UNIVERSIDADE FEDERAL DO RIO DE JANEIRO

CARLA PAULO VIEIRA

CONJUGATED LINOLEIC ACID (CLA): ELABORATION OF ENRICHED FERMENTED MILK AND EVALUATION OF CLA-ISOMERS EFFECTS ON NEUROINFLAMMATORY DISEASE *IN VITRO* MODEL

RIO DE JANEIRO

2020

Carla Paulo Vieira

CONJUGATED LINOLEIC ACID (CLA): ELABORATION OF ENRICHED FERMENTED MILK AND EVALUATION OF CLA-ISOMERS EFFECTS ON NEUROINFLAMMATORY DISEASE *IN VITRO* MODEL

1 volume

Ph.D thesis presented to Food Science Postgraduate Program, Instituto de Química, Universidade Federal do Rio de Janeiro, as part of necessary requirements for achievement of Doctor of Philosophy degree in Sciences (Food Science).

Advisor: Professor Doctor Carlos Adam Conte Junior

RIO DE JANEIRO

2020

CIP - Catalogação na Publicação

Vieira, Carla Paulo
V658c Conjugated Linoleic acid (CLA): elaboration of enriched fermented milk and evaluation of CLA isomers effects on neuroinflammatory disease in vitro mode / Carla Paulo Vieira. -- Rio de Janeiro, 2020.
139 f.

Orientador: Carlos Adam Conte Junior.
Tese (doutorado) - Universidade Federal do Rio de Janeiro, Instituto de Química, Programa de Pós Graduação em Ciência de Alimentos, 2020.

1. Ácido liniléico conjugado (CLA). 2. Leite fermentado enriquecido. 3. Microglia. 4. Neuroinflamação. 5. Viabilidade neuronal. I. Conte Junior, Carlos Adam , orient. II. Título.

Elaborado pelo Sistema de Geração Automática da UFRJ com os dados fornecidos pelo(a) autor(a), sob a responsabilidade de Miguel Romeu Amorim Neto - CRB-7/6283.

Carla Paulo Vieira

CONJUGATED LINOLEIC ACID (CLA): ELABORATION OF ENRICHED FERMENTED MILK AND EVALUATION OF CLA-ISOMERS EFFECTS ON NEUROINFLAMMATORY DISEASE *IN VITRO* MODEL

Ph.D thesis presented to Food Science Postgraduate Program, Instituto de Química, Universidade Federal do Rio de Janeiro, as part of necessary requirements for achievement of Doctor of Philosophy degree in Sciences (Food Science).

Rio de Janeiro, April 29, 2020.

Prof. Dr. Carlos Adam Conte Junior, IQ-UFRJ and FV-UFF (advisor)

Prof. Dr(a). Debora Foguel, UFRJ (outer member)

Prof. Dr. Paulo Renato Zuquim Antas, Fiocruz (outer member)

Prof. Dr. Luiz Anastácio Alves, Fiocruz (outer member)

Prof. Dr(a). Marion Pereira da Costa, UFBA (outer member)

ACKNOWLEDGEMENTS

Firstly, I would like to thank my advisor prof. Dr. Carlos Adam Conte Junior for years of teaching and support and for being ready for the challenge, which was the theme of this thesis for us.

I would like to thank all the collaborators of the research team (UFRJ, UFF and Fiocruz) for their commitment, dedication and solicitude. Without you, it would be impossible to develop this project. Therefore, I express my sincere gratitude to professors Dr. Paulo R. Z. Antas, Renata Frauches, Rafael M. Meyer, Flavia R. S. Lima, Luciana Romão, Debora Foguel, Luiz A. Alves, Débora D. R. Lima, Celio G. F. de Lima, and other team members. I thank you for contributing to improve the impact of this work.

I would like to thank the public Universities (UFRJ and UFF), Fiocruz and Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq) for the financial support, which allowed me to improve my scientific knowledge and skills.

Finally, I would like to thank my parents and brother for the encouragement and support since my childhood. I also would like to thank my friends for affection. I express my sincere gratitude to all you for love, comprehension and support during my PhD development.

ABSTRACT

Vieira, Carla Paulo. Conjugated linoleic acid (CLA): elaboration of enriched fermented milk and evaluation of CLA-isomers effects on neuroinflammatory disease *in vitro* model. Rio de Janeiro, 2020. Thesis (Ph.D.'s degree in Sciences). Conjugated linoleic acid (CLA) is a mixture of geometric and positional conjugated isomers of linoleic acid (LA). CLA isomers are naturally present in foods, however, generally in small concentrations. Therefore, their intake values through natural sources are below of level recommended for achieving health beneficial effects. Thus, to increase the CLA content in foods is a technological challenge. Currently, the milk fermentation with CLA-producing bacteria is one of main technologies strategies studied with this objective. Nevertheless, the previous screening of lactic acid bacteria (LAB) by ability to produce CLA from LA in esterified way, similar those found in milk fat, still is a bottleneck in literature. Regard to bioactive effect of CLA, although CLA can freely get through the blood-brain barrier, it being metabolized in the brain, reports on CLA effects in the central nervous system are still scarce. Studies revealed CLA as potent anti-inflammatory modulator of immune response of peripheral macrophages and these effects of CLA are thought to be mainly via inhibition of NF-κB pathway in human *in vivo* models. Recently, microglial NF-κB was proposed as a pivotal therapeutic target in neurodegenerative disorders as Parkinson´s disease. CLA also primes macrophage M2 polarization in human *in vitro* models, and this would allow the targeting microglia polarization by CLA as a therapeutic avenue in neurodegenerative diseases. However, the effects of CLA on microglia are still unknown. In this context, the original hypothesis of first step of this work was that previous screen of LAB by ability to produce CLA from sterified lipid produces CLA-enriched fermented milk. In the second step of work, the major hypothesis was that CLA has antiinflammatory effect on microglial activation inhibiting the NF-κB pathway. The results of the first step of the present work showed that the previous selection of probiotic LAB strain by the ability to produce CLA from sunflower oil was a differential step to produce CLA-enriched fermented milk. Therefore, the probiotic *Lactococcus lactis* ssp. *cremoris* MRS 47 strain isolated from Brazilian kefir grains can increase the CLA content in the fermented milk. In the second step, CLA mitigated the mice primary microglia activation by LPS to reduce NO secretion. CLA completely prevented the suppression of arginase activity by LPS in primary microglia, driven it from M1 to M2 phenotype. This polarization was isomer-specific and, at least in part, due to inhibition of NF-κB and iNOS pathways through expression of IκBα inhibitor. Finally, co-treatment with CLA and LPS completely prevented cortical neuronal death induced by the endotoxin, being this neuroprotection also isomer-specific. Thus, CLA has potential as anti-inflammatory and neuroprotective agents against microglia-mediated neuroinflammatory disorders, in what seems to have isomer-specific particularities.

Keywords: CLA-producer strain screening, enriched fermented milk, LPS-activated microglia, microglial polarization, neuroinflammation, neuronal viability

LIST OF ABBREVIATIONS AND ACRONYMS

Aβ	Aβ
CLA	Conjugated linoleic acid
CVD	Cardiovascular disease
FA	Fatty acids
FAME	Fatty acid methyl ester
GC	Gas chromatography
HDL	High density lipoprotein
HPLC	High-performance liquid chromatography
IBD	Inflammatory bowel disease
IκBα	Inhibitory Subunit of NF-κBα
IL	Interleukin
iNOS	Inducible nitric oxide synthase
LA	α-linolenic acid
LA	Linoleic acid
LAB	Lactic acid bactéria
LAI	Linoleic acid isomerase
LDL	Low density lipoproteins
MUFA	Monounsaturated fatty acid
NF-κB	Nuclear factor kappa B
PPAR	Peroxisome proliferator-activated receptors
PUFA	Polyunsaturated fatty acid
SCD	Stearoyl-CoA desaturase
SFA	Saturated fatty acid
TNF-α	Tumor necrosis factor alpha
TVA	*trans*-11 vaccenic acid
UHT	Ultra-high temperature

LIST OF FIGURES

LIST OF TABLES

LIST OF FRAMES

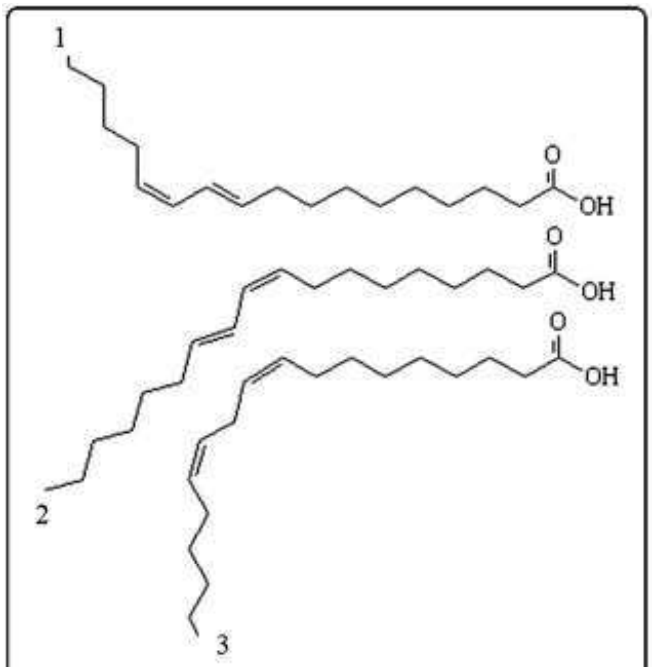
SUMMARY

1 LITERATURE REVIEW

1.1 CHEMICAL STRUCTURE AND SYNTHESIS OF CLA ISOMERS

Conjugated linoleic acid (CLA) is a collective term for a series of conjugated dienoic positional and geometrical isomers of linoleic acid - LA (C18:2, *cis*-9, *cis*-12) (KIM *et al.*, 2016A), as illustrated in Figure 1. CLA-isomers are naturally present in food, mainly those of ruminant origin, such as milk, dairy products, meat and processed meat products from ruminants (MONDRAGÓN, 2016). The CLA-isomers predominate in food from ruminants due to their biosynthesis pathways that occur in these animals: ruminal biosynthesis and endogenous biosynthesis (SHINGFIELD; WALLACE, 2014), which are schematized in Figure 2.

Figure 1 - Isomeric structure. (1) *trans*-10, *cis*-12-CLA (2) *cis*-9, *trans*-11-CLA (3) *cis*-9, *cis*-12-LA.

