104± 0.033	0.165± 0.002	0.124± 0.007*	0.176± 0.016	0.165± 0.011	0.456± 0.007	0.428± 0.028	0.809± 0.015	0.812± 0.044
C18:1 c9	0.140± 0.030	0.130± 0.013	0.449± 0.019	0.326± 0.065	0.592± 0.009	0.448± 0.026*	0.709± 0.045	0.651± 0.026	2.090± 0.038	2.020± 0.132	3.691± 0.067	3.945± 0.217

Average values ± standard deviation (n=3). * Significant differences ($p<0.05$) from 0 h to 24 h.

FA – Fatty acid; Control – No substrate; S1LNA – 1 mg/mL α-linolenic acid (α-LNA); S1.5LNA – 1.5 mg/mL α-LNA; S2LNA – 2 mg/mL α-LNA; S5LNA – 5 mg/mL α-LNA; S10LNA – 10 mg/mL α-LNA; *c* – *cis* double bond.

Table 3.24 Free fatty acids profile of semi-skimmed milk cultured with *Bifidobacterium breve* DSM 20091 and different α-linolenic acid concentrations from hydrolyzed flaxseed oil at 0 h and 24 h of incubation (*continued*).

FA (mg/g)	Control		S1LNA		S1.5LNA		S2LNA		S5LNA		S10LNA	
	0 h	24 h	0 h	24 h	0 h	24 h	0 h	24 h	0 h	24 h	0 h	24 h
C18:2 c9c12 (LA)	0.026± 0.005	0.024± 0.001	0.257± 0.004	0.160± 0.025*	0.372± 0.004	0.274± 0.019*	0.474± 0.013	0.406± 0.022	1.422± 0.028	1.324± 0.093	2.557± 0.008	2.678± 0.171
C18:3 c9c12c15 (α-LNA)	0.005± 0.001	0.004± 0.001	0.803± 0.021	0.139± 0.072*	1.211± 0.014	0.339± 0.098*	1.565± 0.029	0.530± 0.053*	4.508± 0.093	3.566± 0.248*	8.145± 0.048	7.822± 0.593
C18:2 c9t11 (CLA)	0.003± <0.001	0.003± <0.001	0.005± 0.001	0.042± 0.013*	0.006± <0.001	0.045± 0.008*	0.006± 0.001	0.060± 0.006*	0.007± 0.001	0.036± 0.002*	0.011± 0.001	0.041± 0.001*
C18:2 CLA t,t	ND	ND	ND	0.013± 0.002	ND	0.015± 0.002	ND	0.017± 0.004	0.004± <0.001	0.013± 0.001*	0.006± <0.001	0.015± 0.001*
C18:3 c9t11c15 (CLNA)	ND	ND	ND	0.354± 0.053	ND	0.500± 0.063	ND	0.722± 0.072	ND	0.564± 0.041	ND	0.638± 0.015
C18:3 t9t11c15 (CLNA)	ND	ND	ND	0.083± 0.005	ND	0.112± 0.010	ND	0.154± 0.027	ND	0.135± 0.014	ND	0.130± 0.014
SFA	0.597± 0.111	0.557± 0.039	0.787± 0.080	0.517± 0.218	0.836± 0.049	0.510± 0.038*	0.794± 0.114	0.677± 0.082	1.375± 0.022	1.252± 0.071	2.193± 0.036	2.152± 0.071
MUFA	0.168± 0.035	0.154± 0.015	0.480± 0.022	0.345± 0.073	0.622± 0.012	0.466± 0.027*	0.735± 0.049	0.673± 0.029	2.119± 0.038	2.046± 0.132	3.726± 0.067	3.979± 0.216
PUFA	0.033± 0.004	0.031± 0.001	1.065± 0.025	0.792± 0.037*	1.589± 0.017	1.284± 0.075*	2.045± 0.043	1.889± 0.018*	5.942± 0.123	5.638± 0.399	10.719± 0.041	11.324± 0.761
Σ FA	0.798± 0.150	0.742± 0.055	2.332± 0.093	1.654± 0.328	3.047± 0.076	2.260± 0.140*	3.575± 0.204	3.240± 0.128	9.436± 0.173	8.936± 0.594	16.638± 0.066	17.456± 1.047

Average values ± standard deviation (n=3). * Significant differences ($p<0.05$) from 0 h to 24 h.

FA – Fatty acid; Control – No substrate; S1LNA – 1 mg/mL α-linolenic acid (α-LNA); S1.5LNA – 1.5 mg/mL α-LNA; S2LNA – 2 mg/mL α-LNA; S5LNA – 5 mg/mL α-LNA; S10LNA – 10 mg/mL α-LNA; *c* – *cis* double bond; *t* – *trans* double bond; LA – Linoleic acid; CLA – Conjugated linoleic acid; CLNA – Conjugated linolenic acid; SFA – Saturated fatty acids; MUFA – Monounsaturated fatty acids; PUFA – Polyunsaturated fatty acids; Σ FA – Total fatty acids; ND – not detected.

Table 3.25 Esterified fatty acids profile of semi-skimmed milk cultured with *Bifidobacterium breve* DSM 20091 and different α-linolenic acid concentrations from hydrolyzed flaxseed oil at 0 h and 24 h of incubation.

FA (mg/g)	Control		S1LNA		S1.5LNA		S2LNA		S5LNA		S10LNA	
	0 h	24 h	0 h	24 h	0 h	24 h	0 h	24 h	0 h	24 h	0 h	24 h
C4:0	0.241± 0.008	0.266± 0.075	0.263± 0.052	0.232± 0.043	0.262± 0.009	0.227± 0.036	0.286± 0.038	0.261± 0.019	0.196± 0.027	0.190± 0.019	0.181± 0.009	0.165± 0.010
C6:0	0.208± 0.004	0.214± 0.040	0.239± 0.039	0.219± 0.057	0.245± 0.002	0.203± 0.044	0.275± 0.029	0.241± 0.012	0.170± 0.026	0.161± 0.016	0.162± 0.014	0.142± 0.010
C8:0	0.130± 0.014	0.130± 0.034	0.154± 0.022	0.135± 0.017	0.163± 0.009	0.131± 0.011*	0.172± 0.015	0.157± 0.009	0.121± 0.026	0.114± 0.018	0.125± 0.014	0.106± 0.015
C10:0	0.317± 0.038	0.305± 0.086	0.332± 0.015	0.288± 0.018*	0.341± 0.023	0.282± 0.008	0.356± 0.020	0.313± 0.015	0.282± 0.050	0.269± 0.026	0.281± 0.018	0.245± 0.036
C12:0	0.404± 0.052	0.393± 0.116	0.408± 0.022	0.354± 0.021*	0.418± 0.029	0.345± 0.013	0.439± 0.026	0.380± 0.017	0.351± 0.066	0.340± 0.033	0.356± 0.023	0.309± 0.046
C14:0	1.292± 0.124	1.267± 0.373	1.275± 0.072	1.105± 0.070*	1.313± 0.093	1.081± 0.041	1.361± 0.083	1.190± 0.058	1.155± 0.214	1.123± 0.113	1.188± 0.076	1.028± 0.157
C14:1 c9	0.123± 0.013	0.121± 0.039	0.126± 0.007	0.111± 0.006*	0.129± 0.014	0.108± 0.003	0.135± 0.010	0.115± 0.005	0.122± 0.021	0.119± 0.012	0.131± 0.007	0.113± 0.019
C15:0	0.119± 0.016	0.118± 0.042	0.129± 0.014	0.113± 0.006	0.132± 0.010	0.110± 0.006	0.138± 0.010	0.118± 0.006	0.119± 0.022	0.116± 0.014	0.124± 0.009	0.108± 0.015
C16:0	4.604± 0.475	4.396± 1.344	4.456± 0.274	3.805± 0.220*	4.559± 0.281	3.762± 0.152	4.786± 0.299	4.155± 0.198	3.981± 0.732	3.870± 0.419	4.182± 0.278	3.643± 0.559
C16:1 c9	0.232± 0.027	0.222± 0.071	0.222± 0.011	0.190± 0.007*	0.230± 0.018	0.187± 0.006	0.236± 0.017	0.206± 0.008	0.229± 0.040	0.219± 0.023	0.246± 0.018	0.210± 0.033
C17:0	0.059± 0.006	0.054± 0.012	0.063± 0.002	0.059± 0.005	0.069± 0.006	0.058± 0.005	0.067± 0.005	0.058± 0.002	0.060± 0.010	0.055± 0.007	0.061± 0.005	0.051± 0.008
C18:0	0.949± 0.074	0.852± 0.222	0.979± 0.056	0.826± 0.035*	1.005± 0.088	0.816± 0.035	1.044± 0.056	0.902± 0.044	0.884± 0.162	0.855± 0.087	0.965± 0.079	0.833± 0.126
C18:1 c9	2.145± 0.196	1.947± 0.530	2.106± 0.123	1.773± 0.093*	2.201± 0.164	1.811± 0.095	2.359 ±0.159	2.021± 0.101	2.642± 0.490	2.527± 0.304	3.707± 0.223	3.166± 0.383

Average values ± standard deviation (n=3). * Significant differences ($p<0.05$) from 0 h to 24 h.

FA – Fatty acid; Control – No substrate; S1LNA – 1 mg/mL α-linolenic acid (α-LNA); S1.5LNA – 1.5 mg/mL α-LNA; S2LNA – 2 mg/mL α-LNA; S5LNA – 5 mg/mL α-LNA; S10LNA – 10 mg/mL α-LNA; *c* – *cis* double bond.

Table 3.26 Esterified fatty acids profile of semi-skimmed milk cultured with *Bifidobacterium breve* DSM 20091 and different α-linolenic acid concentrations from hydrolyzed flaxseed oil at 0 h and 24 h of incubation (*continued*).

FA (mg/g)	Control		S1LNA		S1.5LNA		S2LNA		S5LNA		S10LNA	
	0 h	24 h	0 h	24 h	0 h	24 h	0 h	24 h	0 h	24 h	0 h	24 h
C18:2 c9c12 (LA)	0.342± 0.033	0.327± 0.095	0.318± 0.022	0.275± 0.014*	0.336± 0.028	0.287± 0.024	0.357± 0.031	0.315± 0.016	0.373± 0.070	0.366± 0.040	0.507± 0.040	0.450± 0.041
C18:3 c9c12c15 (α-LNA)	0.056± 0.018	0.054± 0.032	0.100± 0.018	0.103± 0.009	0.135± 0.024	0.120± 0.018	0.166± 0.031	0.155± 0.021	0.362± 0.061	0.363± 0.055	0.753± 0.043	0.689± 0.064
C18:2 c9t11 (CLA)	0.048± 0.006	0.039± 0.012	0.047± 0.007	0.044± 0.004	0.051± 0.005	0.045± 0.007	0.059± 0.009	0.054± 0.008	0.084± 0.009	0.068± 0.010*	0.122± 0.005	0.092± 0.015
C18:2 CLA t,t	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	0.032± 0.006	0.040± 0.003
SFA	8.322± 0.803	7.995± 2.346	8.297± 0.564	7.135± 0.433*	8.506± 0.535	7.014± 0.285	8.926± 0.577	7.775± 0.373	7.318± 1.334	7.094± 0.747	7.627± 0.519	6.630± 0.982
MUFA	2.500± 0.237	2.290± 0.639	2.454± 0.141	2.074± 0.105*	2.560± 0.196	2.106± 0.104	2.731± 0.185	2.343± 0.114	2.993± 0.551	2.865± 0.338	4.083± 0.247	3.488± 0.433
PUFA	0.446± 0.058	0.420± 0.139	0.465± 0.047	0.422± 0.026	0.522± 0.055	0.453± 0.049	0.582± 0.069	0.525± 0.041	0.820± 0.137	0.796± 0.104	1.413± 0.088	1.272± 0.115
Σ FA	11.269± 1.097	10.705± 3.124	11.216± 0.752	9.631± 0.546*	11.588± 0.781	9.573± 0.431	12.239± 0.821	10.642± 0.528	11.131± 2.013	10.755± 1.187	13.123± 0.847	11.390± 1.520

Average values ± standard deviation (n=3). * Significant differences ($p<0.05$) from 0 h to 24 h.

FA – Fatty acid; Control – No substrate; S1LNA – 1 mg/mL α-linolenic acid (α-LNA); S1.5LNA – 1.5 mg/mL α-LNA; S2LNA – 2 mg/mL α-LNA; S5LNA – 5 mg/mL α-LNA; S10LNA – 10 mg/mL α-LNA ; *c* – *cis* double bond; *t* – *trans* double bond; LA – Linoleic acid; CLA – Conjugated linoleic acid; SFA – Saturated fatty acids; MUFA – Monounsaturated fatty acids; PUFA – Polyunsaturated fatty acids; Σ FA – Total fatty acids; ND – not detected.

Table 3.27 Fatty acid profile of pasteurized semi-skimmed cows' milk.

FA (mg/g)	FFA	EFA
C4:0	0.020 ± 0.002	0.287 ± 0.023
C6:0	0.018 ± 0.002	0.258 ± 0.017
C8:0	0.015 ± 0.002	0.170 ± 0.012
C10:0	0.035 ± 0.006	0.336 ± 0.035
C12:0	0.043 ± 0.006	0.412 ± 0.046
C14:0	0.124 ± 0.021	1.291 ± 0.136
C14:1 c9	0.013 ± 0.002	0.128 ± 0.012
C15:0	0.012 ± 0.002	0.129 ± 0.015
C16:0	0.391 ± 0.074	4.552 ± 0.427
C16:1 c9	0.020 ± 0.003	0.221 ± 0.026
C17:0	0.006 ± 0.001	0.065 ± 0.011
C18:0	0.080 ± 0.017	1.003 ± 0.094
C18:1 c9	0.166 ± 0.026	2.039 ± 0.231
C18:2 c9c12 (LA)	0.029 ± 0.003	0.305 ± 0.034
C18:3 c9c12c15 (α-LNA)	0.007 ± 0.002	0.069 ± 0.007
C18:2 c9t11 (CLA)	0.004 ± 0.001	0.048 ± 0.001
SFA	0.743 ± 0.132	8.503 ± 0.768
MUFA	0.199 ± 0.031	2.388 ± 0.269
PUFA	0.040 ± 0.006	0.423 ± 0.036
Σ FA	0.981 ± 0.168	11.314 ± 1.070

Average values ± standard deviation (n=3).

FA – Fatty acid; FFA – Free fatty acids; EFA – Esterified fatty acids; *c* – *cis* double bond; *t* – *trans* double bond; LA – Linoleic acid; α-LNA – alpha-Linolenic acid; CLA – Conjugated linoleic acid; SFA – Saturated fatty acids; MUFA – Monounsaturated fatty acids; PUFA – Polyunsaturated fatty acids; Σ FA – Total fatty acids.

3.2.5. UV spectra of conjugated isomers

To confirm the structural nature of the conjugated isomers detected by GC-FID in the microbially CLNA-enriched milk, it was performed a UV scanning test. Before fermentation with hydrolyzed FSO at 2 mg/mL α-LNA (S2LNA), milk FFA revealed a maximum absorbance at 210 nm (**Figure 3.22**). After fermentation, two maximums of absorbance at 211 and 233 nm were observed (**Figure 3.22**). As for Tonalin CLA, the highest absorption peak was detected at 233 nm, while for PSO three peaks of absorption - 264, 274 and 285 nm - were observed (**Figure 3.22**).

Maximum absorbance at 232-235 nm is specific of conjugated dienes, independent of the total number of double bonds and chain length, as shown by Coakley et al. (2009) in their works regarding the microbial production of C18:3 c9t11c15 and C18:3 t9t11c15 isomers. Moreover, Czauderna et al. (2011) reported that *ct*/*tc* conjugated dienes are characterized by a maximum absorbance at 234 nm, and *ct* isomers were the major ones detected after fermentation (**Table 3.24**). Therefore, according to the obtained results, the CLNA isomers detected in the enriched fermented milk correspond to conjugated dienes.

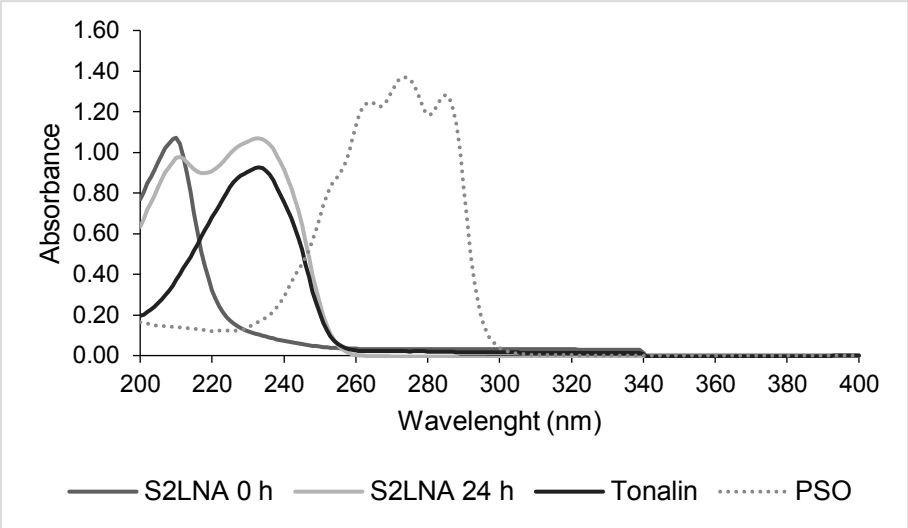


Figure 3.22 UV spectra of fatty acid methyl esters extracts from milk free fatty acids at 0 h and 24 h of fermentation with *Bifidobacterium breve* DSM 20091 and hydrolyzed flaxseed oil at 2 mg/mL of α-linolenic acid (S2LNA), and from esterified fatty acids of Tonalin CLA and pomegranate seed oil (PSO).