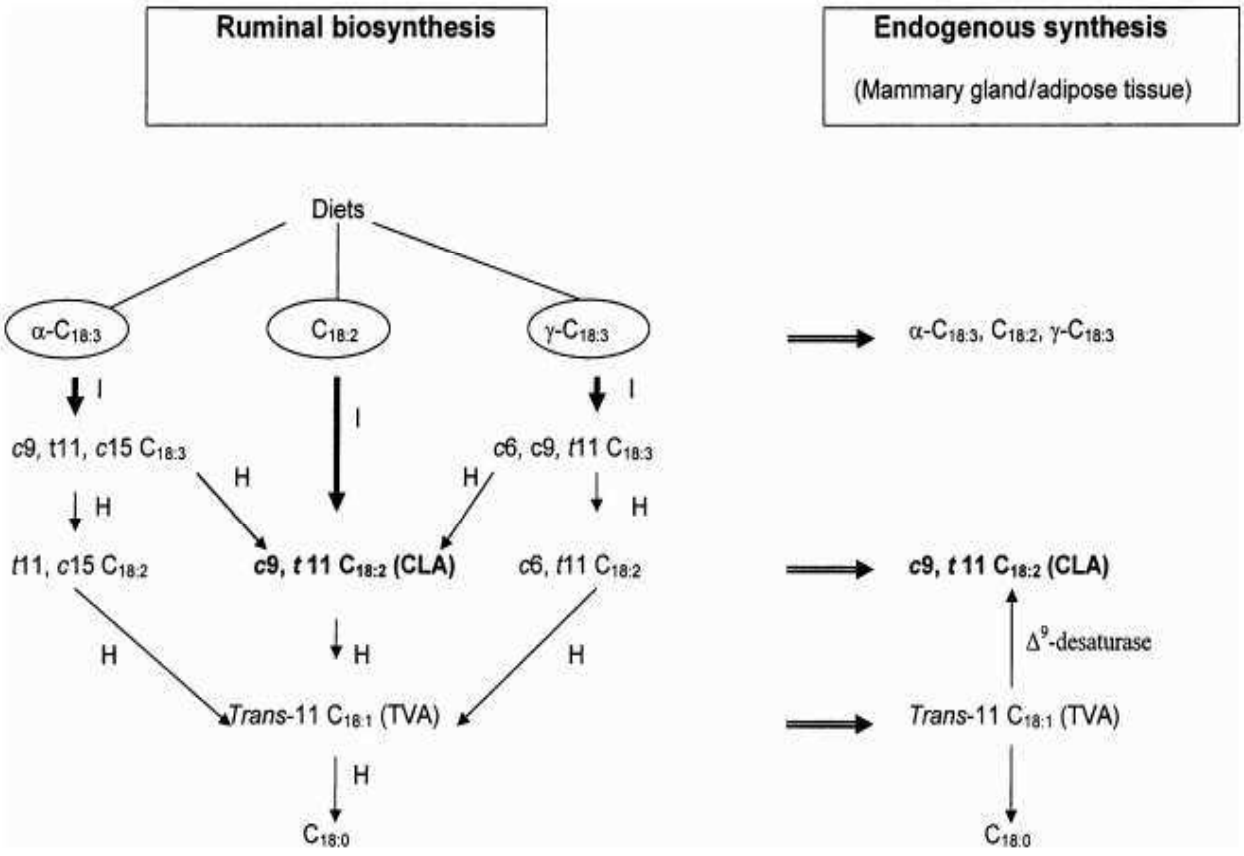


Source: MANCINI; HUNT, 2005.

1.1.1 Ruminal biosynthesis of CLA-isomers

The lipid content in conventional ruminant diets range from 3-7 % on a dietary dry matter basis. Most vegetal origin feed (*e.g.* pasture, silage and plant seeds/oils) are rich in linoleic acid, C18:2 and/or α-linolenic acid (ALA), C18:3 (up to 69.4% and 55.9% of total fatty acids, respectively) (DHIMAN *et al.*, 2007), which are substrates for synthesis of CLA-isomers. Thus, the precursor fatty acids presented in those feed undergo isomerization and biohydrogenation in animal's rumen (SHINGFIELD; WALLACE, 2014). The rumen is a unique organ of ruminants, being responsible for inhabiting microorganisms, which biohydrogenate dietary unsaturated fatty acids as a detoxification mechanism (MAIA *et al.*, 2010). *B. fibrisolvens* was the first shown to be capable of biohydrogenation. However, several other rumen bacterias had been isolated and their biohydrogenation capacity had been acknowledge (LOURENÇO; RAMOS-MORALES; WALLACE, 2010). Biohydrogenation of unsaturated fatty acids in the rumen has several steps (Figure 2). In this way, no single rumen bacterial species can catalyze completely biohydrogenation (DHIMAN *et al.*, 2007).

Figure 2 - Pathways of CLA-isomers production in ruminant species. Pathways of CLA-isomers production in ruminant species: ruminal biosynthesis and endogenous biosynthesis. Conjugated linoleic acid (CLA); TVA, trans vaccenic acid; I, isomerization reaction; H, hydrogenation.



Source: NAM; URE, 2007.

Formerly, the lipids consumed in diet by ruminants on esterified plant lipids or triglycerides way are quickly hydrolyzed to free fatty acids (FA) by lipases of microorganisms being in the rumen. Posteriorly, the unsaturated free FA are hydrogenated by microorganisms in the rumen to produce more highly saturated end products (BUCCIONI *et al.*, 2012). The first intermediate product from biohydrogenation of linoleic acid is *cis*-9*, trans*-11-CLA through linoleate isomerase enzyme, which is produced by the microorganism *Butyrivibrio fibrisolvens*, as well as other bacterial species in the rumen (MIRI; EBRAHIMI; TYAGI, 2015). After, part of the *cis*-9*, trans*-11-CLA produced is rapidly converted into *trans*-11 vaccenic acid (TVA) (CASTILLO; OLIVERA; CARULLA, 2013). Other substrate for CLA production is the α-*cis*-9, *cis*-12, *cis*-15-linolenic acid; C18:3, which also is an unsaturated FA abundant in conventional ruminal diet, also being isomerized and reduced, ending in *cis*-9*, trans*-11-CLA, TVA or with the formation of stearic acid (C18:0) in case of complete biohydrogenation (LEE, 2013). As ruminal biohydrogenation is fast, only small portion of *cis*-9*, trans*-11-CLA and TVA escapes of complete biohydrogenation in the rumen. Therefore, ruminal biohydrogenation is not the major pathway contributing for the *cis*-9*, trans*-11-CLA content in milk and meat fat (DHIMAN *et al.*, 2007).

1.1.2 Endogenous biosynthesis of CLA-isomers

Regarding the endogenous synthesis of CLA-isomers, the TVA produced by microorganisms of rumen reaches the mammary gland and adipose tissue through blood. In these tissues, the TVA is converted to CLA-isomers, mainly cis-9, trans-11-CLA (Jin *et al.*, 2012). Therefore, TVA, which is a positional and geometric isomer of *cis*-9-oleic acid (C18:1), acts as precursor of *cis*-9, *trans*-11 CLA mainly in mammary and adipose tissues of ruminants (SALSINHA *et al.*, 2018), as showed in Figure 2. As result, *cis*-9, *trans*-11 CLA is the predominant CLA-isomer in milk and dairy products as well as in meat and processed meat products from ruminants (MONDRAGÓN, 2016).

Additionally, Stearoyl-CoA desaturase (SCD; Δ9-desaturase) is the enzyme that plays a key role in conversion from TVA to *cis*-9, *trans*-11 CLA in mammary and adipose tissues (MIERLITA *et al.*, 2011). It catalyzes Δ9-cis desaturation of a range of fatty acyl-CoA substrates, being the SCD1 isoform those that predominates in mammary and adipose tissue of ruminant species (JACOBS *et al.*, 2011).

1.1.3 Chemical synthesis of CLA-isomers

Commercial synthetic CLA isomer mixtures present *cis*-9, *trans*-11 and *trans*-10, *cis*-12-CLA as major isomers, although the CLA isomer distributions ranges significantly amongst commercial samples: *cis*-9, *trans*-11 (24.33 to 37.73%); *trans*-10, *cis*-12 (34.93 to 38.92%); *trans*-9, *trans*-11/*trans*-10, *trans*-12 (trace amount to 3.64%) isomers. In addition, those differences in isomeric composition of commercial CLA could be attributed to differences in fatty acid composition of the primal oils used to make the CLA and the isomerization reactions conditions (YU; ADAMS; WATKINS, 2003). Supplements made of CLA isomers are synthesized in the laboratory from linoleic acid derivate from sources such as sunflower, safflower, soybean and corn oils. This reaction implicates alkaline water isomerization (NIEZGODA *et al.*, 2016) and isomerization in propylene glycol (SILVA-RAMÍREZ *et al.*, 2016). Most of the aforementioned CLA isomers are present in food in very tiny amounts apart from have physiological effects of unknown or little biological relevance (DHIMAN; NAM; URE, 2007). Its chemical synthesis also produces various toxic substances. Although substantial amounts of 9- and 10-CLAs can be produced commercially by chemical isomerization, it is necessary to purify the product (which makes its costly and limited) (MITCHELL; MCLEOD, 2008).

1.2 INCIDENCE OF CLA-ISOMERS IN FOOD

A total of 17 natural CLA-isomers were described in milk, dairy products and beef through high-performance liquid chromatography (HPCL) and gas chromatography (CG). These isomers present naturally in foods from ruminants were identified as *cis*-9, *trans*-11; *cis*-8, *trans*-10; *cis*-7, *trans*-9; *cis*-9, *cis*-11; *cis*-11, *cis*-13; *trans*-12, *trans*-14; *trans*-11, *trans*-13; *trans*-10, *trans*-12; *trans*-9, *trans*-11; *trans*-8, *trans*-10; *trans*-7, *trans*-9; *trans*-7, *cis*-9; *trans*-6, *trans*-8; *cis*-12, *trans*-14; *trans*-11, *cis*-13; *cis*-11, *trans*-13; and *cis*-10, *trans*-12 (DHIMAN; NAM; URE, 2007). As previously reported, *cis*-9, *trans*-11-CLA is the predominant CLA-isomer in food of ruminant origin, presenting ratio of 69-79% in relation to total CLA in milk and derivatives and of 59-78% in beef. On the other hand, *trans*-10, *cis*-12-CLA represents less than 2% of total CLA in these food (MARTINS *et al.*, 2007). Trans-10, cis-12-CLA demonstrated a therapeutic potential effect, as lipogenesis reduction (ARIAS *et al.*, 2014), cancer prevenction *in vivo* model (BASSAGANYA-RIERA *et al.*, 2012) and some

human clinical trials, which has drawn the attention of the scientific academy to its study nowadays (McGOWAN *et al.*, 2013).

The variations of CLA levels in food fat from ruminants are due to influence of animal's diet, breed, age, non-nutritive feed additives (such as ionophores) and by the use of synthetic mixtures of CLA supplements (FERNANDEZ; RODRIGUEZ, 2012). Among these factors, the animal's diet is considered more relevant to influence the CLA content, since the more substrates are provided, the greater the CLA synthesis (SIURANA; CALSAMIGLIA, 2016).

1.2.1 Content of CLA-isomers in raw meat and processed meat from ruminants and non-ruminants

The content of CLA (only *cis*-9, *trans*-11-CLA or *cis*-9, *trans*-11-CLA more *trans*-10, *cis*-12-CLA) measured from raw meats is showed in Table 1. The highest concentrations of CLA are in lamb (4.3–19.0 mg/g lipid) and beef (1.2–10.0 mg/g lipid), while for veal and turkey meat the CLA values were 2.7 and 2.5 mg/g lipid, respectively (MONDRAGÓN, 2016). On the other hand, pork, chicken, and meat from horses present the lowest mean concentrations of CLA: 0.65, 0.9 and 0.6 mg/g lipid, respectively. Meat from fish and seafood also are relatively poor in CLA-isomers (0.1-0.8 and 0.3-0.6 mg/g lipid, respectively) (MONDRAGÓN, 2016). Based on those findings, it could be concluded that highest values of CLA are reported for meat from ruminants than non-ruminants. Moreover, data on CLA content in animal's meat that are less usual in human diets, such as meat from elk (1.3–2.1 mg/g fatty acid methyl ester - FAME), bison (2.9–4.8 mg/g FAME), water buffalo (1.83 mg/g fatty acids), and zebu-type cattle (1.47 mg/g fatty acids) are also available (de MENDOZA *et al.*, 2005; RULE *et al.*, 2002). Changes in CLA levels in meat are attributed to seasonal variations, animal genetics, and production practices (ALDAI *et al.*, 2011). In spite of that, for all cases reported, *cis*-9, *trans*-11-CLA is the predominant CLA-isomer in meat. Indeed, *cis*-9, *trans*-11-CLA accounted 78% of the total CLA in beef fat from ruminants (BADIANI *et al.*, 2004). Chin *et al.* (1992) reported that between 76% and 92% (depending on the species) of the total CLA is of *cis*-9, *trans*-11-CLA in beef.

Table 1 - Content of CLA-isomers in raw meats from ruminants and non-ruminants.

Reference	Lamb	Beef	Veal	Pork	Chicken	Turkey	Horse
				(in mg/g fat)			
Chin et al. (1992)	5.6	2.9–4.3[b]	2.7	0.6	0.9	2.5	
Shantha et al. (1994)		5.8–6.8[b]					
Dufey (1999)	11.0[c]	3.6–6.2[a,c]		0.7[c]			0.6[c]
Ma et al. (1999)		1.2–3.0[b,c]					
Raes et al. (2003)		4.0–10.0[a,c]					
Badiani et al. (2004)	4.32						
				(in mg/g FAME)			
Fritsche and Steinhardt (1998)	12.0[c]	6.5[c]		1.2 / 1.5[b,c]	1.5[c]	2.0[c]	
Rule et al. (2002)		2.7–5.6[a,b,d]			0.7[d]		
Wachira et al. (2002)	8.8–10.8[c]						
Knight et al. (2004)	19.0[c]						

FAME = fatty acid methyl ester.
[a] Meat from different production systems/countries.
[b] Different pieces of carcass (and eventually different animals).
[c] Only *c*9,*t*11-18:2 measured.
[d] Only *c*9,*t*11-18:2 and *t*10,*c*12-18:2 measured.

Source: SCHMID; BEE, 2006.

Large variations in the CLA content within muscles of the same species also might occur. However, in some case, the large animal-to-animal variations whithin same specie did not allow to observe differences in the CLA content between breeds or beef muscles (SHANTHA *et al.*, 1994; RAES *et al.*, 2003). Additionally, variations in CLA content of meat from different origins might occur, as reported in beef ribeye steaks purchased from several different stores in western Canada, in which the CLA content varied from 6.18 to 68.50 mg/100 g meat (PREMA *et al.*, 2015). Consistently, differences on CLA concentration in beef from different countries are described. For example, CLA concentration in beef from Argentine, Brazil and USA varied by 70% (3.6–6.2 mg/g lipid), such as Argentine and Brazil presented higher CLA levels than USA. These differences were attributed to differences in animal feeding regime between different regions (DUFEY, 1999).

Regarding to meat products, the CLA content ranged from 0.8 to 6.6 mg/g FAME, as showed in Table 2. Among processed meats, corned beef presented the highest CLA content of 6.6 mg/g FAME, followed for smoked german sausage (4.4 mg/g FAME) and salami (4.2 mg/g FAME). On the other hand, lowest CLA values were found in turkey frank (1.6 mg/g FAME) up to 1.5 and 0.8 mg/g FAME in wiener and smoked bacon, respectively (SCHMID; BEE, 2006). However, it is important to highlight that data on CLA content in meat products are scarce. Meat products were analysed by Chin *et al.* (1992) as well as Fritsche e Steinhardt (1998). CLA amount in meat products is comparable to raw meat. It indicates that the processing method does not influence the CLA content in product (CHIN *et al.,* 1992; FRITSCHE; STEINHARDT, 1998). This corroborates the animal´s diet as most important

factor influencing the CLA content in meat, since it provides the substrates for the CLA synthesis (KHANAL; OLSON, 2004).

Table 2 - Content of CLA-isomers in processed meats (mg/g FAME).

Meat product	*N*	CLA content
Salami	2	4.2
Knackwurst	2	3.7
Black pudding	2	3.0
Mortadella	2	2.9
Wiener	4	1.5/3.6
Liver sausage	2	3.3
Cooked ham	2	2.7
Beef frank	2	3.3
Turkey frank	2	1.6
Beef smoked sausage	2	3.8
Smoked bacon[a]	7	0.8–2.6
Smoked bratwurst	3	2.4
Smoked German sausage for spreading	2	4.4
Smoked ham	2	2.9
Smoked turkey	2	2.4
Minced meat	2	3.5
Corned beef	2	6.6
Potted meat	2	3.0

[a] Different brands.

Source: SCHMID; BEE, 2006.

Regarding to CLA stability to cook and storage, some studies demonstrated that CLA has greater stability than other polyunsaturated fatty acids (PUFA). CLA concentrations (mg/g FAME) did not show reductions in beef patties cooked either rare (60 °C) or well done (80 °C) applying several cooking methods, such as frying, baking, broiling or microwaving (SHANTHA *et al*., 1994). Consistently, Maranesi *et al.* (2005) reported that both broiling and microwave cooking did not influence total CLA (mg/g FAME) in lamb rib-loins. Shantha *et al*. (1994) also examined the effect of storage on CLA concentration in beef patties cooked (60 °C or 80 °C) and submitted to aforementioned cooking methods. No significant changes were observed in beef during 7 days of storage at 4 °C. Newly, it was demonstrated that storage (at -20 °C) of dry aged or wet aged beef during 24 months did not have deleterious effect on the CLA concentrations (MUNGURE *et al.*, 2017). Therefore, CLA is stable to oxidative deterioration during both cook and storage (refrigerated and frozen).

1.2.2 Content of CLA-isomers in milk and derivatives

Milk and dairy products are the most important human dietary sources of CLA-isomers, providing about 70% of the total CLA daily intake (SERAFEIMIDOU *et al.*, 2012). Although the original CLA concentration in raw milk is the most relevant factor influencing the concentration of CLA in dairy product, CLA variations among dairy products elaborated from same milk indicates that technological processing also could have significant role on CLA content (RUIZ *et al.*, 2016). The content of average CLA in whole cow´s milk is of 3.4 mg/g lipid (DHIMAN *et al.*, 2005), but it could achieve up to 30 mg/g lipid depending from source (LAWSON, 2001), as showed in Table 3. This variation is mostly attributed to the dietary regime offered to the animals (GUTIÉRREZ, 2016). The animal´s specie also influences the CLA content in milk. In broad, CLA levels is higher in goat´s (5.30 mg/g lipid) than cow´s milk (3.4 mg/g lipid) (PECOVÁ *et al.*, 2019). Differences in CLA content of dairy products, when milk from various sources is utilized, is also observed. For example, for yogurts, its values ranging from 1.28–15.01, 4.05–12.50 and 4.33–9.76 mg/g lipid in cow, sheep and goat´s milk yogurt, respectively (SERAFEIMIDOU *et al.*, 2012).

Table 3 - Content of CLA in food, including milk and derivatives.

Food	Total CLA (g/kg fat)	*cis*-9,*trans*-11 CLA (g/100 kg total CLA)
Butter	9·4–11·9	91·0
Processed cheese	3·2–8·9	17·0–90·0
Natural cheese	0·6–7·1	17·0–90·0
Yoghurt	5·1–9·0	82·0
T-bone steak (cooked)	4·7–9·9	65·0
T-bone steak (raw)	4·4–6·6	59·0
Vegetable oils	0·2	45·0
Milk fat	2·0–30·0	90·0

CLA, conjugated linoleic acid.

Source: LAWSON *et al.*, 2001.

In broad, dairy products present higher total CLA level than raw milk probably because of manufacture processing (RUIZ *et al.*, 2016). However, more researches are needed to elucidate the accurate effect of the processing conditions of dairy products in CLA content (GUTIÉRREZ, 2016). Among dairy products, butter is the richest in CLA, range from 8.09 to 11.9 mg/g lipid (PESTANA *et al.*, 2009; LAWSON *et al.*, 2001). Although they have smaller amounts, cheese, yogurt and dairy beverage are also good sources of CLA. In cheese, CLA content was reported to ranges from 0.6 to 7.1 and 3.2 to 8.9 mg/g lipid in natural and processed cheese, respectively (LAWSON *et al.*, 2001). Consistently, Luna *et al.* (2007)

reported CLA values of 6.0-9.5, 5.2-6.5 and 3.0-3.8 mg/g lipid in Mahón, Manchego and Cabrales cheese, respectively. However, CLA content is up to 14 mg/g lipid in cheese from different brands was also reported (PESTANA *et al*., 2009). Formation detecting of new CLA-isomers during ripening cheese bring to conclusion that biohydrogenation of ALA could lead to the synthesis of CLA isomers as intermediates during cheese processing (GNÄDIG *et al*., 2004). CLA levels in yogurts was described to vary from 5.1 to 9.0 mg/g lipid (LAWSON *et al.*, 2001). In line, Jéssica *et al*. (2015) found out mean CLA values of 6.31 mg/g lipid in commercial yogurts. However, CLA content reaching up to 15.01 mg/g lipid was also reported in commercial Greek cow´s milk yogurts (SERAFEIMIDOU *et al.*, 2012). CLA concentration raising in fermented dairy products could be explained by the usage of starter or probiotic LAB, which may contain linoleate isomerase enzyme activity (VIEIRA *et al.*, 2017). Dairy beverages showed slightly lower CLA amount (5.46 mg/g lipid) than the average CLA value reported for yogurt (6.31 mg/g lipid) (JÉSSICA *et al*., 2015). CLA content (7.3 mg/g lipid) in powdered milk was higher than in beverages but lower than in yogurts (AVILEZ; MEYER, 2014). On the other hand, sweetened condensed milk (4.5 mg/g lipid) had lower CLA content. Therefore, the technological processing involved in elaboration of this product seems to affect negatively the transferring of CLA from fresh milk into the final product (RUIZ *et al*., 2016). Additionally, in all cases, for both milk and derivatives, the *cis*-9, *trans*-11-CLA was the major CLA-isomer present in food. This agrees with Gutiérrez (2016), which reported *cis*-9, *trans*-11-CLA representing between 72.6 to 91.2 % of total CLA in milk and dairy products. Conversely, *trans*-10, *cis*-12-CLA represents only 0.1-1.5 % of total CLA in these products, as showed in Frame 1.

Frame 1 - Distribution of CLA-isomers (% of total CLA isomers) in milk and derivatives.

Isomer	Percentage of the total isomers
C18:2 *cis*9, *trans*11	72.6-91.2
C18:2 *trans*7, *cis*9	1.2-8.9
C18:2 *trans*9, *trans*11	0.8-2.0
C18:2 *trans*11, *trans*13	0.3-4.2
C18:2 *trans*12, *trans*14	0.3-2.8
C18:2 *trans*10, *trans*12	0.3-1.3
C18:2 *cis*11, *trans*13	0.2-4.7
C18:2 *trans*8, *trans*10	0.2-0.4
C18:2 *trans*11, *cis*13	0.1-8.0
C18:2 *trans*9, *trans*9	<0.1-2.4
C18:2 *trans*8, *cis*10	<0.1-1.5
C18:2 *trans*10, *cis*12	<0.1-1.5
C18:2 *cis*12, *trans*14	<0.01-0.8
Other *cis-cis*	0.1-4.8

Source: GUTIÉRREZ, 2016.

About oxidative stability of CLA, Martínez-Monteagudo *et al.* (2015) demonstrated that fractions of CLA-isomers are more than 78 % remaining after treatment of CLA-enriched milk with ultra-high temperature (UHT) at 125–145 °C for 2–20 s; similarly, CLA remained stable in milk after 15 days of storage both at 4 °C (60-70 %) and at 25 °C (67-75 %). Addition to that, Lynch *et al*. (2005) showed that CLA in enriched pasteurized milk exposed to light-induced oxidative treatment remains stable after 14 days of storage at 4 °C. In CLA-enriched butter, the shelf life was similar to the conventional butter during storage (5–6 °C for 6-8 weeks) (MALLIA *et al*., 2008). In this way, CLA present in food products exhibit improved stability probably due to the bonded structure and the presence of other anti-oxidants (GONG *et al*., 2019).

1.3 INCREASE OF CLA-ISOMERS CONTENT IN FOOD: A TECHNOLOGICAL CHALLENGE

Among the identified CLA-isomers, the *cis*-9, *trans*-11-CLA and *trans*-10, *cis*-12-CLA are described as biologically active, showing protective effects against several diseases, including chronic inflammatory diseases (BENJAMIN; SPENER, 2009). However, CLA intake values through its natural sources are below of level recommended for achieving beneficial effects on human health (DILZER; PARK, 2012). Overall CLA daily intake in human diet ranges from 70 to 430 mg/day (BAUMAN; LOCK, 2006). Considering the

conversion of TVA from diet to CLA by the Δ9-desaturase enzyme occurs in the human body, the mean CLA intake of 650 mg/day could be achieved (GORISSEN *et al.*, 2015).

A diet can provide up to 21.7% of CLA levels recommended for achieving health benefits (about 3-4 g CLA/per day) (DILZER; PARK, 2012). Thus, a considerable research effort have been made in order to increase the CLA concentration in milk and dairy products and raise CLA population intake. Currently, handing animal's diet (SIURANA; CALSAMIGLIA, 2016) and milk fermentation with CLA-producing bacteria (alone or in combination with starter cultures) are the main technological strategies studied to goal this objective (GORISSEN *et al.*, 2015).