Part III - Evaluation of fatty acid metabolites in microbial CLA/CLNA-enrichment of milk

In Part II it was possible to obtain a fermented milk with up to 1 mg/g of CLNA isomers by *B. breve* DSM 20091, using FSO (previously hydrolyzed with CRL lipase) as α-LNA source. However, discrepancies between substrate reduction and conversion percentages still occurred, as observed from results of Part I when assaying in culture medium. Therefore, the aim of Part III was to study possible FA side-metabolites during LA/α-LNA bioconversion. To do so, the profile of milk fermented by *B. breve* DSM 20091 with pure LA and/or α-LNA or hydrolyzed FSO was analyzed by GC-MS.

3.3.1. Bacterial growth and pH

To confirm that *B. breve* DSM 20091 growth was not affected by any tested condition, which might impair milk enrichment with CLA/CLNA isomers, viable cell numbers and pH values were determined. Concerning viable cell numbers, there was a considerable increase after 24 h, with no differences between substrates (from 7.42-7.54 to 9.48-9.58 log CFU/mL; **Figure 3.23**). Accordingly, milk pH value decreased considerably after fermentation (from pH 6.37-6.50 to pH 4.19-4.39) (**Figure 3.24**), and no differences were detected between substrates. This is in accordance with previous results, where the growth of this strain was not affected by pure precursor substrates at 0.5 mg/mL in semi-skimmed milk (Fontes et al., 2018) and could tolerate higher concentrations when these were delivered via vegetable oil (**Section 3.2.4 Microbially CLNA-enriched milk**; in Results and Discussion of Part II). Moreover, viable cell numbers of *L. casei* 1.574 in milk fermented at increasing LA concentrations tended to be higher than control (no substrate), even though differences were not >1 log CFU/mL (Qing et al., 2023). Dairy matrices effectively exhibit a protective effect on probiotic bacteria viability (de Almeida et al., 2018; Meira et al., 2015).

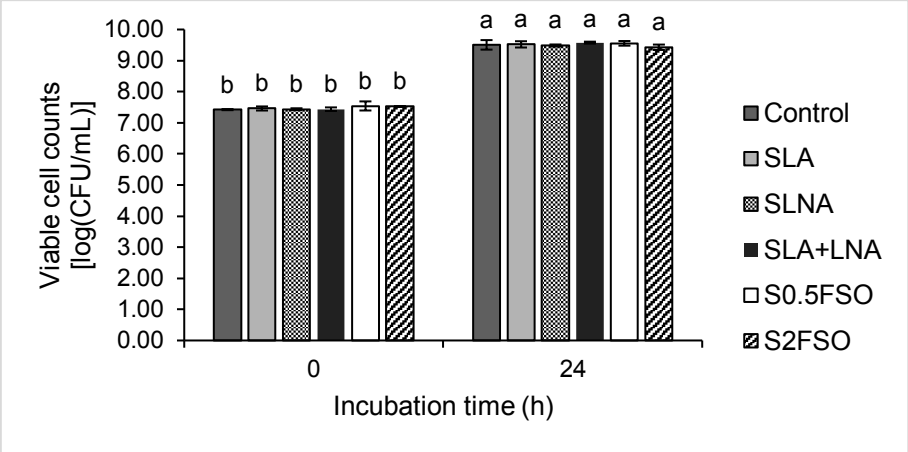


Figure 3.23 Viable cell counts of *Bifidobacterium breve* DSM 20091 grown in pasteurized semi-skimmed milk at different substrate conditions: Control – no substrate; SLA – 0.5 mg/mL linoleic acid (LA); SLNA – 0.5 mg/mL α-linolenic acid (α-LNA); SLA+LNA – 0.15 mg/mL LA + 0.5 mg/mL α-LNA; S0.5FSO – 0.5 mg/mL α-LNA from hydrolyzed flaxseed oil (FSO); S2FSO – 2 mg/mL α-LNA from hydrolyzed FSO. Different superscript letters indicate ≥1 log cycle difference between incubation times.

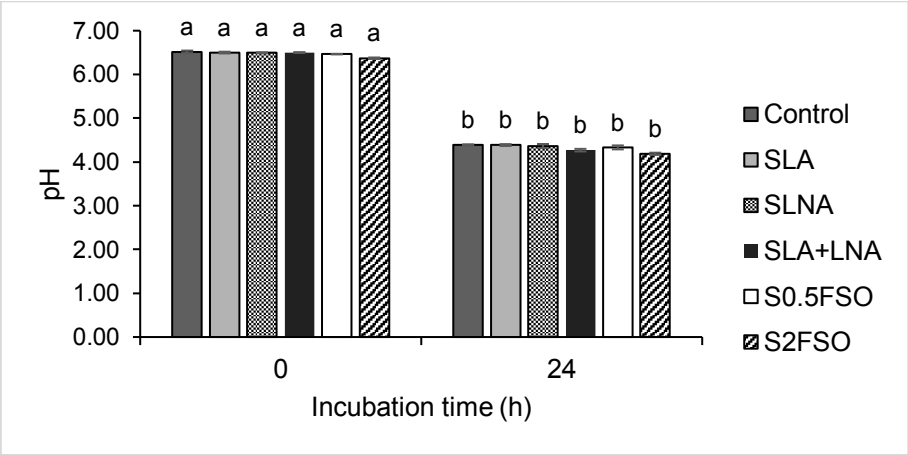


Figure 3.24 pH of pasteurized semi-skimmed milk inoculated with *Bifidobacterium breve* DSM 20091 at different substrate conditions: Control – no substrate; SLA – 0.5 mg/mL linoleic acid (LA); SLNA – 0.5 mg/mL α-linolenic acid (α-LNA); SLA+LNA – 0.15 mg/mL LA + 0.5 mg/mL α-LNA; S0.5FSO – 0.5 mg/mL α-LNA from hydrolyzed flaxseed oil (FSO); S2FSO – 2 mg/mL α-LNA from hydrolyzed FSO. Different superscript letters indicate ≥0.5 units difference between incubation times.

3.3.2. Fatty acid byproducts

Regarding milk FA composition, some alterations were found in the FFA profile, beyond the expected reduction of CLA/CLNA precursor substrates (i.e., LA/α-LNA) (**Table 3.28** and **Table 3.29**). The obtained results pinpoint a decrease in myristic acid (C14:0) proportion when using pure α-LNA, alone (SLNA; from 7.24% to 5.70%, $p<0.05$) or in combination with LA (SLA+LNA; from 6.24% to

4.80%, $p<0.05$) (**Table 3.28**), as in control (from 12.66 to 10.83%), even though not statistically different ($p=0.074$). Moreover, although not statistically significant ($p>0.05$), the stearic acid (C18:0) percentage increased with pure LA (SLA; from 4.52% to 7.30%) and α-LNA (SLNA; from 4.59% to 6.01%) (**Table 3.28**), similarly to control, where it increased significantly in ca. 30% (from 8.55% to 11.02%, $p<0.05$), while changes were less noticeable when applying both substrates (SLA+SLNA, S0.5FSO or S2FSO). Stearic acid increase in control and SLNA (i.e., using pure α-LNA alone) could be related to myristic acid decrease, which might have been elongated into palmitic acid (C16:0) and this further elongated into stearic acid. In SLA (i.e., using pure LA alone), part of the LA could have been metabolized into stearic acid, as this FA is suggested as the final product of LA metabolization pathway (Kishino et al., 2013). These results show that *B. breve* DSM 20091 enzymatic behavior differs depending on the type of substrate that it is exposed to. As for EFA composition, no considerable differences were observed after fermentation at the different substrate conditions (**Table 3.30** and **Table 3.31**).

No further compounds were found in the milk FA profile that could have derived from LA or α-LNA metabolization, like oxo or hydroxy FA. This suggests that for *B. breve* DSM 20091 i) biotransformation of LA and α-LNA into CLA and CLNA might occur in one single-step or ii) intermediates biotransformation is very fast. Moreover, the lack of alterations in the EFA fraction shows that this strain has no capacity to transform the free substrates into the complex triglycerides or phospholipids, for instance. In fact, produced metabolites were only detected in the FFA fraction, being observed interesting results on yielded conjugated FA isomers (**Table 3.29**).

Table 3.28 Free fatty acid composition of pasteurized semi-skimmed milk before (0 h) and after (24 h) fermentation with *Bifidobacterium breve* DSM 20091 at different substrate conditions.

FA (%)	Control		SLA		SLNA		SLA+LNA		S0.5FSO		S2FSO	
	0 h	24 h	0 h	24 h	0 h	24 h	0 h	24 h	0 h	24 h	0 h	24 h
C8:0	0.71±0.04	0.68±0.08	0.36±<0.01	0.31±0.02	0.41±0.03	0.38±0.01	0.35±0.02	0.30±0.04	0.28±0.01	0.25±0.02	0.08±0.01	0.08±0.01
C10:0	2.58±0.15	2.41±0.27	1.33±0.06	1.14±0.06*	1.52±0.15	1.33±0.08	1.25±0.10	1.05±0.15	0.97±0.01	0.88±0.05	0.30±0.03	0.28±0.02
C12:0	3.93±0.18	3.26±0.29	2.04±0.12	1.64±0.08	2.31±0.17	1.78±0.11	1.93±0.09	1.34±0.25	1.45±0.06	1.24±0.07	0.46±0.05	0.42±0.01
C13:0	0.19±<0.01	0.16±0.02	0.10±<0.01	0.08±<0.01	0.10±<0.01	0.09±<0.01	0.09±<0.01	0.08±0.01	0.07±<0.01	0.06±<0.01	0.02±<0.01	0.02±<0.01
C14:0	12.66±0.29	10.83±0.62	6.64±0.53	5.90±0.03	7.24±0.40	5.70±0.36*	6.24±0.15	4.80±0.17*	4.49±0.37	3.82±0.06	1.47±0.12	1.32±0.02
C14:1 c9	1.13±0.04	0.92±0.07	0.58±0.05	0.48±0.03	0.63±0.05	0.49±0.03	0.54±0.01	0.37±0.07	0.39±0.03	0.36±0.02	0.13±0.01	0.12±0.01
iC15	0.19±0.01	0.16±0.02	0.10±0.01	0.10±0.01	0.11±<0.01	0.09±<0.01*	0.09±0.01	0.06±0.02	ND	ND	0.02±<0.01	ND
aiC15	0.36±0.02	0.32±0.02*	0.19±0.02	0.18±<0.01	0.21±0.01	0.16±0.02*	0.18±0.01	0.13±0.03	0.12±0.01	0.11±0.01	0.04±<0.01	0.03±<0.01
C15:0	1.11±0.02	0.99±0.01*	0.58±0.06	0.61±<0.01	0.62±0.03	0.52±0.04	0.54±0.02	0.39±0.08	0.39±0.04	0.36±0.01	0.14±0.01	0.13±<0.01
iC16	0.19±<0.01	0.21±0.01	0.10±0.02	0.12±<0.01	0.11±0.01	0.13±0.01	0.10±<0.01	0.09±0.01	0.09±0.01	0.08±<0.01	0.03±<0.01	0.03±<0.01
C16:0	39.86±0.20	40.45±0.16	21.04±1.90	24.63±0.55	22.13±0.34	21.65±0.92	19.54±0.56	18.14±0.35	16.98±1.13	16.71±0.82	9.20±0.36	8.92±<0.01
C16:1 c7	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	0.03±<0.01	0.03±<0.01
C16:1 c9	2.12±0.03	1.90±0.06*	1.12±0.12	1.12±0.01	1.19±0.06	1.03±0.06*	1.04±0.04	0.82±0.12	0.78±0.08	0.78±0.02	0.32±0.02	0.32±0.01
aiC17	0.30±0.02	0.34±0.02	0.16±0.03	0.23±<0.01	0.16±0.01	0.18±0.01	0.15±0.02	0.12±0.02*	0.10±0.02	0.11±0.01	0.03±0.01	0.03±<0.01
C17:0	0.35±0.03	0.42±0.03	0.19±0.02	0.30±0.01	0.19±<0.01	0.23±0.02	0.17±0.01	0.16±0.03	0.14±0.02	0.16±0.03	0.08±0.01	0.07±<0.01
C17:1 c9	ND	ND	ND	ND	ND	ND	ND	ND	ND	0.06±<0.01	0.04±<0.01	0.04±0.01
C18:0	8.55±0.14	11.02±0.64*	4.52±0.50	7.30±0.15	4.59±0.09	6.01±0.21	4.08±0.22	4.51±0.52	5.59±0.29	6.35±0.45	5.03±0.05	4.97±0.10
C18:1 c9	21.32±0.35	21.48±0.64	12.07±1.00	14.82±0.41	12.35±0.09	12.93±0.55	11.02±0.51	11.04±0.07	22.56±0.05	22.64±0.17	23.13±0.02	22.59±0.18
C18:1 t11	1.67±0.17	1.84±0.17	0.88±0.12	1.42±0.02	0.95±0.01	1.03±0.10	0.86±0.07	0.92±0.14	1.21±0.08	1.33±0.06	1.13±0.02	1.12±<0.01

Average values ± standard deviation (n=3). * Significant differences ($p<0.05$) from 0 h to 24 h.

FA – Fatty acid; Control – no substrate; SLA – 0.5 mg/mL linoleic acid (LA); SLNA – 0.5 mg/mL α-linolenic acid (α-LNA); SLA+LNA – 0.15 mg/mL LA + 0.5 mg/mL α-LNA; S0.5FSO – 0.5 mg/mL α-LNA from hydrolyzed flaxseed oil (FSO); S2FSO – 2 mg/mL α-LNA from hydrolyzed FSO; i – iso; ai – anteiso; *c* – *cis* double bond; *t* – *trans* double bond; ND – not detected.

Table 3.29 Free fatty acid composition of pasteurized semi-skimmed milk before (0 h) and after (24 h) fermentation with *Bifidobacterium breve* DSM 20091 at different substrate conditions (*continued*).

FA (%)	Control		SLA		SLNA		SLA+LNA		S0.5FSO		S2FSO	
	0 h	24 h	0 h	24 h	0 h	24 h	0 h	24 h	0 h	24 h	0 h	24 h
C18:2 c9c12 (LA)	2.78±0.08	2.59±0.04	47.76±4.60	26.32±1.02*	1.82±0.07	1.66±0.07*	13.99±0.38	11.98±0.92	11.48±0.33	8.20±0.31*	14.77±0.16	13.21±0.21*
C18:2 c,c	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	0.09±<0.01	0.08±<0.01
C18:3 c9c12c15 (α-LNA)	ND	ND	0.11±0.01	0.09±<0.01	43.37±1.16	18.08±0.97*	37.86±1.47	13.69±1.10*	32.92±1.75	8.51±0.99*	43.34±0.55	25.69±1.86*
C18:2 c9t11 (CLA)	ND	ND	0.14±0.01	8.34±0.18*	ND	ND	ND	2.28±0.30	ND	2.22±0.03	ND	1.01±0.15
C18:2 t10c12 (CLA)	ND	ND	ND	0.32±0.02	ND	ND	ND	ND	ND	ND	ND	ND
C18:2 CLA c,c	ND	ND	ND	0.41±0.01	ND	ND	ND	ND	ND	ND	ND	ND
C20:0	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	0.11±<0.01	0.10±0.01
C18:3 c9t11c15 (CLNA)	ND	ND	ND	ND	ND	17.64±0.08	ND	21.39±2.79	ND	16.74±0.29	ND	14.88±1.55
C18:2 CLA t,t	ND	ND	ND	4.17±0.03	ND	ND	ND	0.89±0.09	ND	1.00±0.03	ND	ND
C18:3 CLNA (Unk1)	ND	ND	ND	ND	ND	0.44±0.01	ND	0.45±0.02	ND	0.56±0.03	ND	0.33±0.02
C18:3 CLNA (Unk2)	ND	ND	ND	ND	ND	0.78±0.02	ND	0.89±0.11	ND	0.83±0.01	ND	0.56±0.05
C18:3 t9t11c15 (CLNA)	ND	ND	ND	ND	ND	6.39±0.09	ND	6.98±0.65	ND	6.63±0.23	ND	3.64±0.27
SFA	70.98±0.54	71.26±0.70	37.34±3.28	42.52±0.51	39.70±1.06	38.24±1.76	34.69±1.22	31.18±1.68	30.67±1.94	30.13±1.11	17.01±0.65	16.40±0.18
MUFA	26.25±0.45	26.15±0.68	14.65±1.29	17.84±0.39	15.11±0.03	15.49±0.75	13.46±0.63	13.15±0.26	24.93±0.14	25.17±0.24	24.78±0.07	24.21±0.15
PUFA	2.78±0.08	2.59±0.04	48.01±4.57	39.64±0.90	45.19±1.09	44.99±0.69	51.85±1.85	58.53±5.98	44.40±2.08	44.69±1.35	58.21±0.72	59.40±0.03

Average values ± standard deviation (n=3). * Significant differences ($p<0.05$) from 0 h to 24 h.

FA – Fatty acid; Control – no substrate; SLA – 0.5 mg/mL linoleic acid (LA); SLNA – 0.5 mg/mL α-linolenic acid (α-LNA); SLA+LNA – 0.15 mg/mL LA + 0.5 mg/mL α-LNA; S0.5FSO – 0.5 mg/mL α-LNA from hydrolyzed flaxseed oil (FSO); S2FSO – 2 mg/mL α-LNA from hydrolyzed FSO; *c* – *cis* double bond; *t* – *trans* double bond; CLA – Conjugated linoleic acid; CLNA – Conjugated linolenic acid; Unk – Unknown; SFA – Saturated fatty acids; MUFA – Monounsaturated fatty acids; PUFA – Polyunsaturated fatty acids; ND – not detected.

Table 3.30 Esterified fatty acid composition of pasteurized semi-skimmed milk before (0 h) and after (24 h) fermentation with *Bifidobacterium breve* DSM 20091 at different substrate conditions.

FA (%)	Control		SLA		SLNA		SLA+LNA		S0.5FSO		S2FSO	
	0 h	24 h	0 h	24 h	0 h	24 h	0 h	24 h	0 h	24 h	0 h	24 h
C8:0	ND	ND	0.02±0.01	ND	ND	0.04±0.01	ND	0.04±<0.01	ND	0.04±0.01	0.08±0.01	0.06±0.01*
C10:0	0.11±0.01	0.22±0.02*	0.73±0.18	0.13±0.09	0.16±0.04	0.38±0.12	0.33±0.10	0.25±0.11*	0.04±0.01	0.33±0.10	1.00±0.06	0.40±0.13
C12:0	1.67±0.10	1.90±0.03	2.49±0.15	2.47±0.17	2.27±0.48	2.21±0.36	2.06±0.19	1.78±0.24	1.23±0.44	1.92±0.43	2.79±0.03	2.50±0.34
C13:0	0.09±0.01	0.09±<0.01	0.11±0.01	0.08±0.02	0.10±0.01	0.10±0.01	0.09±<0.01	0.10±<0.01	0.10±0.02	0.09±0.02	0.12±0.01	0.11±0.01
iC14	0.06±<0.01	0.06±0.01	0.06±0.01	0.06±0.01	0.07±<0.01	0.06±<0.01	0.06±<0.01	0.07±<0.01	0.07±0.01	0.06±0.01	0.07±<0.01	0.07±<0.01
C14:0	10.28±0.56	10.29±0.12	10.71±0.28	10.06±1.37	10.60±0.21	10.53±0.33	10.41±0.18	10.50±0.07	10.69±0.45	10.35±0.57	10.94±0.05	10.65±0.01
C14:1 c9	0.89±0.07	0.91±0.01	0.96±0.02	0.84±0.16	0.96±0.04	0.94±0.02	0.91±0.04	0.95±<0.01	0.97±0.09	0.89±0.10*	1.01±0.01	0.98±0.01
iC15	0.23±<0.01	0.25±<0.01*	0.23±0.02	0.22±0.03	0.25±0.01	0.25±<0.01	0.20±0.01	0.23±0.01	0.23±0.02	0.22±0.02	0.23±<0.01	0.23±<0.01
aiC15	0.39±0.03	0.40±<0.01	0.39±0.01	0.36±0.05	0.41±<0.01	0.41±<0.01	0.38±0.01	0.41±0.01	0.41±0.02	0.39±0.04	0.41±<0.01	0.40±0.02
C15:0	1.18±0.07	1.18±0.01	1.17±0.02	1.08±0.12	1.20±<0.01	1.20±0.01	1.14±0.04	1.25±0.03	1.23±0.04	1.16±0.11	1.20±0.01	1.20±0.05
iC16	0.23±0.01	0.24±0.01	0.22±0.02	0.24±0.01	0.23±0.01	0.23±<0.01	0.22±<0.01	0.24±0.01	0.24±0.01	0.23±<0.01	0.24±<0.01	0.23±0.01
C16:0	38.62±0.05	37.82±0.12	37.48±0.46	38.84±2.09	37.43±0.70	37.37±0.28	38.72±0.83	37.88±0.07	37.80±0.98	38.18±0.45	36.86±0.10	36.45±0.67
C16:1 c7	0.17±0.02	0.18±0.01	0.17±0.02	0.16±0.02	0.18±<0.01	0.18±0.01	0.16±0.01	0.18±<0.01	0.18±0.01	0.16±0.02	0.18±0.01	0.18±0.01
C16:1 c9	2.13±0.09	2.13±0.04	2.08±0.02	2.00±0.12	2.15±0.04	2.14±0.02	2.08±0.03	2.19±0.05	2.18±0.02	1.95±0.04	2.12±<0.01	2.10±0.10
iC17	0.37±0.03	0.34±0.03*	0.32±0.07	0.30±0.03	0.32±<0.01	0.34±0.04	0.30±0.01	0.33±0.01	0.30±0.02	0.33±0.04	0.31±0.04	0.32±<0.01
aiC17	0.46±<0.01	0.47±0.01	0.45±0.02	0.44±<0.01	0.48±<0.01	0.49±0.01	0.41±0.02	0.48±0.02	0.46±0.02	0.43±0.06	0.45±0.01	0.45±0.03
C17:0	0.51±0.01	0.55±<0.01	0.50±0.06	0.50±0.01	0.53±0.05	0.55±0.01	0.52±0.02	0.58±0.02	0.57±0.02	0.55±0.04	0.54±<0.01	0.55±0.04
C17:1 c9	0.20±0.01	0.23±0.01	0.21±0.02	0.18±0.04	0.22±0.02	0.24±<0.01	0.19±0.01	0.26±0.01	0.24±0.02	0.22±0.03	0.22±<0.01	0.23±0.02
C18:0	12.83±0.07	12.74±0.02	12.36±0.11	12.79±0.72	11.89±0.08	12.05±0.30	13.16±0.23	13.13±0.19	12.78±0.69	15.74±0.08	11.54±0.28	11.68±0.16

Average values ± standard deviation (n=3). * Significant differences ($p<0.05$) from 0 h to 24 h.

FA – Fatty acid; Control – no substrate; SLA – 0.5 mg/mL linoleic acid (LA); SLNA – 0.5 mg/mL α-linolenic acid (α-LNA); SLA+LNA – 0.15 mg/mL LA + 0.5 mg/mL α-LNA; S0.5FSO – 0.5 mg/mL α-LNA from hydrolyzed flaxseed oil (FSO); S2FSO – 2 mg/mL α-LNA from hydrolyzed FSO; i – iso; ai – anteiso; *c* – *cis* double bond; ND – not detected.

Table 3.31 Esterified fatty acid composition of pasteurized semi-skimmed milk before (0 h) and after (24 h) fermentation with *Bifidobacterium breve* DSM 20091 at different substrate conditions (*continued*).

FA (%)	Control		SLA		SLNA		SLA+LNA		S0.5FSO		S2FSO	
	0 h	24 h	0 h	24 h	0 h	24 h	0 h	24 h	0 h	24 h	0 h	24 h
C18:1 c9	23.35±0.67	23.34±0.05	23.05±0.37	23.82±1.34	23.50±0.28	23.59±0.44	22.94±0.11	22.99±0.34	22.85±0.39	22.45±0.49	22.97±0.11	22.91±1.04
C18:1 t11	2.21±0.06	2.27±0.03	2.21±0.03	2.13±0.06	2.33±0.15	2.27±0.15	2.27±0.02	2.30±0.05	2.18±0.05	2.11±0.17	2.18±0.01	2.19±0.08
C18:1 c12/13	0.15±<0.01	0.15±0.01	0.14±<0.01	0.12±0.02	0.15±<0.01	0.15±0.01	0.14±0.01	0.15±0.01	0.15±0.01	0.15±0.03	0.15±<0.01	0.15±0.02
C18:2 c9c12 (LA)	3.11±0.37	3.46±0.12	3.25±0.25	3.03±0.41	3.44±0.04	3.53±0.11	2.69±0.11	2.91±0.10	2.88±0.09	2.71±0.25	2.94±0.04	2.94±0.19
C18:3 c9c12c15 (α-LNA)	0.23±0.01	0.24±0.01	0.21±0.02	0.19±0.04	0.25±<0.01	0.25±0.01	0.20±0.03	0.25±0.01	0.40±0.01	0.36±0.05	0.89±0.01	0.98±0.12
C18:2 c9t11 (CLA)	0.30±0.03	0.29±0.01	0.30±0.01	0.22±0.04	0.32±<0.01	0.29±0.01	0.27±0.02	0.31±0.01	0.34±0.03	0.28±0.06	0.35±0.01	0.33±0.03
C20:0	0.10±0.03	0.11±<0.01	0.08±<0.01	0.10±<0.01	0.08±<0.01	0.11±0.03	0.16±0.12	0.10±0.01	0.13±0.04	0.16±0.03	0.10±<0.01	0.11±0.02
C20:4 c5c8c11c14	0.11±0.03	0.14±0.03*	0.10±0.02	ND	0.10±<0.01	0.11±0.01	0.08±0.01	0.13±<0.01	0.12±0.02	0.11±0.04	0.11±0.01	0.12±0.01
SFA	67.14±0.72	66.65±0.01	67.32±0.74	67.67±0.92	66.04±0.02	66.31±0.75	68.17±0.60	67.37±0.59	66.29±1.59	70.18±1.03	66.88±0.16	65.42±0.45
MUFA	29.10±0.42	29.21±0.11	28.83±0.43	29.25±1.03	29.49±0.46	29.50±0.60	28.68±0.23	29.03±0.47	28.75±0.28	27.93±0.79	28.83±0.11	28.74±1.28
PUFA	3.75±0.30	4.14±0.10	3.86±0.30	3.43±0.49	4.10±0.04	4.19±0.15	3.25±0.17	3.60±0.12	3.75±0.15	3.45±0.40	4.28±0.05	4.37±0.35

Average values ± standard deviation (n=3). * Significant differences ($p<0.05$) from 0 h to 24 h.

FA – Fatty acid; Control – no substrate; SLA – 0.5 mg/mL linoleic acid (LA); SLNA – 0.5 mg/mL α-linolenic acid (α-LNA); SLA+LNA – 0.15 mg/mL LA + 0.5 mg/mL α-LNA; S0.5FSO – 0.5 mg/mL α-LNA from hydrolyzed flaxseed oil (FSO); S2FSO – 2 mg/mL α-LNA from hydrolyzed FSO; *c* – *cis* double boud; *t* – *trans* double bond; CLA – Conjugated linoleic acid; SFA – Saturated fatty acids; MUFA – Monounsaturated fatty acids; PUFA – Polyunsaturated fatty acids; ND – not detected.

3.3.3. Conjugated fatty acid isomers

From pure LA (SLA), *B. breve* DSM 20091 was able to produce four different CLA isomers, namely C18:2 c9t11 and C18:2 t10c12. The other two isomers were identified as C18:2 CLA c,c and C18:2 CLA t,t (**Figure 3.25** and **Figure 3.26**), based on previous results by GC-FID (Fontes et al., 2018). In fact, these FA shared typical fragmentation pattern of conjugated dienes, with fragments at *m*/*z* 67, 81, 95, 109 and 123 (Christie & Han, 2010). According to Christie and Han (2010), the only distinction from their precursor substrate (LA) spectrum is the higher intensity of molecular ion signal (i.e., 294 *m*/*z*), which was indeed verified (**Figure 3.26**).

Other research works have reported the production of C18:2 t9t11 by different *B. breve* strains (Raimondi et al., 2016), including the one studied herein (Hennessy et al., 2012), thus, the *trans*/*trans*-CLA isomer detected could be C18:2 t9t11. It was shown that different probiotic strains (e.g., *L. casei*, *L. sakei, Lc. lactis* or *B. animalis* subsp. *lactis*) can convert LA mainly into C18:2 c9t11, C18:2 t10c12 and/or C18:2 t9t11 CLA isomers (Terán et al., 2015). Despite not being very common for probiotic strains to produce *cis*/*cis*-CLA isomers, the ruminal bacteria *B. fibrisolvens*, which possesses a C12 LAI (catalyzes LA transformation into C18:2 c9t11) similarly to probiotic species (Kepler & Tove, 1967; Luo et al., 2013), can further produce the isomer C18:2 c9c11 (Wallace et al., 2007). Moreover, *L. plantarum* strains have shown the capacity to produce C18:2 c9c11 isomer, in addition to the ones mentioned above (Ribeiro et al., 2018). Therefore, the *cis*/*cis*-CLA isomer produced by *B. breve* DSM 20091 could be C18:2 c9c11. Recently, a C12-like LAI has been described and heterologously expressed from different *Bifidobacterium* species, including *B. breve* (Mei et al., 2023). Thus, it makes sense that the assayed strain produces the above-mentioned set of CLA isomers like other probiotic strains with similar LAI, as stated previously.

Fermentation with pure α-LNA (SLNA), revealed the production not only of the two isomers previously reported for this strain by GC-FID (Fontes et al., 2018), namely C18:3 c9t11c15 and C18:3 t9t11c15, but also two other compounds with a fragmentation pattern and molecular ion (*m*/*z* 292) similar to the above-mentioned ones (**Figure 3.25** and **Figure 3.27**), which most probably are other CLNA isomers. In fact, these compounds shared typical fragmentation spectra of trienoic FA with conjugated dienes, with fragments at *m*/*z* 67, 81, 95, 107 and 121 (Christie & Han, 2010). Moreover, fragmentation spectra match with RLA (C18:3 c9t11c15), for instance, was further confirmed by CFM-ID spectra prediction tool (Wang et al., 2021) for both presumed CLNA isomers, with scores of 0.39 (CLNA Unk1; **Figure 3.28**) and 0.37 (CLNA Unk2; **Figure 3.29**), where match score of detected RLA spectrum with predicted one was 0.56 (**Figure 3.30**). Yang et al. (2020) observed that *L. plantarum* ZS2058 could produce a third CLNA isomer, although at lower concentrations than C18:3 c9t11c15 and C18:3 t9t11c15. Nonetheless, the mass fragments formed were mostly different from these former CLNA isomers. In the current work, unknown CLNA isomers mass spectra revealed few different fragment ions at *m*/*z* 73, 79, 109, 207 and/or 263 *m*/*z*, compared to C18:3 c9t11c15 and C18:3 t9t11c15 (**Figure 3.27**).

Considering the proposed CLA isomers configuration and elution order (C18:2 c9t11, C18:2 t10c12, C18:2 c9c11 and C18:3 t9t11), it is suggested that CLNA isomers might consist of C18:3 c9t11c15, C18:3 t10c12c15 (Unk1), C18:3 c9c11c15 (Unk2) and C18:3 t9t11c15. In fact, CLNA Unk2

shared a particular feature of all-*cis* CLA isomer, i.e., a lower intensity at ion 81 *m*/*z*, close to 79 *m*/*z* (**Figure 3.26** and **Figure 3.27**). However, further analyses have to be performed in the future to confirm the structural configuration of these unknown CLNA isomers, like DMOX derivatization, since such information is not possible to retrieve from mass spectra alone (Christie & Han, 2010). Despite this, to the best of our knowledge, this is the first work to report the production of other CLNA isomers, besides C18:3 c9t11c15 and C18:3 t9t11c15, by a *Bifidobacterium* strain.

In the presence of both precursor substrates, either in pure form (SLA+LNA) or from hydrolyzed FSO (S0.5FSO), among the reported CLA isomers, only C18:2 c9t11 and C18:2 CLA t,t were detected, while all four above-mentioned CLNA isomers were observed (**Figure 3.25**). This seems to support the previously discussed fact (**Section 3.2.2 Microbially CLA/CLNA-enriched milk**; in Results and Discussion of Part II), where *B. breve* DSM 20091 might have a preference to convert α-LNA than LA, either because of a toxicity matter or LAI enzyme specificity (Fontes et al., 2018). Even though the initial proportion of LA was lower than α-LNA, these results also suggest that *B. breve* DSM 20091 enzymatic activity is affected by the type of substrate, expressing probably a higher specificity for α-LNA. At higher substrate concentration (S2FSO) C18:2 CLA t,t isomer could not be detected. This was probably because this isomer co-eluted with the C18:3 c9t11c15, as retention times were very close (21.15 *vs*. 21.27 min) (**Figure 3.25**), and at this condition a higher amount of C18:3 c9t11c15 was produced when comparing with lower substrate concentrations (**Sections 3.2.2 Microbially CLA/CLNA-enriched milk** and **3.2.4 Microbially CLNA-enriched milk**; in Results and Discussion of Part II), enlarging peak area and retention time (21.19 min).

Ruminal bacteria have shown the ability to produce CLA isomers directly from α-LNA (Lee & Jenkins, 2011). In the current work, no CLA isomers were detected when using α-LNA alone, then, the assayed strain could only produce CLA from LA. In fact, to the best of our knowledge, no probiotic strains have demonstrated such above-mentioned capacity.