1.3.1 Manipulation of animal´s diet to increase CLA content in dairy products

The major substrates for biohydrogenation are LA and ALA and after being subjected to a sequences of isomerizations result in formation of TVA in rumen, being TVA the main precursor of endogenous synthesis of *cis*-9, *trans*-11 CLA in the mammary gland (GARCIA *et al*., 2017). Besides, vegetable fats are rich sources in LA and ALA substrates (MURUZ; ÇETINKAYA, 2019). Siurana e Calsamiglia (2016) demonstrated that, among feeding strategies in order to increase the CLA concentration in milk (vegetable fats, fish oils or the combination), the best are those supplemented with vegetable fats rich in LA and diets supplemented with fish oils plus vegetable fats. The latter increased the CLA content in 2.1 times (395 mg of *cis*-9, *trans*-11 CLA/L versus 188 mg of *cis*-9, *trans*-11 CLA/L in conventional milk). Additionally, green pasture is rich in ALA (GÓMEZ-CORTÉS *et al*., 2009) and it is great to increase CLA content in milk (1.8 fold), being cheaper than fat supplements (MURUZ; ÇETINKAYA, 2019). As long as green pasture intake increased, the content of ALA ($R^2 = 0.69$) and CLA ($R^2 = 0.79$) enhance linearly in milk (de RENOBALES *et al*., 2012). Pasture plant species also has a meaning effect on CLA content in milk. For example, legume-based pastures are associated with higher levels of CLA and ALA and lower levels of saturated FA than ryegrass pasture (ADDIS *et al*., 2005).

Vegetable fats could be supplemented as free oils, protected oils, raw seeds or processed seeds. However, extruded seeds or oils are those that greater enhance CLA content (1.95 and 1.93 fold, respectively) (SIURANA; CALSAMIGLIA, 2016). This could be attributed to the fact that when oil is inside intact seeds, it is released gradually, whereas when it is given as free oil or through processed seeds it is immediately available in the rumen (STANTON *et al*., 2003), being biohydrogenated to CLA-isomers (GARCIA *et al.*, 2017).

Similar to observed for cow´s diet, addition of vegetable oils in the diet of dairy sheep and goat is a useful strategy when the diet of these animals has a poor content of CLA precursors, which could be occur when stored forages are used (NUDDA *et al*., 2014). In fact, correlations between the daily intake of ALA through animal's diet and the daily cis-9 trans-11 CLA content in milk was found (R^2 = 0.80) (CABIDDU *et al*., 2006). A milk CLA concentration of 3.44 g/100 g of fat was reached with a very high dose of soybean oil (140 g/day) in diet (KUCUK *et al*., 2004). Moreover, supplementation with soybean oil (2.58 g/100 of FA) is more effective than linseed oil (1.59 g/100 of FA) in raising *cis*-9, *trans*-11 CLA content in sheep´s milk and cheese (BODAS *et al*., 2010). This suggests that complete ruminal biohydrogenation is higher in diets supplemented with oils rich in ALA (linseed) than oils rich in LA (soybean), and this behavior is similar those observed in cows (BU *et al*., 2007). Fish oil plus vegetal fat was more effective than vegetal fat alone to increase *cis*-9, *trans*-11 CLA in sheep milk; fish oil supplemented at 0.50% plus 3.5% of soybean oil per day caused a *cis*-9, *trans*-11 CLA increase of 1.2 fold in sheep milk compared to diet supplemented with soybean oil alone (MOZZON *et al*., 2002). Despite being poor in LA and ALA, fish oil inhibits the reduction of TVA to stearic acid (C18:0) by rumen bacteria (FERREIRA *et al*., 2011). Finally, a high pasture diet increased in 2.56 fold the content of *cis*-9, *trans*-11 CLA in sheep milk compared to a low pasture diet (2.15 g/100g of FA against 0.84 g/100g of FA, respectively). It is worth noting that pasture is a cheaper alternative to increase CLA in sheep milk (NUDDA *et al.*, 2003). Furthermore, the development and usage of protected fat sources, such as calcium salts of FA, have been proposed as way to decrease completely biohydrogenation of unsaturated FA in the animal´s rumen, in order to enhance CLA and PUFA content in milk (BETTERO *et al*., 2017).

Considering the average milk and dairy products intake in human diet, the ingestion of CLA in these foodstuffs from animals exposed to feed handling would increase the intake from 0.21 g/day to 0.44 g/day of CLA. Plus, the mean of CLA effective intake (ingested plus endogenous) would be 0.62 g/day of CLA (SIURANA; CALSAMIGLIA, 2016). This indicates that those CLA-enriched dairy products would be unable to achieve the recommended therapeutic level of 3-4 g/day (DILZER; PARK, 2012). It is important to highlight that CLA therapeutic levels in humans was extrapolated from animal studies (WHIGHAM *et al*., 2007) and therefore could be overestimated, therefore other supplementation alternatives may be needed to reach CLA content in food (SIURANA; CALSAMIGLIA, 2016).

1.3.2 Fermentation with probiotic LAB to increase the CLA content in dairy products

Some studies investigated the role of LAB in CLA production due to reports of higher CLA content in fermented dairy products than in no fermented milk. Since then, several LAB strains of food grade have been identified as potential producers of CLA, such as *Bifidobacterium*, *Enterococcus*, *Lactobacillus*, *Lactococcus*, *Propionibacterium* and *Streptococcus* (ANDRADE *et al*., 2012). It should be noted that many of these LAB strains have probiotic potential (CAMPANA *et al*., 2017). Probiotics were defined by the FAO / WHO as "living organisms that, when ingested in defined doses, have health beneficial effects" (FAO/WHO, 2006). Probiotics colonize the intestine by competitive exclusion (by either nutrients, adhesion sites or production of antimicrobials) and prevent intestinal colonization by potentially pathogenic microorganisms. In addition, these microorganisms have role as modulators of the immune system (CONTE-JÚNIOR *et al*., 2007). Besides probiotics also prevent chronic gastrointestinal inflammatory disorders (RITCHIE; ROMANUK, 2012), including colon cancer (KUMAR *et al*., 2015; KUUGBEE *et al*., 2016), alleviate diarrheal diseases (DOMINGO *et al*., 2017) and regulate diabetes (GOMES *et al*., 2014; SAMAH *et al*., 2016). Since probiotics offer health benefits much more than traditional nutritional foods, they are examples of functional foods. However, the current knowledge of potential effects of probiotics on neurological diseases are still poor and incomplete (BEGUM *et al*., 2017).

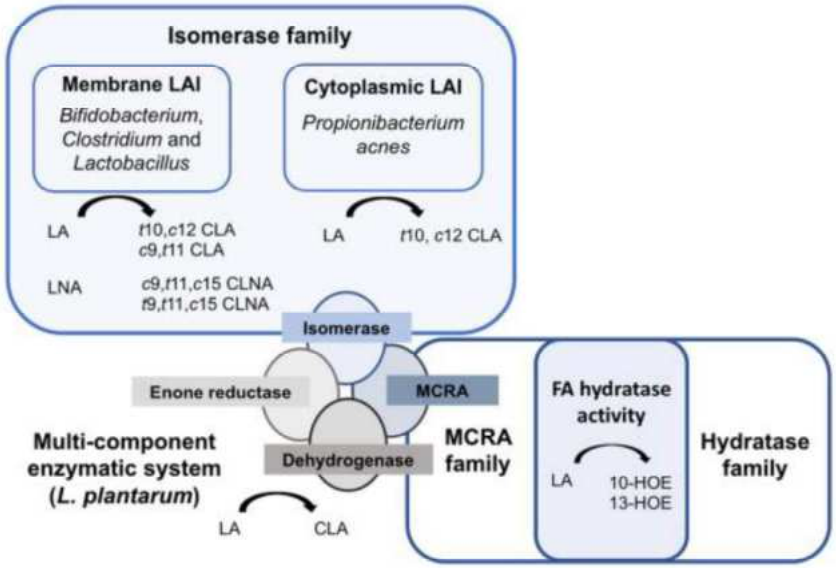
These findings motivate a concern in production of foods through inoculation of probiotic strains with CLA production ability. The inoculation in milk of probiotic starter strains belonging to the group of LAB with potential for CLA production can be used to obtain probiotic fermented dairy product enriched in CLA (KHOSRAVI-DARANI *et al.*, 2014; RIBEIRO *et al.*, 2018).

Studies have also demonstrated the production of CLA by bacteria present inside the intestine. When LA was provided to germ-free mice, CLA production did not occur in them, but only in conventional mice (DRUART *et al.*, 2015). This demonstrates that microorganisms in the intestinal tract are capable of producing CLA and that the concentration of CLA in host tissues could be increased not only by ingesting CLA-rich fermented dairy products, but also by modifying the intestinal microbiota with probiotic LAB with ability to produce CLA present in those foods (DRUART *et al*., 2014).

By means of utilization of *Lactobacillus plantarum* ZS2058 as a representative strain, a pathway for the isomerization of LA in CLA by LAB has been proposed (YANG *et al.*,

2014). The LAI (linoleic acid isomerase) involved in detoxifying mechanism of PUFA in bacterial cells seems to be a multicomponent enzyme system localized in both cell's membrane and soluble fractions; this multicomponent enzymatic system consists of multiple reactions where four enzymes perform different functions, as illustrated in Figure 3 (SALSINHA *et al.*, 2018). The single membrane fraction, denominated CLA-hydratase, initially catalyzes the hydration of LA to 10-hidroxi-18:1. Then, the dehydrating isomerization of 10-hydroxy FA occurs as follows: CLA-dehydrogenase plays a role in the oxidation of hydroxyl groups and the reduction of oxo groups. After, CLA-isomerase performs the migration of double bonds, and the final saturation step of 10-hidroxi FA is carried out by CLA-enone reductase (YANG *et al*., 2014; SALSINHA *et al.*, 2018).

Figure 3 - Multienzymatic system proposed for conjugation of linoleic acid in lactic acid bacteria. LAI: linoleic acid isomerase; LA: linoleic acid; LNA: linolenic acid; CLA: conjugated linoleic acid; FA: fatty acid; MCRA: myosin-cross-reactive antigen.



Source: SALSINHA *et al*., 2018.

Kefir is a dairy drink, which is elaborated by adding kefir grains to milk for fermentation (VIEIRA *et al.*, 2020). Kefir grains are a symbiotic microbial community composed of several species of LAB, acetic acid bacteria and yeast, so that there is a wide diversity among these microorganisms (PLESSAS *et al*., 2017). The grains have an irregular multilobular structure (Figure 4), being joined by a single central section and their size range from 0.3 to 3.5 cm in diameter (LEITE *et al*., 2013). They are also composed of proteins and polysaccharides that cover this complex microbiota, which may have its specific probiotic microorganisms characterized and isolated (KAKISU *et al*., 2011). This is because food

industry prefers to apply isolated probiotic microorganisms from kefir grains rather than kefir grains in order to produce respective fermented products with added value (HAMET *et al.*, 2015). Furthermore, kefir is considered a probiotic drink with functional compounds and has many beneficial physiological effects associated with its consumption, such as improved digestion and tolerance to lactose, antibacterial effect, hypocholesterolaemic effect, control of plasma glucose, anti-hypertensive effect, anti-inflammatory effect, antioxidant activity, anti-carcinogenic activity, anti-allergenic activity and healing effects (ROSA *et al.*, 2017).

Figure 4 - Kefir grains. Grains with irregular multilobular structure joined by a cross section.

Source: CARNEIRO *et al.,* 2010.

Preliminary studies carried out by our research group have shown that fermented milk with kefir grains from different origins had varying levels of CLA. The fatty acid profile and CLA levels at the end of the fermentation of milk seemed to be related to the species that constituted the grains (VIEIRA *et al.*, 2015). Thus, the isolation of probiotic strains with a high potential for CLA formation from this matrix would be enable to future use of these strains to obtain enriched dairy products, or even to obtain the concentrated CLA produced by these strains.