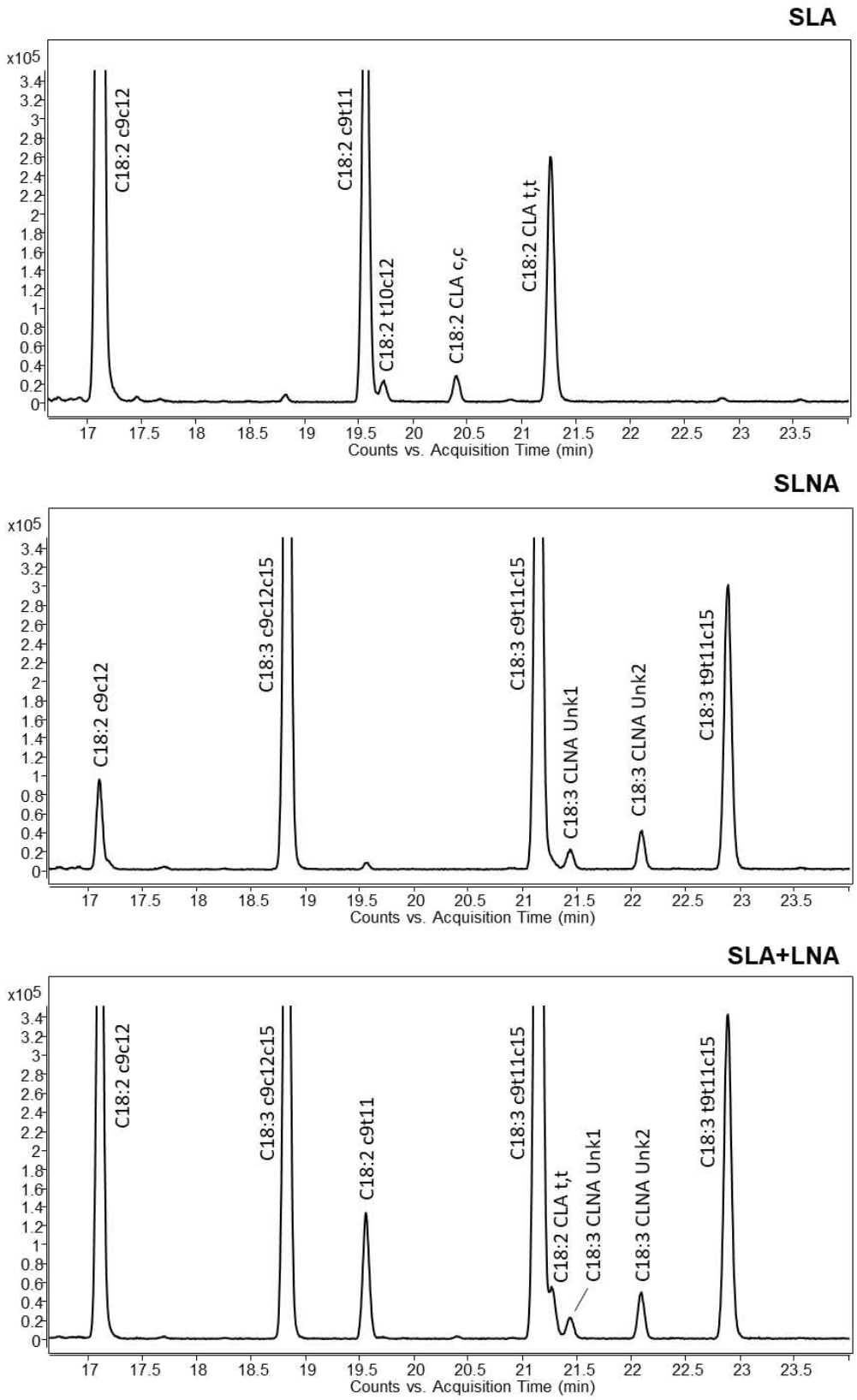


Figure 3.25 Chromatograms of SLA (0.5 mg/mL linoleic acid – LA), SLNA (0.5 mg/mL α-linolenic acid – α-LNA) and SLA+LNA (0.15 mg/mL LA + 0.5 mg/mL α-LNA) pasteurized semi-skimmed milk after fermentation (24 h) with *Bifidobacterium breve* DSM 20091. CLA – Conjugated linoleic acid, CLNA – Conjugated linolenic acid, Unk – Unknown.

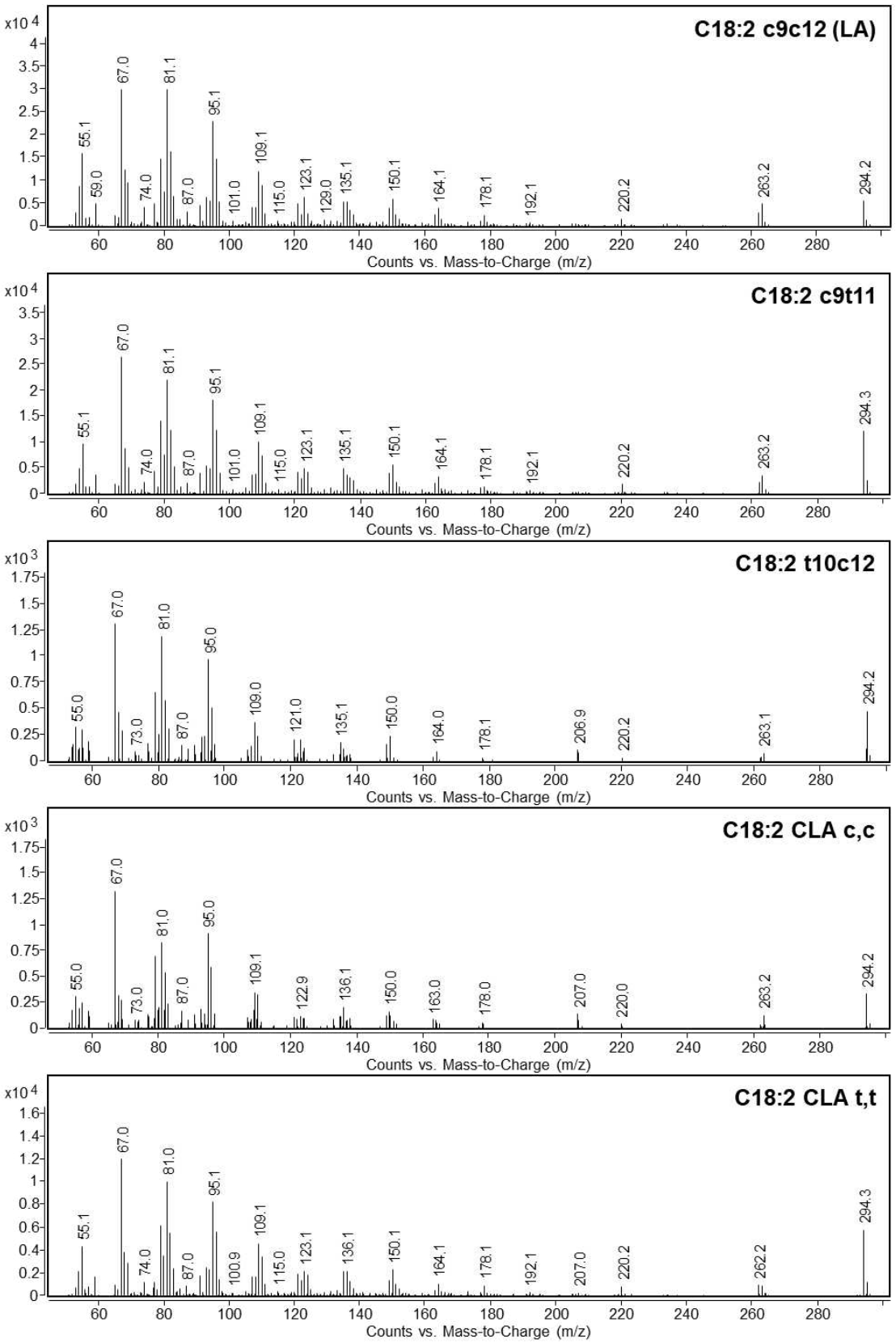


Figure 3.26 Mass spectra of linoleic acid (LA) and conjugated linoleic acid (CLA) isomers detected in SLA (0.5 mg/mL LA) pasteurized semi-skimmed milk after fermentation (24 h) with *Bifidobacterium breve* DSM 20091.

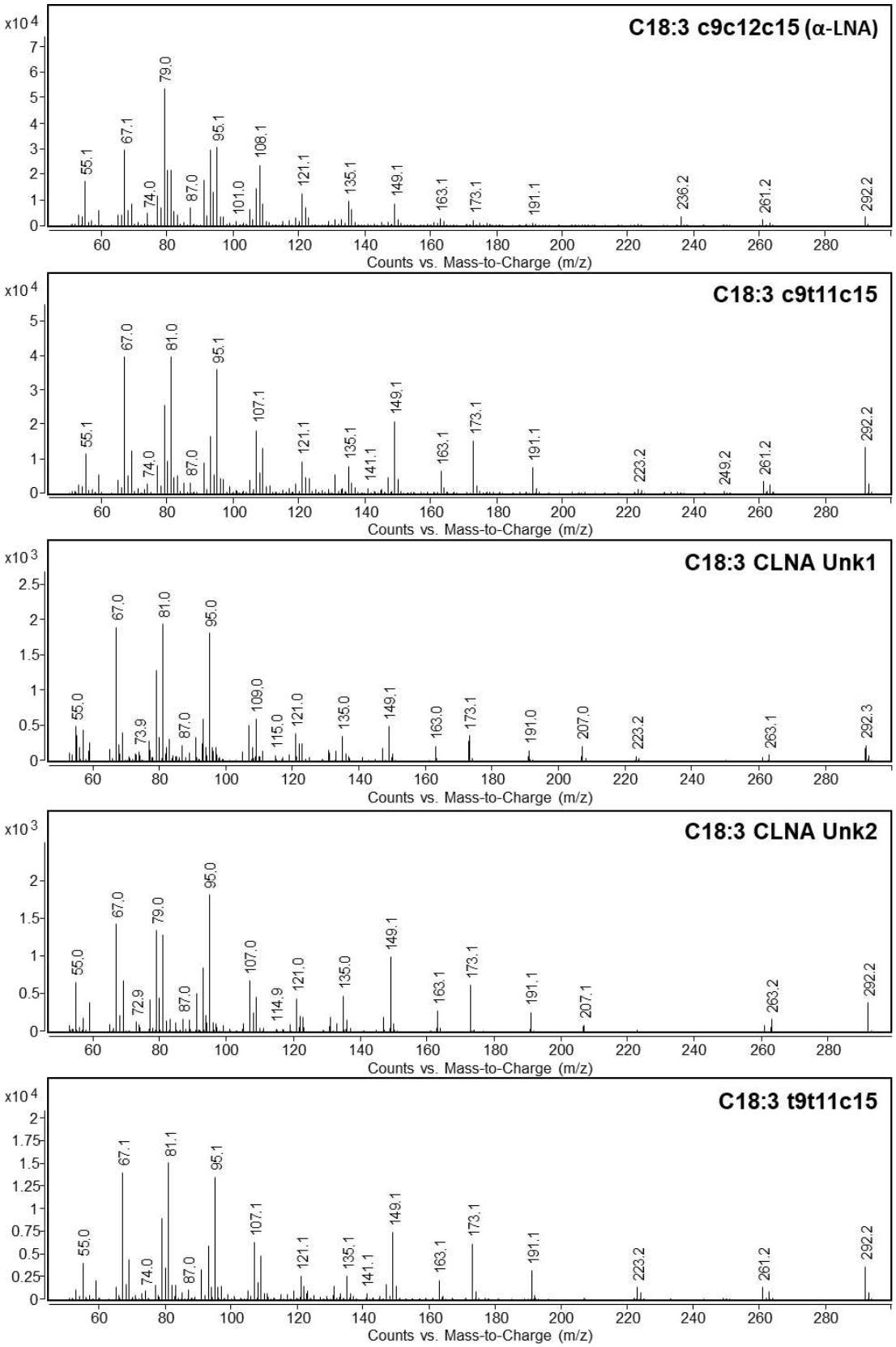


Figure 3.27 Mass spectra of α-linolenic acid (α-LNA) and conjugated linolenic acid (CLNA) isomers detected in SLNA (0.5 mg/mL of α-LNA) pasteurized semi-skimmed milk after fermentation (24 h) with *Bifidobacterium breve* DSM 20091. Unk – Unknown.

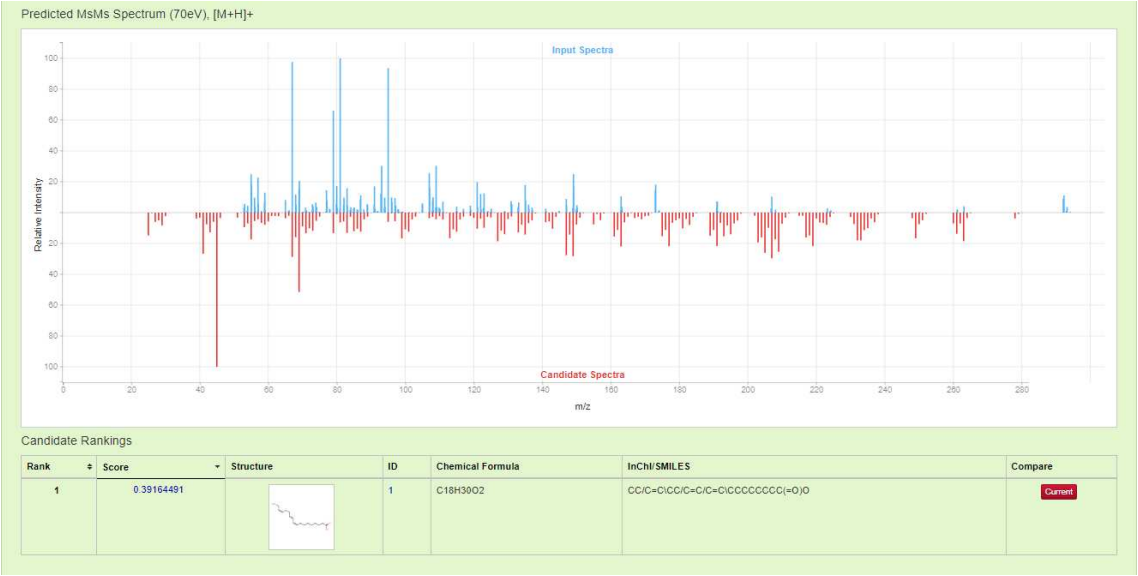


Figure 3.28 Results from CLNA Unk1 spectrum comparison with Rumelenic acid (C18:3 c9t11c15) predicted spectrum by CFM-ID.

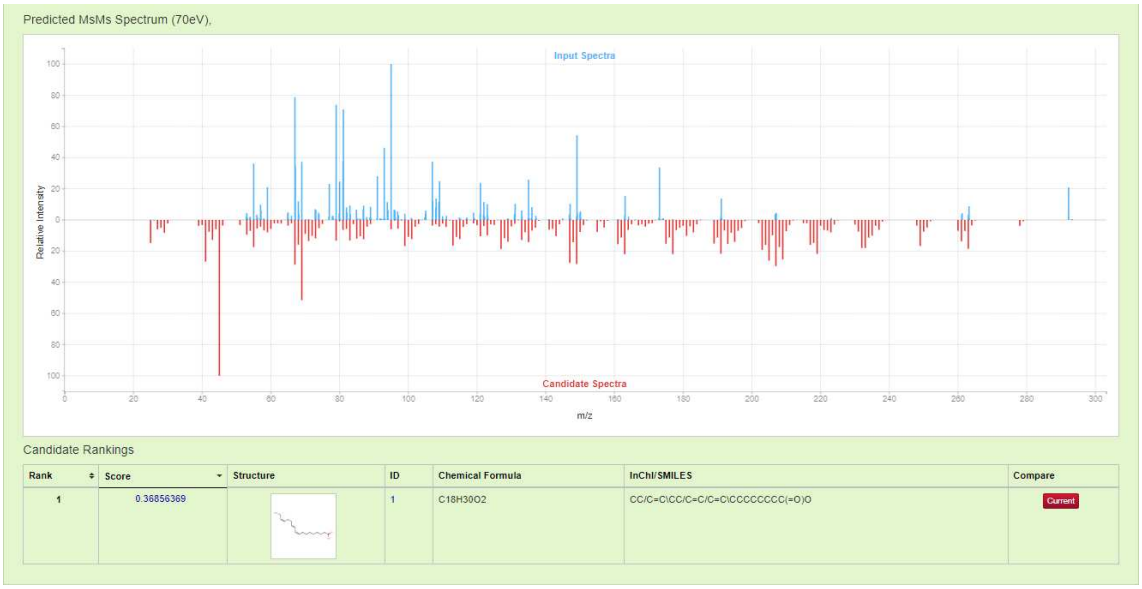


Figure 3.29 Results from CLNA Unk2 spectrum comparison with Rumelenic acid (C18:3 c9t11c15) predicted spectrum by CFM-ID.

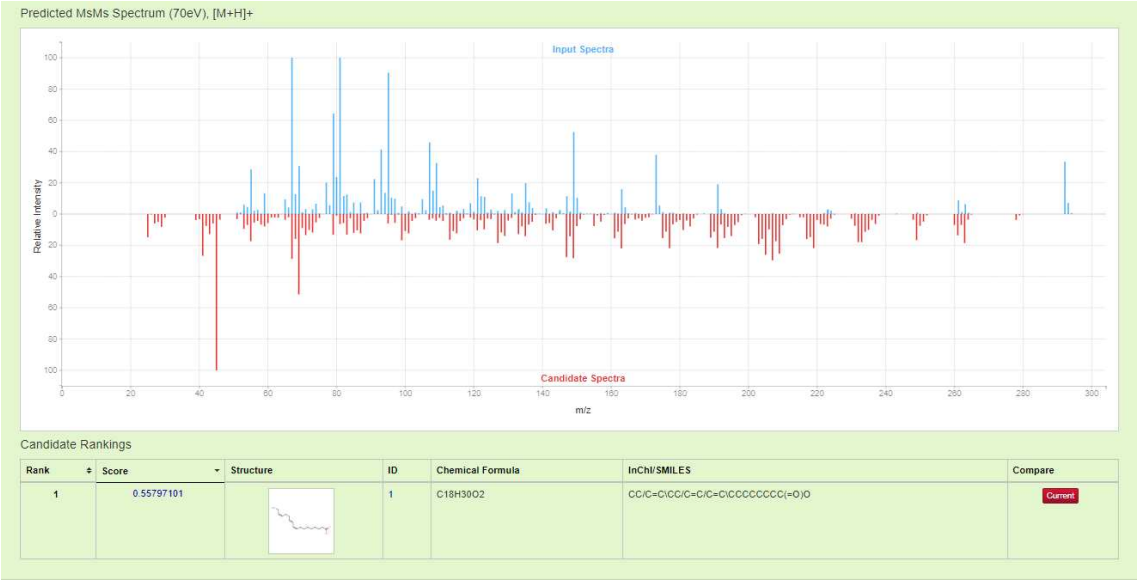


Figure 3.30 Results from detected Rumelenic acid (C18:3 c9t11c15) spectrum comparison with the predicted spectrum by CFM-ID.