LA in its purest form is an expensive substrate for the production of CLA by food grade microbial cultures; thus, vegetable oils rich in LA have been used as an economical source of substrate achievable to be used by food industry (Hosseini et al.., 2015). Sunflower oil, in addition to its low cost, has a high concentration of LA (60-75%), which allows its use as an alternative source of LA for the production of CLA (FUENTES, 2011).

1.4 PHYSIOLOGICAL EFFECTS OF CLA-ISOMERS IN ANIMAL MODELS AND HUMAN CLINICAL TRIALS

CLA has anti-obesity, anticarcinogenic, and anti-inflammatory effects as well as prevents cardiovascular diseases (KOBA; YANAGITA, 2014). Despite wide evidence of health beneficial effects of CLA from animal models, findings in humans are still inconclusive; further studies with defined subject characteristics, experimental times, and CLA doses and preparations will help to understand the role of CLA in human health (DILZER; PARK, 2012).

1.4.1 Health benefits of CLA in peripheral organ systems

Unlike central nervous system, which has few reports on CLA effects, there are a well-developed literature describing the potential for therapeutic effects of CLA in peripheral organs system and disorders (MONACO *et al.*, 2019; Queiroz *et al.*, 2019a).

- Anti-obesity effects of CLA

Supplementation with *trans*-10, *cis*-12 CLA has shown to be effective in reducing body fat in a number of animal models (*e.g.* mice (ROSBERG-CODY *et al.*, 2011), rats (ARIAS *et al.*, 2014), hamsters (SIMON *et al.*, 2006), pigs (QI *et al.*, 2014) and in humans (RACINE *et al.*, 2010). It is important to highlight that there is a wide animal species-specific difference in the magnitude of body-fat reducing effect of CLA, with a prominent response in mice (KOBA; YANAGITA, 2014). On the other hand, *cis*-9, *trans*-11 CLA did not demonstrate significant effect on lean body mass; it was reported that dietary *trans*-10, *cis*-12 CLA but not *cis*-9, *trans*-11 CLA lowered hepatic triglyceride and cholesterol concentration while reduces hepatic levels of fatty acid synthase in obese rats (WANG *et al.*, 2006). On the other hand, despite did not present effect in lean body mass content, *trans*-10, *cis*-12 CLA was able to reduce body weight besides reduce adiposity in obese and lean mices. It suggests that the rate of stem cell to preadipocyte differentiation decreased (YEGANEH *et al.*, 2019).

Decrease in mice body fat (range from 43 to 88%) was obtained following 1 % mixture CLA feeding with either high-fat or low-fat diets for 5 to 6 weeks (WEST *et al.*, 2000; WEST *et al.*, 1998). In Marques *et al.* (2015), standard diet supplemented with *trans*-10, *cis*-12 CLA (0.5%, w/w) for 8 weeks decreased significantly visceral fat mass in mice, although it has not affected body weight. For experimental model of diet-induced obesity and supplemented with equimolar mixture of *trans*-10, *cis*-12 CLA and *cis*-9, *trans*-11 CLA

(0.5%, w/w) for 6 weeks, significant reductions in lipogenesis and fatty acid uptake and the increase in lipolysis in adipose tissue of rats was observed (ARIAS *et al.*, 2014). These effects can be attributed to induction of apoptosis in adipocyte cultures (MARTINEZ *et al.*, 2010), as well as in brown and white adipose tissue, due to TNF-α and uncoupling protein-2 (UCP-2) induction by *trans*-10, *cis*-12 CLA (KIM *et al.*, 2011). In addition, *trans*-10, *cis*-12 CLA inhibits transcription of enzymes involved in *de novo* fatty acid synthesis, desaturation of fatty acids and triacylglycerol synthesis (MA *et al*., 2014). Coherently, CLA was found to enhance fatty acid β-oxidation in muscle and brown adipose tissue, and oxygen consumption and energy expenditure in obese rats (RAHMAN *et al.*, 2001). CLA's anti-obesity properties are thought to be a result of (1) reduced energy intake by suppressing appetite, (2) inhibition of fatty acid metabolism, adipogenesis and lipogenesis, (3) increased lipolysis, (4) decreased adipocyte size and (5) increased fat oxidation and energy expenditure in white adipose tissue, muscle and liver tissue (KENNEDY *et al.*, 2010). Changes in lipid metabolism in mice fed with *trans*-10, *cis*-12 CLA-supplemented diet may be partially mediated by modulating stomach protein expression and exerting a prebiotic effect on benefic gut bacterial species (CHAPLIN *et al.*, 2015).

However, such effects reported in animal models seems to be inconsistent and less distinguished in humans (VILADOMIU *et al*., 2015). Two studies involving either healthy exercising humans (given 1.8 g CLA/day), overweight and obese humans (1.7–6.8 g CLA/day) for 12 weeks showed significant fat mass reduction (4%) without change in body weight (BLANKSON *et al.*, 2000; THOM *et al*., 2001). In addition, 2.5 kg reduction of body weight and 1 kg fat mass reduction in obese volunteers during a trial of supplementation with 2.7 g CLA/day for 6 months was reported (ATKINSON, 1999). Similarly, Risérus, Berglund e Vessby (2001) found a significant reduction (0.6 cm) in sagittal abdominal diameter by 4.2 g CLA/day given to middle-aged obese men during 4 weeks. It was also reported that CLA supplementation (3.4 g/day in triglyceride form) for 1 year decreased body fat mass in healthy overweight humans (GAULLIER *et al.*, 2004). On the other hand, no effects on body weight have been reported in normal weight humans taking 0.6– 6 g/day CLA for periods of 4–14 weeks, regardless of whether the CLA was provided as a mixture of two isomers or as a single isomer (PLOURDE *et al.*, 2008). Still, one study reported no effect of CLA supplementation on body weight and fat in overweight and obese individuals (PLOURDE *et al*., 2008). For patients with metabolic syndrome, a meta-analysis investigated the efficacy of orally administered CLA over a period longer than 4 weeks and showed significant decreasing in body weight (−0.51 kg) and body mass index (−0.18 kg/m^2) when CLA was ingested. CLA

supplementation seems to be efficient to decrease peripheral compartment in overweight children, while visceral fat was maintained. The group received 3g/day of mixed CLA or placebo for 6 months (RACINE *et al*., 2010). However, it is necessary to assess that CLA could adversely reduce HDL. Hence, some cares should be taken with CLA usage in metabolic syndrome patients with low HDL (KIM *et al.*, 2016b). Inconsistent outcomes throughout clinical studies might be due to the large variation of (1) CLA mixture isomers used, (2) dose administered, (3) duration of the treatment, (4) study populations (age, body weight, body fat or metabolic status of the subjects), (5) gut microbiome, and (6) inflammation status (VILADOMIU *et al*., 2015).

- Anticarcinogenic effects of CLA

Both *trans*-10, *cis*-12 CLA and *cis*-9, *trans*-11 CLA have been described to be anticarcinogenic (ABEL; ARENDSE; GELDERBLOM, 2014). The evidence suggests that anticarcinogenic effect of CLA is much stronger than that of ω-3 fatty acids, such as eicosapentaenoic (EPA) and docosahexaenoic (DHA) acids, whose anticarcinogenic effects are well known and require at least 5-10% weight to exert significant effect (NIEZGODA *et al*., 2017). Nevertheless, *trans*-10, *cis*-12 CLA had strongest anti-carcinogenic effect than *cis*-9, *trans*-11 CLA on human colon cancer cells, where caco-2 cells were incubated up to 72 h with 200 μM of each isomer (BEPPU *et al.*, 2006). Similarly, *trans*-10, *cis*-12 CLA derivatives had better anticancer properties against human promyelocytic leukaemia, breast cancer and colon cancer cell lines than *cis*-9, *trans*-11 CLA and a mixture of both isomers (NIEZGODA *et al*., 2017). There are evidences that the two CLA isomers differ in anticancer activity; *cis*-9, *trans*-11 CLA linked mostly to cytotoxicity and apoptosis, while *trans*-10, *cis*-12 CLA possesses a different mechanism mostly involving cell cycle arrest (ABEL; ARENDSE; GELDERBLOM, 2014). CLA in animal's diet showed Delta-6-desaturate (D6D) decreased activity, which may confirm the anticancer properties of these isomers (STARWARSKA *et al*., 2015). D6D is a rate-limiting enzyme used for energy and signaling by cells and is responsible for maintaining many aspects of lipid homeostasis and normal health. This enzyme take part in the synthesis of arachidonic acid, a fatty acid well-known by its pro-inflammatory effect (PENDER-CUDLIP *et al.*, 2013).

A diet supplemented with 1% of a mixture of *trans*-10, *cis*-12 CLA and *cis*-9, *trans*-11 CLA (50:50%) during 42 days prior to the induction of colitis and cancer ameliorates inflammation-driven colorectal cancer, upregulating the levels of regulatory CD4+ T cells in mesenteric lymph nodes of mice (EVANS *et al.*, 2010). Suchlike diet with 1% CLA for 24 days prior the induction of inflammation-related cancer resulted in a faster recovery of acute

inflammation by chemically-induced colorectal cancer in mice; CLA reduced colonic adenoma and adenocarcinoma formation as well as decreased colonic ciclo-oxigenase-2 expression (BASSAGANYA-RIERA *et al*., 2012). Rats fed with diet supplemented with 20% of *cis*-9, *trans*-11 CLA up to 32 weeks after induction of mammary gland carcinogenesis showed 46.07% of tumor incidence against 83.33% of control group (diet with 20% of soybean oil) (KATHIRVELAN *et al.*, 2015). Interestingly, rats fed with CLA-supplemented diet (1% of *trans*-10, *cis*-12 CLA and *cis*-9, *trans*-11 CLA, 50:50%) during pregnancy and breastfeeding inhibited the breast cancer formation in their offspring (BIAŁEK; TOKARZ; ZAGRODZKI, 2014). In canine *in vitro* model of osteosarcoma, *trans*-10, *cis*-12 CLA but not the *cis*-9, *trans*-11 CLA, effectively inhibited cell growth and induced apoptosis osteosarcoma cell line treated with 50 μM of each isomer for 1-4 days. Such inhibition occurred through increased expression of nonsteroidal anti-inflammatory drug (NSAID) activated gene-1 (NAG-1) (WONG; KIM; PARK, 2015). One mechanism by which CLA exerts anticarcinogenesis could be due to an activation of peroxisome proliferator-activated receptors (PPARs). It was shown that CLA was as an activator of PPARγ, and thus, inducing apoptosis and inhibiting proliferation of cancer cells, such as prostate (KUBOTA *et al.*, 1998), breast (MUELLER *et al*., 1998) and colon (SARRAF *et al.*, 1999) in *in vitro* and *in vivo* studies. PPARγ activators can also inhibit NF-κB, decreasing the cellular proliferation of cancerous prostate epithelial cells (KOBA; YANAGITA, 2014). No effect of CLA on inflammation-induced colorectal cancer was observed in immune cell-specific PPARγ null mice, suggesting that CLA is able to modulate the Treg compartment in a PPARγ-dependent fashion (EVANS *et al*., 2010).

CLA has also been shown to inhibit the growth of various human cancer cell lines. *Cis*-9, *trans*-11 CLA inhibited the proliferation of MCF-7 cells (human breast adenocarcinoma cell line) by down-regulating the expression of Bcl-2 as well as procaspase-9. Growth inhibitory ratios of MCF-7 cells treated with 50, 100, and 200 μM of *cis*-9, *trans*-11 CLA for 72h were 13.9%, 22.7%, and 47.6%, respectively (LIM *et al*., 2014). Coherently, Altei *et al.* (2019) reported reduction of phosphocholine level, a cancer malignance biomarker, in MCF-7 cells treated with 100 μM of CLA for 3 days. Oh *et al.* (2014) reported significant suppression of growth rate for different human cancer cell lines; T47D, A549, and SW480, which are lines derived from patients with breast carcinoma, lung nonsmall cell carcinoma, and colorectal adenocarcinoma, respectively, were all inhibited by administration of *cis*-9, *trans*-11 CLA (50-200 μM) for 72h. On the other hand, for this same study and conditions, HT29 cells (cell line of colorectal adenocarcinoma) were not inhibited by CLA. In

addition, the *trans*-10, *cis*-12 CLA isomer (45 μM) had great synergestic effect with 10 μg of Taxol (broader spectrum anticancer drug), reducing at 45.72 % of viability of human prostatic cancer cells (PC3 cell line) against 100 % of control viability after 48 h of exposure (KIZILŞAHIN *et al.*, 2014).