Part IV – Biochemical and organoleptic properties of microbial CLNA-enriched fermented milk

No further FA metabolites were detected from LA or α-LNA metabolization by *B. breve* DSM 20091, according to results from Part III; however, two new CLNA isomers were detected.

The aim of Part IV was to evaluate the biochemical and organoleptic characteristics of the developed CLNA-enriched fermented milk by *B. breve* DSM 20091 with lipase-hydrolyzed FSO as substrate source (as shown in Part II), therefore assessing its suitability for consumption. However, an initial problem arose related with fermented milk pH being low for sensory acceptance. Therefore, first, there was a need to select the most favorable fermentation time, under food grade conditions, in a way to reach a reasonable pH value without compromising CLNA yield by *B. breve* DSM 20091. With all fermentation conditions set, the CLNA-enriched fermented milk was thereafter analyzed for sugars and organic acids composition, titratable acidity, pH, nutritional composition, volatile compounds profile and sensory properties.

3.4.1. Optimization of incubation time

According to results in **Sections 3.2.4 Microbially CLNA-enriched milk** (in Results and Discussion of Part II) and **3.3.1 Bacterial growth and pH** (in Results and Discussion of Part III), the developed CLNA-enriched fermented milk showed pH values of almost 4.00. Fermented dairy products with low pH (4.0-4.5) may incur low sensory acceptance (Granato et al., 2010). Thus, when developing such products, fermentation is normally interrupted by the time matrix reaches pH 4.5 (Oliveira et al., 2012; Šertović et al., 2019). Accordingly, different incubation times (from 16 to 24 h) were tested to verify which one would lead to a pH level above 4.5 without considerably affecting CLNA isomers production. Moreover, since it is being studied the development of a new food product,

this assay was performed under food-grade conditions, as described in **Section 2.4.3 Optimization of incubation time** (in Materials and Methods of Part IV).

Bifidobacterium breve DSM 20091 viable cell numbers increased significantly from 7.28 log CFU/mL to 8.99 log CFU/mL right after 16 h of incubation, presenting stable viable cell numbers until 24 h (9.28 log CFU/mL) (**Figure 3.31**). In terms of pH value, it decreased significantly after 16 hours (from pH 6.27 to 5.01), and it was not much lower than pH 4.74 after 24 hours (**Figure 3.32**). As for CLNA isomers production, no differences ($p>0.05$) were observed between incubation times (0.96-1.17 mg/g) (**Figure 3.33**). Results showed that after 16 h of incubation *B. breve* was already in stationary phase, with no considerable changes in the analyzed parameters from 16 h to 24 h. On the other hand, it was possible to increment CLNA-enriched fermented milk pH from ~4.0 to 4.7 (after 24 h) at the assayed conditions. Somehow, that must be associated with the adopted food-grade conditions, such as replacing the pre-inoculum medium by skim-milk. Nevertheless, to guarantee that the pH level would not reach values lower than 4.5, the incubation period was reduced to 22 h in the following assays.

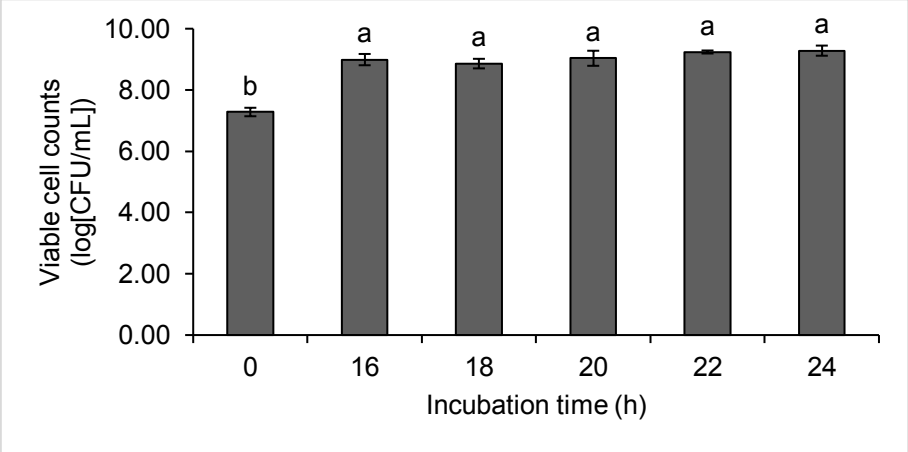


Figure 3.31 Viable cell counts of *Bifidobacterium breve* DSM 20091 grown in pasteurized semi-skimmed milk with 2 mg/mL α-linolenic acid (from hydrolyzed flaxseed oil) for different incubation times. Different superscript letters indicate ≥1 log cycle difference between incubation times.

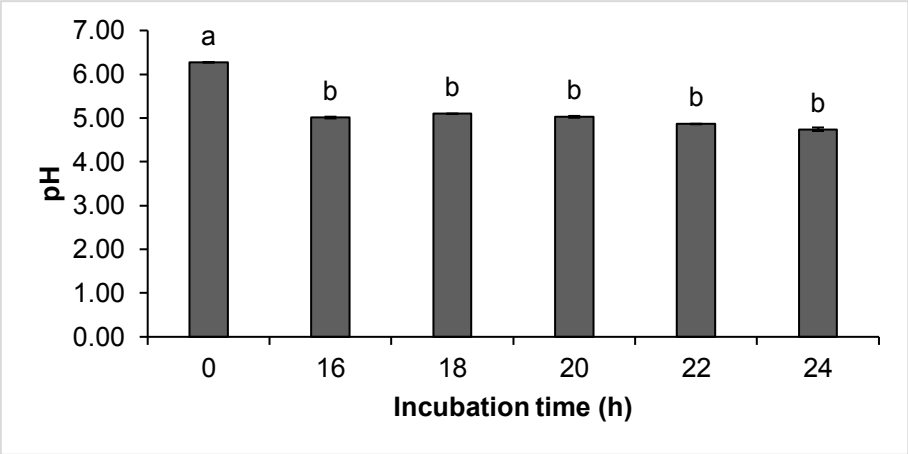


Figure 3.32 pH values of pasteurized semi-skimmed milk inoculated with *Bifidobacterium breve* DSM 20091 at 2 mg/mL α-linolenic acid (from hydrolyzed flaxseed oil) and fermented for different incubation times. Different superscript letters indicate ≥0.5 units difference between incubation times.

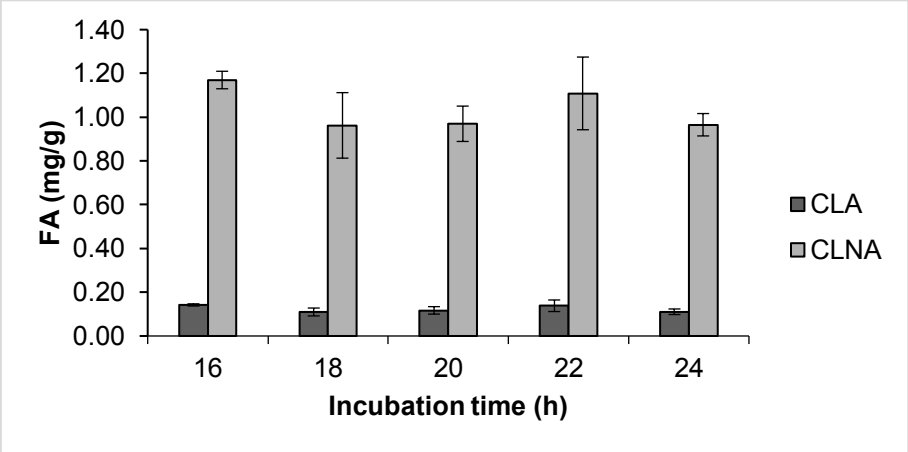


Figure 3.33 Content of free conjugated linoleic acid (CLA) and free conjugated linolenic acid (CLNA) isomers released by *Bifidobacterium breve* DSM 20091 grown in pasteurized semi-skimmed milk with 2 mg/mL α-linolenic acid (from hydrolyzed flaxseed oil) for different incubation times. FA – Fatty acid.

3.4.2. Sugars and organic acids composition, titratable acidity, and pH

In this section the metabolization of milk sugars, the organic acids produced and their quantification, as well as the titratable acidity and pH of the developed CLNA-enriched fermented milk were evaluated. These parameters might have an influence on the organoleptic properties of dairy products.

3.4.2.1. Sugars

Non-fermented milk contained 5.34 g/100 g of lactose (**Table 3.32**), which was further hydrolyzed into glucose (0.89-1.03 g/100 g) and galactose (0.88-1.02 g/100 g) in both fermented

milks, decreasing lactose content ($p<0.05$) to 3.35-3.54 g/100 g. Moreover, no differences ($p>0.05$) were found in the content of lactose or its monomers between control and CLNA-enriched milk (**Table 3.32**).

Table 3.32 Sugars and organic acids composition, titratable acidity and pH of non-fermented pasteurized semi-skimmed milk, and control and CLNA-enriched fermented milks.

Compound (g/100 g)	Non-fermented milk	Fermented Milk	
		Control	CLNA-enriched milk
Sugars			
Lactose	5.34±0.36^{a}	3.35±0.16^{b}	3.54±0.18^{b}
Glucose	ND	1.03±0.04	0.89±0.06
Galactose	ND	1.02±0.04	0.88±0.09
Organic acids			
Citric acid	0.92±0.07	0.86±0.06	0.84±0.04
Lactic acid	ND	0.37±0.03	0.42±0.01
Formic acid	0.29±0.05	0.25±0.05	0.26±0.03
Acetic acid	ND	0.40±0.03	0.44±0.03
Propionic acid	ND	0.88±0.03	0.85±0.04
Titratable acidity (%)	0.13±0.01^{c}	0.50±0.01^{b}	0.69±0.01^{a}
pH	6.79±0.01^{b}	5.10±0.01^{a}	4.93±0.04^{a}

Average values ± standard deviation (n=3). Different superscript letters indicate significant differences within rows ($p<0.05$).

ND – not detected.

Beta-galactosidases, also known as lactases, are responsible for lactose hydrolysis into glucose and galactose (Singh et al., 2019). Such capacity is essential for bacterial metabolism where the released glucose is used for further energy production. In fact, β-galactosidase activity has been reported in different species of bifidobacteria, including *B. breve* (Martínez-Villaluenga & Gómez, 2007), thus, the results presented above are in accordance.

3.4.2.2. Organic acids

Citric and formic acids were present in non-fermented milk (0.92 and 0.29 g/100 g, respectively) (**Table 3.32**), but its contents did not differ ($p>0.05$) from the control or CLNA-enriched fermented milks (citric acid: 0.84-0.96 g/100 g; formic acid: 0.25-0.32 g/100g). This is in accordance with Nguyen et al. (2012), who observed no alteration in citric or formic acid contents in reconstituted skimmed milk after fermentation by a *B. breve* strain.

On the other hand, additional organic acids were detected in both fermented milks (**Table 3.32**), namely, lactic (0.37-0.42 g/100 g), acetic (0.40-0.44 g/100 g) and propionic (0.85-0.88 g/100 g) acids. Bifidobacteria are known as heterofermentative bacteria, producing lactic and acetic acids during growth through the fructose-6-phosphate pathway (Nguyen et al., 2012). The lactic acid/acetic acid ratio obtained was ca. 0.9; such balanced amount of acetic acid versus that of lactic acid is important toward preservation of the sensory quality of the fermented milks. Propionic acid results from the fermentation of glucose or lactic acid (Markowiak-Kopeć & Śliżewska, 2020). To the best of our knowledge, among bifidobacteria, its production has been reported for *B. longum*, *B. bifidum*,

Bifidobacterium infantis and *B. lactis* species (LeBlanc et al., 2017; Usta-Gorgun & Yilmaz-Ersan, 2020). In fact, no propionic acid was detected in milk fermented by *B. breve* ATCC 15700 (Nguyen et al., 2012). The present research work suggests that propionic acid can eventually be produced by *B. breve* species, depending on the strain.

Furthermore, when comparing the control fermented milk with the CLNA-enriched counterpart, no differences ($p>0.05$) were found among organic acids contents (**Table 3.32**).

3.4.2.3. Titratable acidity and pH

As expected from a fermentation process, titratable acidity values in both fermented milks were higher ($p<0.05$) than those of non-fermented milk (0.13%; **Table 3.32**). Moreover, CLNA-enriched fermented milk titratable acidity value was significantly higher ($p<0.05$) than that of the control (0.69% *vs*. 0.50%, respectively; **Table 3.32**). This difference must have resulted from the added hydrolyzed FSO in the case of the CLNA-enriched milk. Titratable acidity of typical fermented milk products is within the range of 0.7-1.1% (Kim et al., 2020; Mani-López et al., 2014), however the bacteria employed in those dairy products consist of starter cultures commercialized for such purpose.

The pH values were accordingly lower in both fermented milks, when comparing with the non-fermented milk (pH 6.79; **Table 3.32**), where CLNA-enriched fermented milk pH was slightly lower than that of the control (pH 4.93 *vs*. 5.10, respectively; **Table 3.32**), and correlated inversely with the titratable acidity values (lower pH associated with higher titratable acidity for CLNA-enriched fermented milk), also associated to the added hydrolyzed FSO.

3.4.3. Nutritional composition

The physico-chemical composition of the developed control and CLNA-enriched fermented milk was evaluated and is listed in **Table 3.33**. All compositional parameters (protein, carbohydrates, sugars and ash contents) were similar between both fermented milks except for fat content, which was significantly higher ($p<0.05$) in CLNA-enriched fermented milk (2.00 g/100 g *vs*. 1.70 g/100 g in control; **Table 3.33**), as expected given the higher FA content resulting from the added hydrolyzed FSO and produced CLNA isomers.

Table 3.33 Physico-chemical composition of control and CLNA-enriched fermented milks.

Parameter (g/100 g)	Control	CLNA-enriched milk
Fat	1.70±<0.01^{b}	2.00±<0.01^{a}
Carbohydrates	4.70±0.01	4.60±0.01
of which Sugars	3.11±0.01	2.95±0.01
Protein	3.23±0.01	3.06±0.12
Dry residue	10.30±0.01	10.42±0.05
Ash	0.70±0.01	0.66±0.01

Average values ± standard deviation (n=2). Different superscript letters indicate significant differences within rows ($p<0.05$).

A comparison with the nutritional composition of the closest product in the Portuguese market – a semi-skimmed natural yogurt (Instituto Nacional de Saúde Doutor Ricardo Jorge I.P. INSA, 2023) – showed comparable values, with 5 g/100 g of carbohydrates, 87.9 g/100 g of water and 0.75 g/100 g of minerals. The fat content of yogurt (1.8 g/100 g) is similar to that of the control fermented milk. The protein content is in line with that listed in the nutritional table of the commercial milk employed (3.4 g/100 g; data not shown).

3.4.4. Volatile compounds profile

After analysis of volatile compounds in the control and CLNA-enriched fermented milks, several compounds were identified (**Table 3.34**): 7 ketones (acetone, 2-butanone, 2,3-butanedieone, 2-heptanone, 3-octanone, acetoin and 2-nonanone), 3 hydrocarbons (decane, undecane and dodecane), 3 alcohols (2-heptanol, 1-hexanol and 2-ethyl-1-hexanol), 5 carboxylic acids or esters (methyl hexanoate, acetic, butanoic, hexanoic and octanoic acids) and 3 sulphur compounds (methyl thiolacetate, dihydro-2-methyl-3(2H)-thiophenone and dimethyl sulfone). Acetaldehyde, diacetyl, acetoin, acetone and 2-butanone constitute the principal compounds responsible for the typical flavor of fermented dairy products like yogurt (Cheng, 2010). The key compound for yogurt aroma is acetaldehyde (Routray & Mishra, 2011), however, this volatile flavor was not detected in any of the fermented milks. Although the acetaldehyde production potential has been reported for different *Bifidobacterium* species, revealing a species- and strain-dependent trait (Mituniewicz-Małek et al., 2017; Salazar et al., 2009), it appears that *B. breve* DSM 20091 does not present such capacity. The major volatile compound detected was acetic acid (83%; **Table 3.34**), similar to the results reported for a fermented milk by another *Bifidobacterium* strain, namely *B. animalis* subsp. *lactis* Bb-12, where ~90% of acetic acid was detected (Zareba et al., 2012).

When comparing with the control fermented milk, differences were observed in total ketones content, which was significantly lower ($p<0.05$) in CLNA-enriched milk (6.05% *vs*. 9.02%; **Table 3.34**). Ketones constitute important flavor compounds in fermented milk (Dan et al., 2017). On the other hand, hydrocarbons amount was higher ($p<0.05$) in CLNA-enriched milk (1.04% *vs*. 0.31%; **Table 3.34**). Nevertheless, hydrocarbons are not considered major flavor contributors due to their high threshold (Li et al., 2020).

Table 3.34 Volatile composition of control and CLNA-enriched fermented milks.