Although there is little evidence of CLA anticarcinogenic effect in clinical studies, some results indicate positive effect of dietary CLA intake protection against cancer in humans. Breast cancer patients (stage I–III) were given CLA supplementation (7.5g/day) prior to surgery. CLA supplementation at least 10 days prior to surgery was associated with reduced S14 levels (correlated with the recurrence of breast cancer) and Ki-67 (tumor proliferation marker) in tumor tissue in patients with higher cancer scores (II), but not in patients with lower cancer scores (I) (MCGOWAN *et al.*, 2013). Mohammadzadeh *et al.* (2013) reported that supplementation of CLA, 3g/day for 6 weeks, to rectal cancer patients (Stage II–III) during chemoradiotherapy significantly reduced matrix metalloproteinase (MMP)-2 and -9, suggesting that CLA potentially reduced angiogenesis and tumor invasion. Patients with rectal cancer treated with preoperative chemoradiotherapy and receiving 3g of CLA/day (*trans*-10, *cis*-12 CLA and *cis*-9, *trans*-11 CLA, 50:50%) during 6 weeks presented physical function, role function, and cognitive function significantly enhanced. Moreover, comparison of changes in fatigue, pain and diarrhea scores and global health status scores between CLA and placebo groups were significant (FARAMARZI *et al.*, 2017). However, with low number of studies, there is insufficient evidence to determine whether CLA ingestion has a significant effect on human cancer. Moreover, further well-designed studies are required, particularly in considering the confounding variables (HARTIGH, 2019).

- Prevention of cardiovascular diseases by CLA

Cardiovascular diseases (CVD) are a major cause of death in most developed countries, being atherosclerosis the primary etiology of most CVD, such as heart attack and stroke (LAITINEN *et al.*, 2015). Atherosclerotic plaques are characterized by the accumulation of lipids and inflammatory cells in the arterial wall, which can trigger a rupture and subsequent ischemic events (XIE *et al.*, 2016). A number of animal studies reported the anti-atherosclerotic effects of CLA via reduction of total cholesterol, triacylglycerides, LDL-cholesterol, and blood pressure, as well as enhancement of HDL-cholesterol (DILZER; PARK, 2012).

The first studies to test the atherogenicity of CLA supplements were performed in rabbits, species that readily develop human-like atherosclerosis when fed with a cholesterol-containing diet (FAN *et al.*, 2015). A mixture of CLA (*trans*-10, *cis*-12 CLA and *cis*-9, *trans*-

11 CLA, 50:50%) at doses ranging from 0.05%–1% (w/w) during 90 to 154 days consistently showed reduced aortic lipid deposition (LEE *et al.*, 1994), regression of established lesions, and improved plasma lipoproteins (KRITCHEVSKY *et al*., 2004) during regression of disease. In addition, CLA was able to significantly increase serum and aortic tissue concentration of IL-10 (an anti-inflammatory and immunosuppressive cytokine) in mice (McCARTHY *et al.*, 2013). The Syrian Golden hamster is another useful model for studies of atherosclerosis because of its similar lipoprotein profile to humans when fed with a diet high in fat and cholesterol (SULLIVAN *et al.*, 1993). Supplementation of 1% (w/w) *cis*-9, *trans*-11-CLA or *trans*-10, *cis*-12-CLA with a high-fat, high-cholesterol diet for 12 weeks did not change atherosclerosis development when compared to 1% (w/w) LA-supplemented controls in hamsters (MITCHELL *et al.*, 2005). However, improvements in the plasma lipids, such as increase of HDL, were reported (MITCHELL *et al.*, 2005; NAVARRO *et al.*, 2007). CLA supplementation may improve some parameters associated with atherosclerosis, although it does not appear to contribute to atherosclerosis regression in this animal model (HARTIGH, 2019). It is also important to highlight that different CLA isomers as well as its ratios could have different atherogenic effects (BRUEN *et al*., 2017).

Two of the most common mouse strains used for the study of atherosclerosis are apoE-deficient mice (apoE−/−) and Ldlr−/− mice. Both models develop atherosclerosis as a result of the accumulation of cholesterol-containing lipoproteins in the plasma due to defective lipoprotein clearance (MEIR; LEITERSDORF, 2004). The majority of CLA supplementation studies in apoE−/− mice showed a suppression or regression of atherosclerosis, with reduced atherosclerotic lesion sizes in the aorta, coupled with decreased macrophage accumulation and expression of pro-inflammatory genes (HARTIGH, 2019). ApoE−/−Ldlr−/− double knockout mice fed eggs supplemented with 0.5% (w/w) mixed CLA (*trans*-10, *cis*-12-CLA and *cis*-9, *trans*-11-CLA) or *trans*-10, *cis*-12-CLA alone during 3 weeks also exhibited protection from atherosclerosis with a coincident decreased plaque macrophage content (MITCHELL et al., 2012). The atheroprotection has been attributed to *trans*-10, *cis*-12 CLA isomer in mice (HARTIGH, 2019). Obese Ldlr−/− mice fed with *trans*-10, *cis*-12 CLA (1% w/w) in a 0.15% cholesterol-containing high-fat high sucrose diet showed significant improvements in aortic and sinus atherosclerosis. Such effect was independent from weight loss, as a weight-matched control group undergoing caloric restriction did not exhibit such improvements in atherosclerosis. Perivascular adipose tissue surrounding the thoracic aorta was also enriched with macrophages “M2 type”, exhibiting high expression levels of Arginase-1, that is, it there was increase of atheroprotetor macrophage phenotype (KANTER

et al., 2018). Consistently, *trans*-10, *cis*-12-CLA polarized *in vivo* macrophages in white adipose tissue towards M2 phenotype, which has shown protection against atherosclerosis (PINI *et al.*, 2015). However, there are few studies suggesting that CLA supplementation has negative (TAYLOR *et al.*, 2005) or no effetcs on atherosclerosis (COOPER *et al.*, 2008). The diferences in results could be attributed to key differences in the lipoprotein profiles and plaque structure among distinct mice models for atheroscleris, so that studies comparing the outcomes from these mouse models should be interpreted with caution (HARTIGH, 2019).

CLA positive effects on parameters related to CVD were attributed to following mechanisms: the involvement of peroxisome proliferator-activated receptors (PPARs, key for lipogenesis), sterol regulatory element-binding proteins (SREBPs, key for fatty acid synthesis and elongation), and/or steroyl-CoA desaturase (SCD, key for triglyceride and cholesterol formation) (DILZER; PARK, 2012).

Human CLA studies focusing on cardiovascular disease risk factors are still inconclusive, because either positive, negative or no effects were reported (HARTIGH, 2019). Multiple studies have shown that CLA supplementation is associated with a decline in serum HDL levels, a lipoprotein usually associated with cardio-protective effects and the lowering of cholesterol (GAULLIER *et al.*, 2004; RACINE *et al.*, 2010; SOFI *et al.*, 2010). On the other hand, two other studies observed that CLA supplementation (3 to 4.5 g/day of a 50:50 isomer blend of *trans*-10, *cis*-12-CLA and *cis*-9, *trans*-11-CLA) during 8 weeks increased HDL cholesterol and the ratio of HDL to LDL compared to control subjects (MOLONEY *et al.*, 2004; ZHAO *et al.*, 2009). A prospective men's study (aged 60 to 79 years) without prevalent heart failure followed up for an average of 13 years showed that CLA percentage in their serum was inversely associated with C-reactive protein and NT-proBNP (N-terminal pro-B-type natriuretic peptide; a marker of ventricular stress); high CLA percentage was also associated with significantly reduced risk of heart failure after adjustment for heart failure risk factors and C-reactive protein (WANNAMETHEE *et al.*, 2018). On the other hand, supplementation (4.5 g/day) with a mixed CLA (50:50 %, *trans*-10, *cis*-12-CLA and *cis*-9, *trans*-11-CLA) to overweight men for 4 weeks did no improve the endothelial function, measured by the peripheral arterial tonometry index determination, nor changed cardiovascular risk factors such as the platelet-activating factor acetylhydrolase activity, C-reactive protein, paraoxonase, LDL or HDL cholesterol, or triglycerides (PFEUFFER *et al.*, 2011). Subjects with diagnosed coronary artery disease submitted to diet supplemented with either 3 g/day mixed CLA (50:50 %, *trans*-10, *cis*-12-CLA and *cis*-9, *trans*-11-CLA) or a placebo for two months did no present significant changes in plasma triglycerides, LDL

cholesterol, or HDL cholesterol (EFTEKHARI *et al.*, 2014). The null effects of CLA on atherosclerosis observed in some of reported studies can be explained by fact that none of these trials conducted to date focused in supplementation exclusive with the *trans*-10, *cis*-12 CLA isomer (HARTIGH, 2019). It is important to highlight that the *trans*-10, *cis*-12-CLA isomer, but not a mixture of the *trans*-10, *cis*-12-CLA and *cis*-9, *trans*-11-CLA or *cis*-9, *trans*-11-CLA alone had positive effect on markers for CVD in animal models (KANTER *et al*., 2018).

A recent meta-analysis concluded that CLA had no positive effects on blood pressure in humans (YANG *et al*., 2015). Other markers associated with increased risk of CVD, such as angiotensin-I converting enzyme (ACE), plasminogen activator inhibitor-1 (PAI-1), or platelet function (including blood clotting) have no significant changes following CLA supplementation (ZHAO *et al.*, 2009). However, synergistic effect between CLA supplementation (4.5 g/day; 50:50 %, *trans*-10, *cis*-12-CLA and *cis*-9, *trans*-11-CLA) and hypertension drug therapy (ramipril drug) for 8 weeks was reported to reduce plasma angiotensinogen and decrease plasma hypertensive adipocytokines in treated obese hypertensive patients. Therefore, is clear that more long-term studies will be required in order to measure fully the applicability of CLA on incidence of CVD (DILZER; PARK, 2012).

- Anti-inflammatory effects of CLA

Beneficial effects of CLA on immune and inflammatory responses have been reported in animal models (DILZER; PARK, 2012; YANG; COOK, 2003) and human research clinical trials (PETERSON *et al.*, 2009). Decreased of colonic inflammation, decreased antigen-induced cytokine production by immune cells, decreased adverse effects of immune challenges and modulation of inflammatory mediators (such as cytokines, prostaglandins, leukotrienes and immunoglobulins) are some of conditions that have been mentioned (OLESZCZUK *et al.*, 2012). Both *cis*-9, *trans*-11 and *trans*-10, *cis*-12-CLA isomers decreased innate immune responses by lowering the activity of monocytes, macrophages, dendritic cells and natural killer cells and diminishing the production of prostaglandins and leukotrienes (O'SHEA *et al.*, 2004). Mixtures (50:50%) of *cis*-9, *trans*-11 and *trans*-10, *cis*-12-CLA dietary in the regulation of bacterially induced colitis in pigs showed the efficacy of CLA in ameliorating disease associated with colitis (HONTECILLAS *et al*., 2002). In this study, both dietary CLA supplementation and systemic bacterial immunization were able to decrease colonic epithelial erosion, yet only CLA treatment was able to prevent the enlargement and thickening of the colonic mucosa. Dietary CLA increased the numbers of TCRγδCD8αα T cells in peripheral blood and maintained numbers of CD4+ and CD8+ T

cells in the colonic mucosa. Supplementation of diet of pigs with *cis*-9, *trans*-11 and *trans*-10, *cis*-12-CLA (50:50%) enhanced the cytotoxic potential of peripheral blood lymphocytes and proliferation of TCRαβCD8αα T cells. Thus, dietary CLA enhanced cellular immunity by modulating phenotype and effector functions of CD8+ T cells, which are involved in both adaptive immunity and can be conditioned to play a role in innate immunity (BASSAGANYA-RIERA *et al.*, 2001). Both *cis*-9, *trans*-11 and *trans*-10, *cis*-12-CLA isomers are able to reduce the production of proinflammatory cytokines by macrophages, and thus, modulate the environment that supports the differentiation of lymphocytes towards a more regulatory phenotype during the antigen presentation phase (JIANG *et al*., 1998).