Compound (Rel. Area %)	Control	CLNA-enriched milk
Acetone	1.22±0.09[a]	0.81±0.05[b]
2-Butanone	1.02±0.16[a]	0.58±0.08[b]
Diacetyl	0.78±0.07	0.68±0.16
2-Heptanone	0.74±0.29	0.63±0.12
3-Octanone	3.97±0.88[a]	2.40±0.10[b]
Acetoin	1.11±0.13	0.95±0.16
2-Nonanone	0.18±0.02	ND
Σ Ketones	9.02±1.42[a]	6.05±0.67[b]
Decane	0.16±0.04[b]	0.39±0.04[a]
Undecane	0.15±0.01	0.12±0.03
Dodecane	ND	0.53±0.19
Σ Hydrocarbons	0.31±0.11[b]	1.04±0.26[a]
2-Heptanol	0.17±0.04	0.14±0.04
1-Hexanol	0.29±0.04[b]	0.71±0.01[a]
2-Ethyl-1-hexanol	0.18±0.03	ND
Σ Alcohols	0.64±0.11	0.85±0.03
Methyl hexanoate	ND	0.53±0.12
Acetic acid	83.02±2.32	83.32±0.77
Butyric acid	0.98±0.04	0.85±0.01
Hexanoic acid	1.79±0.09[a]	1.26±0.16[b]
Octanoic acid	0.78±0.06[a]	0.35±0.02[b]
Σ Carboxylic acids and esters	86.57±2.25	86.31±1.06
Methyl thiolacetate	0.17±0.03	ND
Dihydro-2-methyl-3(2H)-thiophenone	2.13±0.35	2.55±0.20
Dimethyl sulfone	0.09±0.01	0.06±0.01
Σ Sulfur compounds	2.38±0.39	2.61±0.18
Σ Unk	1.09±0.21[b]	3.14±0.34[a]

Average values ± standard deviation (n=2). Different superscript letters indicate significant differences within rows ($p<0.05$).

Rel. Area % – Relative area percentage; Unk – Unknown compounds; ND – Not detected.

3.4.5. Sensory properties

Determination of total microbial and *Enterobacteriaceae* viable cell counts revealed that both fermented milks were safe for consumption, as numbers were below the countable range (Total microbial counts <$3.00x10^3$ CFU/mL; *Enterobacteriaceae* counts <$3.00x10^2$ CFU/mL).

Within the sensory parameters evaluated in the CLNA-enriched fermented milk, the first assessment was the detection of a high syneresis volume (**Figure 3.34**) and a tenuous aroma of reduced sulfur compounds, while in the control fermented milk an intense aroma of reduced sulfur compounds was perceived. Volatile sulfur compounds share common thiol precursors, such as methanethiol or hydrogen sulfide, and these thiols can arise from the biodegradation of the sulfur/carbon bond of methionine or cysteine by bacteria (Landaud et al., 2008). Lactobacilli bacteria showed the production of reduced sulfur compounds, such as, hydrogen sulfide, methanethiol,

dimethyl disulfide and dimethyl trisulfide, when in the presence of cysteine or methionine (Sreekumar et al., 2009). Moreover, Montoya et al. (2009) observed the production of dimethyl trisulfide by a *B. breve* strain in a Swiss cheese curd slurry model system, and associated it with the free methionine naturally present. However, no reduced sulfur compounds were detected in the fermented milks (which contained added cysteine) when volatiles were analyzed, but that could be related to the very low odor threshold of such compounds (van Gemert, 2003).

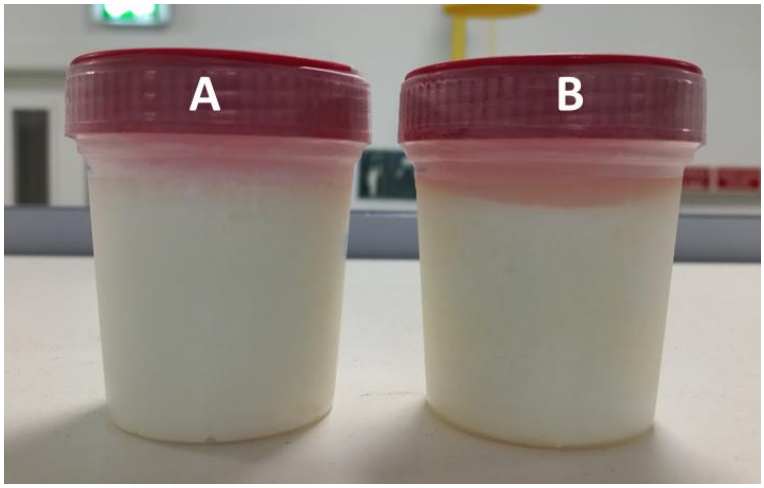


Figure 3.34 Control (A) and CLNA-enriched (B) fermented milks.

Furthermore, the CLNA-enriched fermented milk also revealed an herbal aroma, remembering melon peel, cucumber or aloe vera, but in the control fermented milk a lactic aroma was distinguished, remembering natural yogurt or kefir, and very slight notes of butter. The herbal flavor detected in the CLNA-enriched fermented milk must be associated with the hydrolyzed FSO added, being detected a higher content ($p<0.05$) of 1-hexanol (0.71% *vs*. 0.29% in control; **Table 3.34**), which is characterized as a green aroma (Ali et al., 2022).

In terms of apparent texture, the CLNA-enriched fermented milk was more like a consistent gel whereas the control fermented milk appeared as a very slight gel. Regarding mouthfeel, in the CLNA-enriched fermented milk a cucumber flavor, high astringency, medium-high bitterness and slight acidity predominated, while in the control fermented milk the main features that predominated were reduced sulfur compounds, low acidity and sweetness, and a fine and watery texture (neither creamy nor granular). In what concerns after-taste, astringency, bitterness and cucumber flavor predominated in the CLNA-enriched fermented milk, while in the control counterpart it predominated a sulfur aroma. The high astringency and bitterness of CLNA-enriched fermented milk must be related to the FSO presence.

The poor flavor properties perceived in the CLNA-enriched fermented milk must be in part associated with the fact that the *B. breve* alone was unable to generate enough lactic acid to obtain a fermented dairy product with the appropriate aroma and flavor. In general, bifidobacteria are normally co-cultured with LAB starters, such as *Streptococcus thermophilus* and *Lactobacillus delbrueckii* subsp. *bulgaricus*, which are capable of producing suitable flavors in dairy products (Prasanna et al., 2014).

Part V – Stability assessment of developed CLNA-enriched dairy products throughout storage

The developed CLNA-enriched fermented milk by *B. breve* DSM 20091 with lipase-hydrolyzed FSO, under food-grade conditions (as shown in Part II and Part IV), revealed acceptable biochemical and nutritional properties, however, lacked important dairy flavor contributors, prevailing a certain note of bitterness and astringency, according to results from Part IV.

The aim of Part V was to assess the shelf-life stability of the developed CLNA-enriched fermented milk. To do so, microbiological enumeration, pH and FA profiles were analyzed throughout 28 days of refrigerated storage. To obtain an alternative food delivery system of CLNA isomers, the fermented milk was further lyophilized and the storage stability of the obtained dried CLNA-enriched milk was assessed, by microbiological enumeration and FA profile analysis during 12 weeks at room temperature. This latter delivery system is of interesting application given its versatility of application, longer storage stability and cost-effectiveness (no refrigeration and less transport costs given its dry nature).

3.5.1. Stability of CLNA-enriched fermented milk

3.5.1.1. Microbiological enumeration and pH

Bifidobacterium breve DSM 20091 viable cell numbers, which were found initially (T0d) at 7.41 (control fermented milk) and 8.09 log CFU/mL (CLNA-enriched fermented milk), decreased after 7 days of storage (T7d) at 4 ºC to 5.92 and 4.51 log CFU/mL, respectively. Thereafter, viable cell numbers kept decreasing to levels below the detectable range (i.e., $<5.00x10^3$ CFU/mL) until the end of the storage period (T28d), for both assayed conditions (data not shown). According to Odamaki et al. (2011), microorganisms are often exposed to several stress factors during refrigerated storage of fermented milk, including low pH, low temperature, high osmotic pressure, nutrient starvation and oxidation, leading to loss of viability, some of which could also justify the loss of viability observed for *B. breve* DSM 20091. Odamaki et al. (2011) were able to show that *B. longum* BB536 survival in fermented milk during refrigerated storage could be improved by co-culturing with *Lc. lactis* subsp. *lactis* MCC866, which protected cells from oxidative stress.

As for total microbial counts, these were found to be below the detectable range (i.e., $<3.00x10^3$ CFU/mL) at T0d and during the entire storage period at 4 ºC for both fermented milks conditions (data not shown). In other research work, low and constant counts (<2.00 log CFU/mL) of other microorganisms, like coliforms, yeasts and molds, were also observed for control or CLNA-enriched yogurts – prepared with Pomegranate or Jacaranda seed powders – after 28 days at 4 ºC (Van Nieuwenhove et al., 2019).

Regarding the fermented milk pH value, levels were maintained constant during the whole storage period for both conditions, with values around pH 5.05-5.14 for control and pH 4.86-4.99 for the CLNA-enriched milk. A constant pH value has also been reported for kefir (Turek & Wszołek, 2021) and yogurt (Van Nieuwenhove et al., 2019) products during 14 and 28 days of refrigerated (4 ºC) storage, respectively, whether enriched with CFA or not.

3.5.1.2. Fatty acid profiles

Regarding the FA composition in the control fermented milk, there were variations in the amount of free saturated FA (SFA) and MUFA during refrigerated storage (**Table 3.35**), with a decreasing tendency, as observed for C14:0 (from 0.131 to 0.106 mg/g), C16:0 (from 0.340 to 0.275 mg/g) and C18:1 c9 (from 0.160 to 0.142 mg/g) after 28 days from the beginning of storage. On the other hand, esterified SFA decreased significantly ($p<0.05$) throughout refrigerated storage (**Table 3.36**), namely C16:0 (from 4.145 to 3.694 mg/g) and C18:0 (from 1.329 to 0.995 mg/g). It could be hypothesized that lipolysis led to this reduction, although, no further increases were observed in the FFA fraction. In fact, the opposite seemed to occur, so it is most likely that the FFA got oxidized, since these lipid species are prone to oxidation, generating hydroperoxides leading to EFA oxidation as well (Musakhanian et al., 2022).

As for CLNA-enriched fermented milk, free SFA values ended up being significantly ($p<0.05$) lower by the end of storage, especially because of the C18:0 reduction (from 0.334 to 0.213 mg/g) (**Table 3.35**). Moreover, there were variations in esterified SFA during refrigerated storage, but C18:0 decreased significantly as well ($p<0.05$; from 1.208 to 0.956 mg/g) (**Table 3.36**). Considering all the obtained data, it suggests that oxidation processes have occurred similarly to the control fermented milk.

On the other hand, free MUFA and PUFA increased ($p<0.05$) throughout storage (**Table 3.35**), including C18:1 c9 (from 0.658 to 0.765 mg/g) and the CLNA C18:3 c9t11c15 (from 0.652 to 0.929 mg/g). Some research works have reported the production of CLA/CLNA isomers using bacterial resting cells, involving *B. breve* (Kishino et al., 2009; Oh et al., 2003). Therefore, it is most likely that, even if *B. breve* DSM 20091 cells have lost viability during storage (**Section 3.5.1.1 Microbiological enumeration and pH**; in Results and Discussion of Part V), their enzymatic system responsible for CLNA formation remained active, leading to the observed increase in CLNA isomers. Other studies have observed that major CFA in CLNA-enriched yogurt (Van Nieuwenhove et al., 2019) and in commercial CLA-fortified dairy products (Rodríguez-Alcalá & Fontecha, 2007) were not significantly changed after 28 days or 10 weeks at 4 ºC, respectively. However, in these works, CLA/CLNA isomers were supplemented into the dairy matrices and not microbiologically produced *in situ*.

It has been proposed that the microbial pathway where CLA isomers are formed involves the release of further compounds, including oleic acid (i.e., C18:1 c9) (Kishino et al., 2013). Therefore, the detected increment of this former FA could be related to the above-mentioned CLNA formation.

Table 3.35 Free fatty acid composition of control and CLNA-enriched fermented milks during 28 days of storage at 4 ºC.

FA (mg/g)	Control					CLNA-enriched milk				
	T0d	T7d	T14d	T21d	T28d	T0d	T7d	T14d	T21d	T28d
C4:0	0.018±0.002	0.015±<0.001	0.017±0.001	0.013±0.001	0.015±0.002	0.013±0.001	0.015±0.001	0.016±0.001	0.014±0.002	0.012±0.002
C6:0	0.016±0.002	0.012±0.001	0.015±<0.001	0.010±0.001	0.013±0.002	0.010±0.001	0.013±<0.001	0.013±0.001	0.011±0.001	0.010±0.001
C8:0	0.015±0.002	0.010±0.001	0.014±<0.001	0.008±0.001	0.011±0.003	0.009±0.001	0.011±<0.001	0.012±0.002	0.009±0.001	0.008±0.001
C10:0	0.039±0.004	0.029±<0.001	0.038±0.001	0.024±0.001	0.031±0.008	0.023±0.002	0.031±0.001	0.031±0.003	0.024±0.001	0.023±0.003
C12:0	0.048±0.006	0.037±<0.001	0.047±0.001	0.031±0.002	0.038±0.009	0.030±0.001	0.040±0.001	0.041±0.003	0.032±0.001	0.030±0.004
C14:0	0.131±0.015^{a}	0.102±0.002^{b}	0.128±0.004^{a}	0.091±0.005^{b}	0.106±0.025^{ab}	0.080±0.001^{b}	0.109±0.002^{a}	0.108±0.008^{a}	0.090±0.006^{ab}	0.082±0.010^{ab}
C14:1 c9	0.017±0.001	0.015±<0.001	0.016±0.001	0.012±0.001	0.012±0.002	0.012±<0.001	0.015±0.001	0.014±0.001	0.012±<0.001	0.010±0.001
C15:0	0.013±0.002	0.010±<0.001	0.013±0.001	0.009±0.001	0.010±0.002	0.008±<0.001	0.011±<0.001	0.011±0.001	0.010±0.001	0.009±0.001
C16:0	0.340±0.049^{a}	0.263±0.001^{b}	0.325±0.010^{a}	0.250±0.014^{b}	0.275±0.064^{ab}	0.373±0.008^{c}	0.496±0.020^{a}	0.482±0.028^{a}	0.441±0.032^{ab}	0.401±0.023^{bc}
C16:1 c9	0.020±0.003	0.015±<0.001	0.018±<0.001	0.015±0.001	0.017±0.003	0.013±<0.001	0.019±0.001	0.018±0.001	0.016±0.001	0.015±0.001
C17:0	0.005±0.001	0.004±<0.001	0.005±<0.001	0.004±<0.001	0.004±0.001	0.005±<0.001	0.006±<0.001	0.006±<0.001	0.006±0.001	0.005±<0.001
C18:0	0.104±0.018	0.082±0.001	0.095±0.004	0.075±0.006	0.073±0.018	0.334±0.008^{b}	0.410±0.034^{a}	0.327±0.011^{b}	0.275±0.012^{c}	0.213±0.001^{d}
C18:1 c9	0.160±0.025^{a}	0.115±0.003^{b}	0.149±<0.001^{a}	0.125±0.006^{ab}	0.142±0.033^{ab}	0.658±0.029^{b}	0.821±0.062^{a}	0.810±0.043^{a}	0.776±0.009^{a}	0.765±0.007^{a}
C18:2 c9c12 (LA)	0.025±0.002	0.017±0.001	0.022±<0.001	0.020±0.001	0.025±0.005	0.333±0.002^{b}	0.402±0.034^{a}	0.371±0.031^{ab}	0.396±0.008^{a}	0.389±0.023^{a}
C18:3 c9c12c15 (α-LNA)	0.003±<0.001	0.002±<0.001	0.002±<0.001	0.002±<0.001	0.003±<0.001	0.308±0.030	0.379±0.044	0.307±0.065	0.366±0.024	0.345±0.015
C18:2 c9t11 (CLA)	0.002±<0.001	0.002±<0.001	0.002±<0.001	0.002±<0.001	0.002±<0.001	0.089±0.021	0.103±0.003	0.112±0.009	0.108±0.001	0.139±0.024
C18:2 CLA t,t	ND	ND	ND	ND	ND	0.018±0.004	0.022±0.002	0.023±0.002	0.023±0.001	0.027±0.003
C18:3 c9t11c15 (CLNA)	ND	ND	ND	ND	ND	0.652±0.081^{c}	0.736±0.018^{c}	0.756±0.044^{bc}	0.775±0.002^{b}	0.929±0.013^{a}
C18:3 t9t11c15 (CLNA)	ND	ND	ND	ND	ND	0.116±0.016^{c}	0.137±0.013^{bc}	0.137±0.010^{bc}	0.155±0.004^{ab}	0.171±0.008^{a}
SFA	0.728±0.101^{a}	0.565±0.002^{b}	0.697±0.020^{a}	0.517±0.031^{b}	0.576±0.132^{ab}	0.884±0.023^{b}	1.143±0.057^{a}	1.047±0.058^{a}	0.913±0.059^{b}	0.792±0.045^{c}
MUFA	0.197±0.029^{a}	0.145±0.003^{b}	0.183±0.001^{a}	0.152±0.007^{ab}	0.171±0.039^{ab}	0.683±0.029^{b}	0.854±0.064^{a}	0.843±0.045^{a}	0.804±0.010^{a}	0.791±0.005^{a}
PUFA	0.030±0.003	0.021±0.001	0.026±<0.001	0.023±0.001	0.030±0.006	1.516±0.090^{c}	1.778±0.113^{b}	1.707±0.119^{bc}	1.823±0.037^{b}	1.999±0.023^{a}
Σ FA	0.955±0.133^{a}	0.731±0.002^{b}	0.906±0.020^{a}	0.692±0.039^{b}	0.777±0.177^{ab}	3.083±0.142^{b}	3.776±0.234^{a}	3.597±0.219^{a}	3.540±0.032^{a}	3.589±0.017^{a}

Average values ± standard deviation (n=3). Different superscript letters indicate significant differences between storage times of each fermented milk sample ($p<0.05$).