Several cell culture studies have demonstrated that CLA, particularly the *cis*-9, *trans*-11 isomer, is able to diminish proinflammatory cytokine production, mainly IL-6, TNFα, IFNγ, and IL-1β, which play an important role in the pathogenesis of many chronic inflammation-mediated diseases (BASSAGANYA-RIERAAND; HONTECILLAS, 2010; OLESZCZUK *et al.*, 2012). Experiment *ex vivo* showed decreased NF-κB activation and TNFα mRNA levels after adding *trans*-10, *cis*-12-CLA to LPS-stimulated pig peripheral blood mononuclear cells (PBMCs) (KIM *et al.*, 2011). In contrast, *trans*-10, *cis*-12-CLA treatment in non-stimulated PBMCs by LPS resulted in differing results, since the authors report an increased NF-κB activation and TNFα production. It suggesting that CLA could act as a proinflammatory stuff in environments not stimulated by endotoxin. Besides, CLA may elicit different actions depending on the environment conditions, thus requiring further mechanistic investigations (VILAMIDOU *et al.*, 2015). Pre-treatment with *cis*-9, *trans*-11-CLA delays the LPS-induced NF-κB translocation from the cytoplasm and into the nucleus by preventing IκBα degradation in macrophages and dendritic cells. Interestingly, such CLA effects were reversed in the presence of an IL-10 neutralizing antibody (LOSCHER *et al.*, 2005).

Peroxisome proliferator-activated receptors are nuclear receptors and ligand-activated transcription factors that regulate gene expression involved in energy regulation, glucose homeostasis and immune function. Those fatty acid receptors are widely expressed in the immune system (i.e. macrophages, dendritic cells, T cells, epithelial cells) and regulate the expression of several genes involved in immune cell proliferation, apoptosis and inflammation (O'SHEA *et al*., 2004). PPARs are mostly activated by polyunsaturated fatty acids such as CLA, whereas their interaction with saturated fatty acids results in weak activation (VILADOMIU *et al*., 2015). PPAR activation, and PPARγ activation in particular, has been reported as an important negative regulator of inflammatory responses through

several different mechanisms including the transcriptional regulation of cytokines, chemokines and cell survival factors as well as inhibiting the transcription of proinflammatory factors such as NF-κB, AP-1 and STAT (MACREDMOND *et al*., 2011). Therefore, PPARγ activation represents a promising avenue for developing safer nutritional interventions against inflammation (VILADOMIU *et al.*, 2015). In this context, the anti-inflammatory effects of CLA are partly mediated through its ability to activate PPARγ in imune and epithelial cells (EWASCHUK *et al*., 2006).

Regarding inflammation-mediated diseases, obesity is characterized with a systemic low-grade chronic inflammation (KENNEDY *et al.*, 2010). As previously reported in the present work, several animal studies, especially in rodents, show beneficial metabolic effects of CLA, mainly *trans*-10, *cis*-12-CLA, resulting in reduced fat deposition and increased lean body mass (KOBA; YANAGITA, 2014). Asthma is a chronic airway inflammatory disorder that involves a complex interplay between resident cells, such as epithelial cells and connective tissue, and infiltrating immune cells including eosinophils and activated T lymphocytes (MACREDMOND; DORSCHEID, 2011). Recent meta-analysis of several prospective studies has demonstrated that obesity precedes the development of asthma with a relative risk for incident asthma in obese adults (BEUTHER; SUTHERLAND, 2007). Weight loss and modulation of adipokines following CLA treatment can have a beneficial effect in respiratory inflammation and asthma. CLA treatment has already been shown to reduce airway inflammation and hyper-reactivity through a variety of PPARγ dependent and independent mechanisms (MACREDMOND; DORSCHEID, 2011). Specifically, CLA isomer *cis*-9, *trans*-11 is able to reduce broncho-alveolar inflammatory cell count and lung IL-5 levels in an animal model of allergic asthma through a PPARγ-dependent mechanism (JAUDSZUS *et al.*, 2008). Finally, *cis*-9, *trans*-11 and *trans*-10, *cis*-12-CLA (50:50%) dietary mixtures seem to have beneficial effects related to viral clearance in pigs (BASSAGANYA-RIERA *et al.*, 2003) and reduce rhinovirus infection and duration in humans (BASSAGANYA-RIERA *et al*., 2003). *In vitro* studies suggest that these effects might be due to the ability of *cis*-9, *trans*-11 and *trans*-10, *cis*-12-CLA isomers to inhibit viral entry and virus-induced inflammation (MACREDMOND; DORSCHEID, 2011). In this scenario, the anti-viral action of CLA brings out a question: could CLA-isomers exert preventive and/or therapeutic effects on SARS-CoV-2 (severe acute respiratory syndrome coronavirus 2), coronavirus responsible by the coronavirus disease 2019 (COVID-19) pneumonia pandemic?

Inflammatory Bowel Disease (IBD) is a chronic, recurring, debilitating and widespread immuno-inflammatory illness of unknown etiology with two clinical

manifestations: Ulcerative Colitis (UC) and Crohn's Disease (CD) (CAMILLERI, 2003; LAKATOS, 2006). Even though IBD therapies have improved (CAMILLERI, 2003), there is still a need to develop novel immuno-nutritional interventions due to the significant side effects associated with current treatments (MACONI *et al.*., 2005). A promising avenue for the development of such nutrition-based treatments for IBD is by targeting PPARs. As discussed above, several CLA isomers are able to activate PPARγ in the nucleus of a cell (SCRASCIA *et al*., 2003). By using a *B. hyodysenteriae*-induced colitis pig model, it was found that dietary *cis*-9, *trans*-11 and *trans*-10, *cis*-12-CLA (50:50%) supplementation upregulates colonic PPARγ, which subsequently binds to DNA and regulates transcription of anti-inflammatory genes, thus resulting in the suppression of colonic inflammation; dietary CLA supplementation ameliorated tissue inflammation and weight loss associated with bacterial-induced colitis (HONTECILLAS *et al.*, 2002). In addition, novel *in vivo* evidence showed how VSL#3 (commercial probiotic bacterial mixture) administration changes microbial diversity and local CLA production, which resulted in PPARγ-dependent anti-inflammatory effects during DSS- and IL-10-deficiency-induced experimental colitis in mice (BASSAGANYA-RIERA *et al.*, 2012a). In humans, thirteen patients with mild to moderately active CD were enrolled in an open-label study of CLA. Patients received a capsule containing 6 g of *cis*-9, *trans*-11 and *trans*-10, *cis*-12-CLA daily for 12 weeks. Treated patients expressed a decreased disease activity (calculated using the CD activity index-CDAI) and increased quality of life (assessed using the Inflammatory Bowel Disease Questionnaire-IBDQ), which correlated with a significant suppression of the ability of peripheral blood CD4+ and CD8+ T cell subsets to produce pro-inflammatory cytokines including IFNγ (BASSAGANYA-RIERA *et al.*, 2012B). IBD is among the top three high-risk conditions for the development of colorectal cancer (XIE; ITSKOWITZ, 2008). Dietary *cis*-9, *trans*-11 and *trans*-10, *cis*-12-CLA ameliorates inflammation-driven colorectal cancer in mice (EVANS *et al*., 2010). Particularly, CLA upregulated the levels of regulatory CD4+ T cells in mesenteric lymph nodes of wild type mice. However, no differences were seen in immune cell-specific PPARγ null mice, suggesting that CLA is able to modulate the Treg compartment in a PPARγ-dependent fashion (HONTECILLAS; BASSAGANYA-RIERA 2007; WOHLFERT *et al*., 2007). Consistently, dietary *cis*-9, *trans*-11 and *trans*-10, *cis*-12-CLA (50:50 %) or VSL#3 treatments resulted in a faster recovery during acute inflammation and lowered disease severity during the chronic, tumor-bearing phase of disease during chemically-induced colorectal cancer in mice. Both treatments were able to reduce colonic adenoma and adenocarcinoma formation, since CLA was able to modulate gene expression of COX-2

(enzyme that plays a role in inflammatory process and prostaglandins production) in the colonic mucosa (BASSAGANYA-RIERA *et al.*, 2012c).

1.4.2 Health benefits of CLA in central nervous system

Although CLA can freely get through the blood-brain barrier and being actively incorporated and metabolized in the brain (BINYAMIN *et al.*, 2019; FA *et al.*, 2005), reports on CLA effects in the central nervous system are still scarce and little is known about the possible neuroprotective ability of CLA (MONACO *et al.*, 2019).

Rats receiving diet consisting of 0.5% CLA (50:50 % of *trans*-10, *cis*-12-CLA and *cis*-9, *trans*-11-CLA) for 24 days presented inhibition of angiogenesis in the brain, suggesting that CLA can be explored as a therapeutic treatment for cancer and tumors in the brain (SIKORSKI *et al.*, 2008). A maternal diet containing 7% lipids from goat milk (as a source of CLA) during gestation, lactation or both, has also been found to affect cortical electrical activity in the rat progeny (SOARES *et al.*, 2012). Rats were fed with an experimental diet containing CLA-enriched butter (210 g/kg of diet) for 4 weeks presented improved memory through up-regulation of phospholipase A2 encoding-genes in brain tissue (GAMA *et al.*, 2015). Rats groups that received intraperitoneal injections of 80% CLA dietary oil (25 mg/kg body weight) exhibited exacerbated recovery from functional deficits, such as hypoadrenalism, induced by a controlled cortical impact injury (GEDDES *et al.*, 2017). Diet containing 1-3% of CLA (50:50 % of *trans*-10, *cis*-12-CLA and *cis*-9, *trans*-11-CLA) offered to the mothers from the 7th day of gestation until the end of lactation induced a decrease in the parameters of anxiety and cerebral lipid peroxidation in the offspring in rats (QUEIROZ *et al.*, 2019b). Alike result on anxiety reduction was observed in rat progeny with dams fed a diet containing 7% lipids from goat milk (as a source of CLA) during gestation and lactation (SOARES *et al.*, 2013). Similarly, rats fed a diet containing 1-3% of CLA (50:50 % of *trans*-10, *cis*-12-CLA and *cis*-9, *trans*-11-CLA) during gestation and lactation induced anticipated reflex maturation and improved memory in the offspring (QUEIROZ *et al.*, 2019a). Those finds suggest that CLA administration could be beneficial with regard to cognitive parameters *in vivo.*

In an *in vitro* model, 30 µM of *cis*-9, *trans*-11-CLA was found to protect primary cortical cells from death when given concurrently or up to 5 h after glutamate exposure (3 µM) in stroke simulated by exposing mouse cortical neurons to glutamate (HUNT *et al.*, 2010). Five-week daily supplementation with a mixture 50:50 % of CLA (650 mg/kg^{-1} body

weight) prevented age-dependent neurodegeneration in a mouse model of neuropsychiatric erythematous lupus (MONACO *et al*., 2019). CLA was administered to 3 weeks old mice of experimental groups by adding the self-emulsion formulation to their drinking water (1.6% oil) and prevented age related cognitive deterioration as well as mitochondrial oxidative damage, reducing accumulation of Aβ (amyloid beta) and of p25, a calpain product, demonstrating that CLA is a natural calpain inhibitor (BINYAMIN *et al*., 2019). Coherently, in Alzheimer´s disease human *in vitro* model, CLA (20 μM for 72h) showed neuroprotective effects against neurotoxins, such as H_2O_2 and $A\beta_{1-42,}$ inhibiting Aβ oligomerization/fibrillation and tau phosphorylation in SH-SY5Y (human neuroblastom cell line). This implicated CLA as a new core structure for selective μ-calpain inhibitors with neuroprotective effects (LEE *et al*., 2012). Chronic dietary CLA (3% of a mixture *trans*-10, *cis*-12-CLA and *cis*-9, *trans*-11-CLA, 31.7 % :30.9 %, respectively) during 9 weeks can reduce cerebral prostaglandin E2 (NAKANISH *et al*., 2003). Recently, human cultured astrocytes treated for 6 days with 100 μM of *trans*-10, *cis*-12-CLA presented downregulation of inflammatory markers, such as tumour necrosis factor-α (TNF-α) and interleukin-1β (IL-1β) (SABA *et al.*, 2019).