FA – Fatty acid; T0d – Time zero days; T7d – Time seven days; T14d – Time fourteen days; T21d – Time twenty-one days; T28d – Time twenty-eight days; *c* – *cis* double bond; *t* – *trans* double bond; LA – Linoleic acid; α-LNA – alpha-Linolenic acid; CLA – Conjugated linoleic acid; CLNA – Conjugated linolenic acid; SFA – Saturated fatty acids; MUFA – Monounsaturated fatty acids; PUFA – Polyunsaturated fatty acids; Σ FA – Total fatty acids; ND – Not detected.

Table 3.36 Esterified fatty acid composition of control and CLNA-enriched fermented milks during 28 days of storage at 4 ºC.

FA (mg/g)	Control					CLNA-enriched milk				
	T0d	T7d	T14d	T21d	T28d	T0d	T7d	T14d	T21d	T28d
C4:0	0.213±<0.001	0.202±0.010	0.197±0.017	0.204±0.033	0.210±0.002	0.192±0.007^{ab}	0.217±0.009^{a}	0.175±0.003^{b}	0.215±0.025^{a}	0.200±0.006^{a}
C6:0	0.180±0.006	0.159±0.014	0.163±0.002	0.168±0.019	0.165±0.006	0.161±0.011^{abc}	0.182±0.013^{a}	0.144±0.001^{c}	0.177±0.022^{ab}	0.158±<0.001^{bc}
C8:0	0.113±0.010^{a}	0.103±0.007^{ab}	0.088±0.001^{b}	0.105±0.013^{ab}	0.101±0.001^{ab}	0.096±0.007	0.112±0.010	0.083±0.010	0.106±0.011	0.102±0.003
C10:0	0.362±0.013	0.309±0.034	0.337±0.010	0.326±0.042	0.339±0.001	0.304±0.008	0.329±0.014	0.301±0.013	0.342±0.036	0.317±0.007
C12:0	0.459±0.013	0.404±0.037	0.419±0.011	0.409±0.046	0.419±0.001	0.396±0.009^{b}	0.439±0.024^{a}	0.374±0.018^{b}	0.426±0.049^{ab}	0.401±0.003^{b}
C14:0	1.447±0.045	1.275±0.109	1.307±0.034	1.255±0.146	1.302±0.001	1.257±0.021^{b}	1.376±0.074^{a}	1.168±0.044^{b}	1.314±0.152^{ab}	1.239±0.001^{b}
C14:1 c9	0.132±0.006	0.120±0.009	0.125±0.007	0.120±0.015	0.127±0.002	0.118±0.001	0.125±0.007	0.109±0.004	0.127±0.016	0.118±<0.001
C15:0	0.149±0.002	0.130±0.011	0.135±0.003	0.128±0.015	0.131±0.001	0.130±0.004	0.140±0.010	0.120±0.004	0.134±0.015	0.125±0.001
C16:0	4.145±0.133^{a}	3.623±0.329^{b}	3.743±0.111^{b}	3.562±0.417^{b}	3.694±0.018^{b}	3.632±0.053^{ab}	3.925±0.203^{a}	3.343±0.118^{b}	3.743±0.437^{ab}	3.531±0.025^{b}
C16:1 c9	0.200±0.004	0.173±0.015	0.188±0.012	0.183±0.020	0.201±0.006	0.183±0.024	0.184±0.013	0.164±0.009	0.196±0.021	0.187±0.004
C17:0	0.064±0.003	0.055±0.006	0.057±0.003	0.052±0.006	0.056±0.002	0.057±<0.001	0.060±0.003	0.051±0.001	0.055±0.005	0.054±<0.001
C18:0	1.329±0.043^{a}	1.170±0.118^{ab}	1.135±0.044^{b}	1.051±0.125^{bc}	0.995±0.002^{c}	1.208±0.018^{a}	1.290±0.058^{a}	1.035±0.030^{b}	1.100±0.128^{ab}	0.956±0.022^{b}
C18:1 c9	1.970±0.076	1.712±0.179	1.840±0.060	1.806±0.187	1.936±0.014	1.873±0.025	1.995±0.103	1.766±0.081	2.027±0.249	1.999±0.020
C18:2 c9c12 (LA)	0.268±0.010^{ab}	0.226±0.024^{b}	0.246±0.008^{b}	0.252±0.019^{b}	0.288±0.004^{a}	0.258±0.002^{b}	0.269±0.011^{b}	0.245±0.009^{b}	0.289±0.037^{ab}	0.299±0.006^{a}
C18:3 c9c12c15 (α-LNA)	0.029±0.001	0.025±0.004	0.027±0.001	0.025±0.004	0.032±0.001	0.107±0.003^{a}	0.091±0.004^{ab}	0.079±0.009^{b}	0.093±0.020^{ab}	0.105±0.005^{a}
C18:2 c9t11 (CLA)	0.027±0.003	0.023±0.002	0.025±0.004	0.027±0.002	0.035±0.001	0.028±0.002	0.026±0.001	0.027±0.001	0.032±0.003	0.037±0.001
SFA	8.461±0.266^{a}	7.430±0.672^{b}	7.582±0.223^{b}	7.260±0.863^{b}	7.412±0.016^{b}	7.433±0.121^{b}	8.070±0.418^{a}	6.795±0.242^{b}	7.612±0.876^{ab}	7.082±0.068^{b}
MUFA	2.302±0.084^{a}	2.004±0.202^{b}	2.153±0.079^{ab}	2.109±0.223^{ab}	2.264±0.021^{a}	2.175±0.048	2.304±0.123	2.040±0.093	2.351±0.286	2.305±0.016
PUFA	0.324±0.013^{ab}	0.274±0.030^{b}	0.298±0.013^{b}	0.304±0.025^{b}	0.355±0.006^{a}	0.394±0.004^{b}	0.387±0.016^{b}	0.350±0.020^{b}	0.414±0.058^{ab}	0.440±0.002^{a}
Σ FA	11.086±0.363	9.708±0.904	10.033±0.315	9.673±1.110	10.031±0.031	10.002±0.173^{b}	10.761±0.557^{a}	9.184±0.355^{b}	10.377±1.217^{ab}	9.826±0.086^{b}

Average values ± standard deviation (n=3). Different superscript letters indicate significant differences between storage times of each fermented milk sample ($p<0.05$).

FA – Fatty acid; T0d – Time zero days; T7d – Time seven days; T14d – Time fourteen days; T21d – Time twenty-one days; T28d – Time twenty-eight days; *c* – *cis* double bond; *t* – *trans* double bond; LA – Linoleic acid; α-LNA – alpha-Linolenic acid; CLA – Conjugated linoleic acid; SFA – Saturated fatty acids; MUFA – Monounsaturated fatty acids; PUFA – Polyunsaturated fatty acids; Σ FA – Total fatty acids.

3.5.2. Stability of CLNA-enriched lyophilized milk

In a way to find an alternative, more versatile, delivery system of CLNA isomers for consumers, providing simultaneously a strategy to increment its concentration, the CLNA-enriched fermented milk was lyophilized. After this process, it was obtained a powdered milk that contained 10 and 12-fold higher amounts of total FFA and EFA, respectively, comparing to CLNA-enriched fermented milk at T0d (**Table 3.35** and **Table 3.36** *vs*. **Table 3.37** and **Table 3.38**). Regarding the CLNA isomers specifically, these were concentrated 8-10 times to a total of 6.269 mg/g (**Table 3.37**). These values are in line with what would be expected from the loss of the 89.1% of water present in bovine semi-skimmed milk (Instituto Nacional de Saúde Doutor Ricardo Jorge I.P. INSA, 2023) after a lyophilization process. With a new product developed, it is crucial to determine if it is stable throughout its established shelf-life, therefore, the CLNA-enriched lyophilized milk was further evaluated in terms of microbiological quality and FA content during storage.

3.5.2.1. Microbiological enumeration

Viable cell numbers of *B. breve* DSM 20091 were below the countable range (<5.00x10^3 CFU/mL) at the beginning of storage (T0wk) and maintained this trend throughout storage for both conditions (data not shown). The loss of *B. breve* DSM 20091 viability, initially present in the fermented milk at 7.41 (control) or 8.09 log CFU/mL (CLNA-enriched milk) (**Section 3.5.1.1 Microbiological enumeration and pH**; in Results and Discussion of Part V), might be related to the shock brought about by the freezing process prior to lyophilization, since no cryoprotectant was added. The cryoprotectants can prevent the osmotic shock caused on the microorganisms by the formation of ice crystals during freezing (Morgan et al., 2006; Nakamura et al., 2009).

Concerning the total microbial counts, these were below the countable range (<3.00x10^3 CFU/mL) at T0wk and during the whole 12-wk storage period for both conditions (data not shown).

3.5.2.2. Fatty acid profiles

Regarding the FA composition, in the control lyophilized milk, there were variations in free SFA and MUFA amounts, but values ended up being lower ($p<0.05$) at T12wk than at the beginning of the study (**Table 3.37**), specially for C14:0 (from 0.794 to 0.510 mg/g), C16:0 (from 2.533 to 1.473 mg/g), C18:0 (from 0.845 to 0.433 mg/g) and C18:1 c9 (from 1.071 to 0.737 mg/g). The free PUFA also decreased slightly (from 0.193 to 0.156 mg/g), although not significantly ($p>0.05$; **Table 3.37**). It is most likely that these FA got oxidized, as FFA are susceptible to oxidation (Musakhanian et al., 2022). As for EFA, there was a general increase ($p<0.05$) in FA contents throughout storage (**Table 3.38**). A possible explanation for such a finding is that the *B. breve* DSM 20091 during fermentation could have produced some compound(s) that interfered with lyophilized milk EFA composition.

With respect to CLNA-enriched milk, free SFA varied during storage but their content reach significantly ($p<0.05$) lower values by the end of the study in comparison to T0wk (**Table 3.37**), this includes C14:0 (from 0.466 to 0.299 mg/g) and C16:0 (from 3.186 to 2.608 mg/g), while C18:0 always tended to decrease throughout storage (from 3.440 to 1.818 mg/g). This suggests that an oxidation process has occurred, similar to the control. Free MUFA increased ($p<0.05$) with storage, more

precisely, C18:1 c9 (from 7.357 to 8.049 mg/g) (**Table 3.37**). The same was observed for free PUFA (**Table 3.37**), specifically for the CLA (C18:2 c9t11 – from 0.526 to 1.718 mg/g; C18:2 CLA t,t – from 0.145 to 0.307 mg/g) and CLNA isomers (C18:3 c9t11c15 – from 5.119 mg/g to 10.954 mg/g; C18:3 t9t11c15 – from 1.150 to 1.844 mg/g). On the other hand, the α-LNA decreased ($p<0.05$) after storage (from 6.163 to 3.449 mg/g) (**Table 3.37**), and, although not significantly different ($p>0.05$), the LA content at T12wk (4.341 mg/g) was lower than at the beginning of the assay (4.602 mg/g). Taken all together, it is possible that the remaining LA and α-LNA were further converted into their conjugated forms during CLNA-enriched lyophilized milk storage. According to the previously mentioned results (**Section 3.5.2.1 Microbiological enumeration**; in Results and Discussion of Part V), the *B. breve* DSM 20091 was no longer viable at the start of the stability assay, and, being this a lyophilized product, it is improbable that the strain's enzymatic system is kept active, as suggested for the CLNA increment observed in the enriched fermented milk (**Section 3.5.1.2 Fatty acid profiles**; in Results and Discussion of Part V). This because enzymes need a minimum amount of water to function (Chisti, 2020). So, CLA and CLNA isomers formation seems unlikely of biological nature. However, it has been suggested that LA can isomerize into CLA through oxidative reactions (Rodríguez-Alcalá et al., 2014). Considering the proposed oxidation of free SFA, we hypothesized that this might have generated an oxidative environment that led to LA and α-LNA isomerization in the CLNA-enriched lyophilized milk. Perhaps the increase observed for C18:1 c9 could be of chemical nature as well, resulting from LA or α-LNA transformation somehow.

Regarding the EFA, general SFA maintained similar values, however, C18:0 decreased during storage (from 14.636 to 12.507 mg/g) (**Table 3.38**). Esterified MUFA increased with storage (**Table 3.38**), more precisely, C16:1 c9 (from 1.955 to 2.340 mg/g) and C18:1 c9 (from 21.786 to 25.048 mg/g), as was observed for PUFA (**Table 3.38**), namely, LA (from 3.061 to 3.930 mg/g), α-LNA (from 0.855 to 1.080 mg/g) and C18:2 c9t11 (from 0.338 to 0.482 mg/g). As suggested for the control, some bacterial compound(s) could have affected EFA composition, however that was reflected only in certain MUFA and PUFA of the CLNA-enriched milk, principally FA with an 18-carbon chain. On the other hand, there was a decrease in C18:0, as previously mentioned, which could suggest that this FA was desaturated into C18:1 c9, this subsequently into LA/CLA and then α-LNA, although that seems difficult to occur, since it requires desaturase enzymes (Halim et al., 2022), and being EFA, it is not a common natural reaction.

To the best of our knowledge, no other research work has studied the stability throughout storage of a powder-like dairy product enriched in CFA. Even though, comparing with a similar matrix, Rodríguez-Alcalá et al. (2019) reported no changes on FA composition of a commercial infant formula stored at room temperature until the end of shelf life - 12 weeks. However, being a commercial product, it will contain preservatives, which retard food spoilage caused by microbial growth or oxidation. Thus, it would be important to incorporate antioxidants in CLNA-enriched lyophilized milk formula in the future to avoid the suggested occurring oxidation processes.

Table 3.37 Free fatty acid composition of control and CLNA-enriched lyophilized milks during twelve weeks of storage at room temperature.

FA (mg/g)	Control				CLNA-enriched milk			
	T0wk	T4wk	T8wk	T12wk	T0wk	T4wk	T8wk	T12wk
C4:0	0.191±0.009^{b}	0.245±0.022^{a}	0.243±0.006^{a}	0.212±0.030^{ab}	0.191±0.004^{b}	0.208±0.015^{ab}	0.217±0.003^{a}	0.208±0.001^{ab}
C6:0	0.141±0.004^{b}	0.173±0.016^{a}	0.194±0.011^{a}	0.152±0.022^{ab}	0.139±0.006^{b}	0.144±0.012^{ab}	0.162±0.004^{a}	0.142±0.005^{b}
C8:0	0.092±0.001^{b}	0.101±0.008^{b}	0.131±0.007^{a}	0.090±0.014^{b}	0.082±0.004^{ab}	0.082±0.009^{ab}	0.102±0.001^{a}	0.079±0.002^{b}
C10:0	0.219±0.002^{b}	0.193±0.011^{b}	0.302±0.015^{a}	0.191±0.023^{b}	0.167±0.015^{b}	0.149±0.013^{b}	0.222±0.002^{a}	0.145±0.011^{b}
C12:0	0.261±0.015^{b}	0.169±0.010^{c}	0.326±0.009^{a}	0.199±0.018^{c}	0.174±0.013^{b}	0.137±0.012^{c}	0.264±0.004^{a}	0.151±0.009^{bc}
C14:0	0.794±0.060^{a}	0.393±0.015^{c}	0.898±0.090^{a}	0.510±0.048^{b}	0.466±0.018^{b}	0.300±0.032^{c}	0.752±0.011^{a}	0.299±0.013^{c}
C14:1 c9	0.091±0.005^{ab}	0.075±0.001^{b}	0.102±0.002^{a}	0.056±0.007^{c}	0.068±0.005^{ab}	0.060±0.002^{b}	0.082±<0.001^{a}	0.053±0.002^{b}
C15:0	0.073±0.007^{a}	0.033±0.001^{b}	0.080±0.011^{a}	0.045±0.006^{b}	0.048±0.001^{b}	0.030±0.002^{c}	0.076±0.003^{a}	0.032±<0.001^{c}
C16:0	2.533±0.231^{a}	1.000±0.048^{c}	2.704±0.419^{a}	1.473±0.137^{b}	3.186±0.110^{b}	2.557±0.061^{c}	4.459±0.195^{a}	2.608±0.133^{c}
C16:1 c9	0.106±0.007^{b}	0.066±0.003^{c}	0.143±0.015^{a}	0.088±0.010^{bc}	0.091±0.001^{b}	0.074±0.003^{c}	0.148±0.002^{a}	0.080±0.003^{bc}
C17:0	0.037±0.004^{a}	0.015±0.002^{b}	0.037±0.006^{a}	0.022±0.002^{ab}	0.039±0.001^{b}	0.030±<0.001^{b}	0.052±<0.001^{a}	0.029±0.002^{b}
C18:0	0.875±0.084^{a}	0.330±0.023^{c}	0.770±0.150^{a}	0.433±0.051^{b}	3.440±0.083^{a}	3.315±0.085^{a}	2.378±0.027^{b}	1.818±0.060^{c}
C18:1 c9	1.071±0.092^{a}	0.430±0.022^{c}	1.277±0.149^{a}	0.737±0.073^{b}	7.357±0.257^{c}	7.453±0.191^{c}	9.060±0.228^{a}	8.049±0.225^{b}
C18:2 c9c12 (LA)	0.155±0.011^{b}	0.079±0.005^{c}	0.219±0.011^{a}	0.127±0.007^{b}	4.602±0.346^{ab}	3.957±0.622^{b}	4.804±0.055^{a}	4.341±0.203^{b}
C18:3 c9c12c15 (α-LNA)	0.022±0.005^{ab}	0.013±0.001^{b}	0.035±0.006^{a}	0.017±0.001^{b}	6.163±1.245^{a}	3.523±1.217^{b}	3.572±0.149^{b}	3.449±0.339^{b}
C18:2 c9t11 (CLA)	0.016±0.003	ND	0.019±0.001	0.013±0.001	0.526±0.108^{b}	1.263±0.365^{a}	1.743±0.133^{a}	1.718±0.121^{a}
C18:2 CLA t,t	ND	ND	ND	ND	0.145±0.008^{b}	0.274±0.059^{a}	0.303±0.039^{a}	0.307±0.030^{a}
C18:3 c9t11c15 (CLNA)	ND	ND	ND	ND	5.119±0.729^{c}	8.417±0.820^{b}	11.261±0.131^{a}	10.954±0.021^{a}
C18:3 t9t11c15 (CLNA)	ND	ND	ND	ND	1.150±0.006^{c}	1.588±0.080^{b}	1.843±0.146^{ab}	1.844±0.061^{a}
SFA	5.217±0.390^{a}	2.650±0.108^{c}	5.685±0.645^{a}	3.327±0.351^{b}	7.932±0.135^{b}	6.951±0.241^{c}	8.685±0.179^{a}	5.513±0.235^{d}
MUFA	1.268±0.104^{a}	0.570±0.025^{c}	1.522±0.166^{a}	0.881±0.090^{b}	7.516±0.251^{c}	7.587±0.197^{c}	9.291±0.225^{a}	8.182±0.230^{b}
PUFA	0.193±.0.019^{b}	0.092±0.005^{c}	0.273±0.004^{a}	0.156±0.008^{b}	17.706±0.739^{b}	19.022±0.516^{b}	23.525±0.542^{a}	22.613±0.309^{a}
Σ FA	6.678±0.512^{a}	3.313±0.136^{c}	7.481±0.815^{a}	4.364±0.448^{b}	33.154±1.124^{c}	33.561±0.954^{c}	41.501±0.588^{a}	36.308±0.774^{b}

Average values ± standard deviation (n=3). Different superscript letters indicate significant differences between storage times of each lyophilized milk sample ($p<0.05$).

FA – Fatty acid; T0wk – Time zero weeks; T4wk – Time four weeks; T8wk – Time eight weeks; T12wk – Time twelve weeks; *c* – *cis* double bond; *t* – *trans* double bond; LA – Linoleic acid; α-LNA – alpha-Linolenic acid; CLA – Conjugated linoleic acid; CLNA – Conjugated linolenic acid; SFA – Saturated fatty acids; MUFA – Monounsaturated fatty acids; PUFA – Polyunsaturated fatty acids; Σ FA – Total fatty acids; ND – Not detected.

Table 3.38 Esterified fatty acid composition of control and CLNA-enriched lyophilized milks during twelve weeks of storage at room temperature.

FA (mg/g)	Control				CLNA-enriched milk			
	T0wk	T4wk	T8wk	T12wk	T0wk	T4wk	T8wk	T12wk
C4:0	1.914±0.046^{c}	2.546±0.139^{ab}	2.749±0.039^{a}	2.381±0.027^{b}	2.075±0.086^{b}	2.101±0.113^{ab}	2.348±0.072^{a}	2.181±0.034^{ab}
C6:0	1.782±0.013^{c}	2.342±0.112^{ab}	2.557±0.016^{a}	2.204±0.060^{b}	1.940±0.085	1.938±0.091	2.199±0.073	2.025±0.036
C8:0	1.251±0.032^{c}	1.686±0.052^{b}	1.856±0.008^{a}	1.555±0.007^{b}	1.381±0.071	1.374±0.056	1.564±0.072	1.399±0.022
C10:0	3.425±0.105^{c}	4.566±0.187^{b}	5.097±0.013^{a}	4.350±0.022^{b}	3.790±0.140^{b}	3.782±0.203^{b}	4.329±0.176^{a}	3.993±0.029^{b}
C12:0	4.277±0.150^{c}	5.691±0.229^{b}	6.349±0.043^{a}	5.426±0.019^{b}	4.765±0.170^{b}	4.692±0.260^{b}	5.336±0.211^{a}	4.958±0.029^{b}
C14:0	13.204±0.459^{c}	17.520±0.673^{b}	19.506±0.194^{a}	16.647±0.043^{b}	14.718±0.450	14.471±0.784	16.326±0.647	15.193±0.088
C14:1 c9	1.130±0.038^{c}	1.507±0.059^{b}	1.944±0.044^{a}	1.533±0.005^{b}	1.268±0.039^{b}	1.263±0.078^{b}	1.566±0.077^{a}	1.394±0.028^{ab}
C15:0	1.180±0.040^{c}	1.579±0.052^{b}	1.764±0.015^{a}	1.507±0.003^{b}	1.319±0.043	1.308±0.060	1.472±0.060	1.374±0.009
C16:0	40.838±1.416^{c}	54.243±2.072^{b}	60.314±0.734^{a}	51.438±0.118^{b}	45.764±1.355^{b}	45.044±2.491^{b}	50.619±1.973^{a}	47.134±0.214^{b}
C16:1 c9	1.770±0.078^{d}	2.305±0.074^{c}	3.005±0.072^{a}	2.586±0.033^{b}	1.955±0.064^{b}	2.112±0.363^{ab}	2.495±0.037^{a}	2.340±0.005^{a}
C17:0	0.550±0.025^{d}	0.731±0.027^{b}	0.806±0.008^{a}	0.677±0.001^{c}	0.615±0.014	0.610±0.037	0.674±0.025	0.621±0.009
C18:0	12.823±0.481^{b}	17.071±0.602^{a}	16.264±0.264^{a}	13.662±0.055^{b}	14.636±0.403^{a}	14.418±0.803^{a}	13.777±0.458^{ab}	12.507±0.033^{b}
C18:1 c9	18.515±0.610^{c}	24.589±0.973^{b}	30.334±0.334^{a}	26.009±0.002^{b}	21.786±0.648^{b}	21.503±1.209^{b}	26.602±1.054^{a}	25.048±0.133^{a}
C18:2 c9c12 (LA)	2.531±0.087^{d}	3.563±0.128^{c}	4.662±0.012^{a}	4.013±0.005^{b}	3.061±0.078^{b}	3.123±0.182^{b}	4.144±0.149^{a}	3.930±0.017^{a}
C18:3 c9c12c15 (α-LNA)	0.260±0.010^{d}	0.362±0.018^{c}	0.507±0.017^{a}	0.428±0.006^{b}	0.855±0.040^{b}	0.844±0.062^{b}	1.128±0.017^{a}	1.080±0.013^{a}
C18:2 c9t11 (CLA)	0.225±0.015^{d}	0.341±0.015^{c}	0.521±0.008^{a}	0.432±0.017^{b}	0.338±0.016^{b}	0.325±0.025^{b}	0.520±0.017^{a}	0.482±0.012^{a}
SFA	81.246±2.767^{c}	107.975±4.145^{ab}	117.263±1.303^{a}	99.846±0.354^{b}	91.002±2.817	89.736±4.899	98.646±3.767	91.385±0.503
MUFA	21.416±0.726^{c}	28.401±1.106^{b}	35.283±0.363^{a}	30.129±0.036^{b}	25.009±0.751^{b}	24.877±1.650^{b}	30.663±1.167^{a}	28.782±0.156^{a}
PUFA	3.017±0.112^{d}	4.265±0.161^{c}	5.690±0.021^{a}	4.873±0.028^{b}	4.254±0.102^{c}	4.292±0.269^{c}	5.791±0.149^{a}	5.491±0.016^{b}
Σ FA	105.678±3.605^{c}	140.641±5.413^{b}	158.236±1.687^{a}	134.848±0.419^{b}	120.266±3.670^{b}	118.905±6.818^{b}	135.100±5.083^{a}	125.658±0.676^{ab}

Average values ± standard deviation (n=3). Different superscript letters indicate significant differences between storage times of each lyophilized milk sample ($p<0.05$).

FA – Fatty acid; T0wk – Time zero weeks; T4wk – Time four weeks; T8wk – Time eight weeks; T12wk – Time twelve weeks; *c* – *cis* double bond; *t* – *trans* double bond; LA – Linoleic acid; α-LNA – alpha-Linolenic acid; CLA – Conjugated linoleic acid; SFA – Saturated fatty acids; MUFA – Monounsaturated fatty acids; PUFA – Polyunsaturated fatty acids; Σ FA – Total fatty acids.

CHAPTER 4

General Conclusions

CHAPTER 4 – GENERAL CONCLUSIONS

The CLA and CLNA isomers constitute interesting candidates as functional ingredients with potential anti-carcinogenic and anti-obesity properties. However, limitations in terms of concentration and availability hamper the possibility of achieving an effective dose from their natural sources. Several probiotic strains have been described with LA/α-LNA isomerization capacity so, by promoting *in situ* microbial enrichment of food matrices with those conjugated isomers may constitute an alternative strategy to increment daily consumption of CLA and CLNA. Therefore, in the present PhD thesis, the development of a new dairy product enriched in microbial CLA and CLNA isomers was studied. To do so, in a first stage, genotypic, substrate tolerance and phenotypic screenings were performed for the selection of CLA/CLNA-producing strains. Then, to enable a more economically viable, cost-effective technological process given the fact that pure precursor LA/α-LNA substrates are very expensive, commercially available vegetable oils were initially hydrolyzed to efficiently release free LA/α-LNA substrate sources and these were then studied to obtain a CLA/CLNA-enriched fermented milk. Thereafter, it was evaluated if further FA metabolites were being produced during this enrichment process. The developed CLNA-enriched fermented milk was thoroughly characterized in terms of microbiological, physico-chemical, biochemical and organoleptic properties in order to assess its feasibility as an efficient CLNA delivery food matrix. Finally, the shelf-life stability of this former dairy product was assessed as well as that of an alternative delivery vehicle, i.e. CLNA-enriched lyophilized milk. From the results obtained throughout the outlined research work, the following conclusions were drawn:

- The development of LAI primers based on conserved motifs revealed improved efficiency in the identification of potential CLA-producing strains. However, very few strains possessing putative genes encoding enzymes involved in LA isomerization could yield CLA isomers, even though LA reduction percentages were considerably high. Moreover, no association was found between CLA production potential and LA tolerance that could corroborate the hypothesized detoxifying mechanism behind LA conversion. Therefore, the selection of potentially CLA-producing bacteria cannot be performed relying solely on genotypic screening and/or substrate tolerance. Despite this, *B. breve* DSM 20091 revealed the best CLA and CLNA yields (>50%), being selected for the development of a CLA/CLNA-enriched fermented milk.
- The CRL lipase turned out to have the best efficiency in releasing FFA from any of the edible vegetable oils assayed. Due to cost reasons, SBO and FSO, which are rich in the precursor LA and α-LNA, respectively, were selected to study microbial CLA/CLNA-enrichment with *B. breve* DSM 20091, after previous hydrolysis with CRL. In a first stage, the best outcome was achieved with hydrolyzed FSO at 0.5 mg/mL α-LNA, being mainly produced CLNA isomers (0.34 mg/g). Higher α-LNA concentrations (with hydrolyzed FSO) were further tested, where maximum milk enrichment, of mostly CLNA (0.88 mg/g), was reached at 2 mg/mL α-LNA. The obtained results suggest that it is possible to increase the bioavailability of LA and α-

LNA in milk for *B. breve* DSM 20091 to produce CFA, by using lipase-hydrolyzed commercial edible vegetable oils. However, this strain preferentially produces CLNA than CLA isomers.

- Apart from apparent LA conversion into stearic acid, when applied alone, microbial enrichment of milk with CLA or CLNA isomers was not accompanied by further side-FA metabolites production, either because the biotransformation process does not involve other compounds or due to high enzymatic kinetics. Despite this, the GC-MS analysis corroborated previous studies on the production of CLA and CLNA isomers by *B. breve* DSM 20091 and the influence of substrates on its enzymatic activity, namely, the reduced LA bioconversion due to possible higher specificity for α-LNA. Moreover, this study enabled the discovery of two other CLNA isomers that have never been reported for *Bifidobacterium* strains. The obtained data suggests that those isomers might be C18:3 t10c12c15 and C18:3 c9c11c15.
- The developed fermented milk enriched in microbial CLNA isomers produced by *B. breve* DSM 20091, using hydrolyzed FSO as substrate source, revealed acceptable biochemical and nutritional characteristics, comparable to other similar food products. Regarding its sensory properties, the CLNA-enriched fermented milk lacked important flavor contributors, and a certain astringency and bitterness prevailed.
- During a 28-day refrigerated storage period of CLNA-enriched fermented milk, microbiological enumeration and pH values brought no concerns. Moreover, CLNA isomers (the bioactive compound) increased throughout storage, most likely due to active enzymatic systems from the *B. breve* strain. On the other hand, results suggested the occurrence of an oxidation process of some saturated FA, which could have contributed to quality loss and compromised the organoleptic properties of the developed fermented milk. Furthermore, although being microbiologically stable, there were FA alterations during CLNA-enriched lyophilized milk storage over 12 weeks at room temperature that seemed of oxidative nature as well, including the apparent conversion of LA and α-LNA into conjugated forms.

In conclusion, the identification of efficient CLA/CLNA-producing strains for the development of enriched food matrices showed some drawbacks, since genotypic and substrate tolerance screening techniques are not reliable, especially when other factors might be interfering, such as, inhibition of gene or enzyme(s) expression, production of other compounds or non-optimal growth conditions. With the selected bacterium – *B. breve* DSM 20091 – it was not possible to simultaneously enrich fermented milk with both CLA and CLNA, since this strain seems to have a preference to isomerize α-LNA. Despite this, it was possible to obtain a fermented milk with up to 1 mg/g of CLNA isomers by using a previously hydrolyzed commercially available vegetable oil (i.e., FSO). To the best of our knowledge, this is the first study to employ edible vegetable oils as substrate sources in microbial CLNA-enrichment and to attain such amounts of CLNA isomers in a dairy product. No further FA metabolites were detected from the fermentation process, on the other hand, two additional CLNA isomers never reported before for bifidobacteria were discovered. The CLNA-enriched fermented milk revealed reasonable compositional properties, but there are organoleptic traits to tackle. Moreover, the CLNA content increased during storage, but oxidation processes seemed to occur.

The CLNA-enriched lyophilized milk, which contained almost 10-times more CLNA than the fermented product, revealed oxidation concerns throughout storage as well that would require development of further strategies (i.e. addition of an antioxidant compound).

Therefore, the findings from this research work revealed that the application of *in situ* microbial production of CLA and CLNA isomers to increment the daily intake of these potential bioactive compounds faces several challenges to become an even more feasible strategy.

CHAPTER 5

Future Prospects

CHAPTER 5 – FUTURE PROSPECTS

Through the work conducted during this PhD thesis it was observed that the *in situ* strategy to develop new dairy products enriched in microbial CFA isomers is challenging to achieve, and according to the gathered results several aspects remain to be improved.

The identification of CLA/CLNA-producing strains through culturing assays with precursor substrates followed by FA analysis by chromatographic methods becomes laborious and time-consuming, especially when the number of strains to screen is extensive. Therefore, faster techniques for the selection of potential CLA/CLNA-producers have been studied. The substrate tolerance screening was not reliable, as no association was found between the high-tolerant strains and the genotypically-positive ones, i.e., with CLA/CLNA-production potential. The best approach would be through the genotypic screening of enzymes involved in LA/α-LNA isomerization, however, not all positive strains showed the ability to produce conjugated isomers. Nevertheless, there are some features that could be addressed in a way to improve its reliability. First, the designed primers based on conserved motifs revealed better results than reported species-specific primers. Therefore, the design of primers of involved enzymes based on conserved motifs of the largest number of species possible should be studied to obtain more comprehensive primers for the selection of potential CLA/CLNA-producing strains. Then, the lack of substrate isomerization into conjugated forms could be a matter of no gene or enzyme expression, so these aspects should be evaluated along with the factors that could be interfering, such as, pH.

Discrepancies were observed between substrate reduction and actual conversion into conjugated isomers, so it was further hypothesized that LA/α-LNA might have been metabolized into other compounds, since the formation of CLA/CLNA may involve intermediate FAs. Therefore, GC-MS analysis of FA profile and hydroxy FA analysis was performed, but no further FA compounds were found. Analysis of other lipid classes might elucidate what happens to the fraction of substrate that is not isomerized, thus, comprehensive lipidomic analysis must be further undertaken. Perhaps, it could be found other metabolites with bioactive interest.

During GC-MS analysis of the developed CLNA-enriched fermented milk it was detected the presence of two further CLNA isomers never described for other bifidobacteria strains. Thus, it would be of interest to confirm its structural configuration in the future, through appropriate techniques, like DMOX derivatization.

Sensory analysis of the CLNA-enriched fermented milk revealed that some organoleptic attributes were not the most appropriate for a dairy product, thus, further studies to improve such properties need to be performed, namely, co-culturing with conventional yogurt or lactobacilli starter cultures and addition of an aroma. Moreover, its stability throughout storage revealed potential occurrence of oxidation processes, so the inclusion of an antioxidant in the microbial CLNA-enriched fermented milk formulation should also be addressed in the future.

Through the lyophilization of the CLNA-enriched fermented milk it was possible to increment the amount of CLNA and develop an alternative delivery system for consumers, however, the incorporation of cryoprotectants (to maintain the probiotic strain viability) and of natural antioxidant

compounds (to prevent oxidation) needs to be further assessed. Moreover, it could be further evaluated the possibility of compacting this powdered dairy product to develop a nutraceutical supplement of CLNA isomers.

With the above-mentioned properties optimized, it would be of interest to validate the bioactive potential of the developed CLNA-enriched dairy products, thus, in a first stage, products should be tested in animal models of health conditions such as cancer or obesity, and, in a second stage, in humans through clinical trials.

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