1.4.3 Safety consideration for CLA use by humans

The European Food Safety Authority (EFSA) demonstrated that the administration of an equimolecular mixture of the *cis*-9,*trans*-11-CLA and *trans*-10, *cis*-12-CLA at a dose of 3.5 g for six months had no adverse effects on insulin sensitivity levels and blood glucose control or liver function in humans with normal weight or overweight with no history of diabetes (EFSA, 2010). In addition, when high quality CLA preparations (containing approximately 80–90% of the 2 biologically active isomers: *cis*-9, *trans*-11 and *trans*-10, *cis*-12-CLA) are consumed in a range of 0.5–7 g/day no adverse effects were observed in short-term in human subjects for several studies reported (DILZER; PARKER, 2012; GAULLIER *et al.*, 2002). Furthermore, the FDA allows since 2008 that CLA (*cis*-9, *trans*-11 and *trans*-10, *cis*-12-CLA) be added to foods and gave it a GRAS (generally regarded as safe) status for doses up to 6 grams/day (HARTIGH, 2019). Further studies, however, are needed to ascertain its long-term effects (time over 6 months), as well as its safety in patients with type 2 diabetes. In fact, as concluded by the EFSA expert panel, in these specific patients, the equimolecular mixture of CLA isomers appeared to negatively affect dynamic (ISI, OGIS) and static (HOMA-IR) markers of insulin sensitivity and increased some subclinical

inflammation markers (15-keto-dihydroprostaglandin F2 and C-reactive protein) (EFSA, 2010).

In this context from above introductory section, it is possible conclude that the production of CLA-rich food is still a technological challenge. Therefore, for the first step of present work, it was hypothesized that previous screening of LAB able to synthetize CLA from esterified LA (sunflower oil) increases the CLA content produced from milk fat in fermented milk. In addition, CLA has been described as a potent anti-inflammatory, inhibiting the NF-κB pathway in macrophage as well as priming macrophage into a M2 (anti-inflammatory) phenotype. Thus, for the second step of this work, the major original hypothesis was that CLA, similarly as in macrophages, has anti-inflammatory effect on microglial activation inhibiting the NF-κB pathway. The additional hypothesis was that CLA primes microglia from M1 (proinflammatory) to drive into a M2 (anti-inflammatory) phenotype, and that this microglial polarization is neuroprotective.

2 OBJECTIVES

2.1 OVERALL OBJECTIVE

Produce CLA-enriched milk through fermentation with previously screened LAB strain and investigate the neuroprotective and anti-neuroinflammatory effects of CLA in the *in vitro* animal model of neuroinflammatory disease.

2.2 SPECIFIC OBJECTIVES

Investigate if the previous selection of LAB with ability to produce CLA from esterified LA (sunflower oil) may enhance the CLA production from milk fat during fermentation;

Assess the effect of fermentation with the screened LAB on the fatty acids profile and nutritional quality indices in fermented milk;

Assess the potential of CLA to inhibit microglial *in vitro* activation by LPS;

Assess the potential of CLA to drive microglia from M1 to M2 phenotype;

Assess the neuroprotector potential of CLA on direct neuronal injury.

3 RESULTS AND DISCUSSION

This section was written in chapters, where each one is related to an original manuscript, either published (Chapter I) or in process of submission (Chapter II) to international scientific journals. In each chapter can be found detailed information about the experimental design, methodology and further discussion.

4 CHAPTER I

Journal of Functional Foods
Volume 31, April 2017, Pages 172-178

Short communications

Lactococcus lactis ssp. *cremoris* MRS47, a potential probiotic strain isolated from kefir grains, increases *cis*-9, *trans*-11-CLA and PUFA contents in fermented milk

Carla Paulo Vieira [a, b], Claudius Couto Cabral [a, b], Bruno R.C. da Costa Lima [c], Vânia Margaret F. Paschoalin [b], Kátia Christina Leandro [d], Carlos Adam Conte-Junior [a, b]

https://doi.org/10.1016/j.jff.2017.01.047

Qualis: A1

Impact Factor: 3.197

Lactococcus lactis ssp. *cremoris* MRS47, a potential probiotic strain isolated from kefir grains, increases *cis*-9, *trans*-11-CLA and PUFA contents in fermented milk

Short Title: A potential probiotic strain increases CLA and PUFA in fermented milk

Carla Paulo Vieira[a,b], Claudius Couto Cabral[a,b], Bruno R. C. da Costa Lima[c], Vânia Margaret F. Paschoalin[b], Kátia Christina Leandro[d], Carlos Adam Conte-Junior[a,b*]

[a] Department of Food Technology, Faculty of Veterinary, Fluminense Federal University, 24230-340, Rio de Janeiro, Brazil.

[b] Chemical Institute, Federal University of Rio de Janeiro, 21941-909, Rio de Janeiro, Brazil.

[c] Department of Animal and Food Sciences, University of Kentucky, 40546, Lexington, USA

[d] Chemistry Department, Oswaldo Cruz Foundation, 21040-900, Rio de Janeiro, Brazil.

*Corresponding author:
Professor Carlos Adam Conte Junior, D.V.M., M.Sc., Ph.D.
Rua Vital Brazil Filho, n. 64. Santa Rosa
CEP: 24.230-340
Niterói – RJ
Brazil
Phone: +55 21 2629-9545
E-mail address: carlosconte@id.uff.br

Abstract

Conjugated linoleic acid (CLA) has a number of beneficial health effects, including decreases in cancer incidence and atherosclerosis severity. However, dietary CLA intake is relatively low to promote the desired physiological effects. The CLA bacterial production in foods is a technological challenge besides the ability of probiotics to produce CLA is currently inconclusive. Therefore, the aim of the present study was to screen potential probiotic LAB strains isolated from Brazilian kefir grains able to produce CLA during the fermentation of whole cow milk. The selected strain, *Lactococcus lactis* ssp. *cremoris* MRS 47, increased CLA and polyunsaturated fatty acid content while at the same time significantly reducing saturated fatty acids and the thrombogenic index in fermented milk. These findings highlight the possibility of enriching CLA content in a dairy-fermented product if LAB were pre-selected by their capacity to produce CLA from milk fat, in what seems to be a strain-dependent ability.

Keywords: fatty acids, fermented milk, conjugate linoleic acid, polyunsaturated fatty acids, probiotic strain screening, gas chromatography analysis.

4.1 INTRODUCTION

Kefir grain starter culture is used to ferment milk into an acidic and ethanolic viscous beverage. Previous studies have reported the isolation of lactic acid bacteria (LAB) strains with probiotic potential from kefir grains (BOLLA *et al.*, 2013). The regular consumption of foodstuffs containing live probiotic bacteria decreases blood cholesterol levels, improves the immune system and potentially promotes anti-carcinogenic protective effects (SHAH, 2007).

Conjugated linoleic acid (CLA) is a mixture of geometric and positional conjugated isomers of C18:2, *cis*-9*, cis*-12 (linoleic acid). Rodent-animal *in vitro* and *in vivo* models and different human cell line studies have demonstrated a variety of beneficial health effects, such as anti-carcinogenic, anti-atherosclerotic, anti-oxidative, anti-obesity and anti-inflammatory activities, and stimulation of lean muscle mass deposition (YANG *et al*., 2015). Moreover, intake of CLA-rich diets in human have shown anti-inflammatory (PENEDO *et al*., 2013) and anti-carcinogenic potential about several human cancer strains (De LA TORRE *et al.*, 2006). The *trans*-10*, cis*-12-CLA isomer is thought to be antiobesity and antidiabetic, whereas *cis*-9*, trans*-11-CLA isomer is mainly anti-inflammatory. Both CLA isomers are reported to inhibit cancer through different pathways (YANG *et al*., 2015). CLA is naturally found in food matrices. The *cis*-9*, trans*-11-CLA isomer accounts for up to 90% of all the CLA isomers found in natural sources (CHIN *et al.,* 1992). Nonetheless, the intake of CLA in human diets is below the recommended levels considering that human consumption should be of 3.0 g of

CLA per day to achieve maximum health benefits (IP *et al.,* 1994). Although studies have been published using food-grade bacteria as starter, adjunct or probiotic culture to develop functional fermented dairy with increased levels of CLA, the results described in literature so far are below expectations due to technological bottlenecks. Thus, more research studies are needed to assess if CLA bacterial production kinetics can be increased and thus can match food processing requirements. Therefore, CLA bacterial production in foods still is a technological challenge (GORISSEN *et al*., 2015). In addition, a linoleic acid isomerase enzyme activity in LAB has been reported (GORISSEN *et al.,* 2011). However, the ability of probiotic bacteria to produce CLA is still inconclusive (MANZO *et al.,* 2015). Thus, taking into account the beneficial health effects attributed to probiotic microorganisms (SHAH, 2007), efforts are made to develop a milk fermentation process by probiotic LAB to increase CLA content in dairy derivatives.

In this context, the aim of the present study was to screen probiotic LAB strains isolated from kefir grains for CLA production potential and to test their ability to produce CLA from cow milk fat during milk fermentation.

4.2 MATERIAL AND METHODS

4.2.1 Material

Sunflower seed oil and whole cow milk were purchased from a retail market in Southeastern Brazil (state of Rio de Janeiro). All organic solvents were of chromatographic grade (Tedia, São Paulo, Brazil). Commercially available De Man, Rogosa and Sharpe (MRS) broth was purchased from Sigma-Aldrich Chemical Co, MO, USA. A commercial mixture of fatty acid methyl esters (FAME mix, ref.47885-U, Supelco, Co., PA, USA) was used for identification of the FAME peaks. All other reagents were of analytical grade (Sigma-Aldrich Chemical Co.).

4.2.2 Screening of potential probiotic lactic acid bacteria strains for the ability to synthesize conjugated linoleic acid (CLA) from sunflower seed oil

The sunflower seed oil micellar solution, used to improve sunflower oil bioavailability to bacterial strains, was prepared according to the method described by Wang *et al.* (2007).

The nine LAB strains tested in present work (Table 4) for CLA production potential were previously selected regarding to probiotic potential by our research group (LEITE *et al.*, 2015). The criteria used were: acidic pH tolerance, bile salts resistence, antagonistic activity against model food pathogens, bacteriocin-like inhibitory substances against indicator organisms, total antioxidative activity, undesirable enzymatic or hemolytic activities, resistance to clinically relevant antibiotics and level of adhesion to human Caco-2 epithelial cells.

Each LAB strain was individually activated in 10 mL of MRS broth at 37 ºC for 24 h (Thermo Scientific, USA). After this, 1 mL were transfered to sunflower seed oil micellar solution in 10 mL of MRS broth (1.7% v/v) and incubated at 37 ºC for 6 h. After incubation, 1 mL of the MRS broth was centrifuged (Sorvall ST16R, Thermo Scientific) at 20,800 × *g* for 1 min at 4 ºC and the supernatant was transfered to a fresh test tube. The fatty acids were extracted with n-hexane as described by Barret, Ross, Fitzgerald e Stanton (2007).

CLA in the n-hexane extract was detected by a characteristic absorption peak at 233 nm (EL-SALAM *et al.,* 2010), using MSR broth containing sunflower seed oil micelles without bacterial strains as blank. An increase in the CLA concentration coincides with a linear increase in absorbance, up to absorbance values of 1.0 (BARRET *et al.,* 2007; WANG *et al.,* 2007).

4.2.3 Chemical parameters of unfermented and fermented milk

Titratable acidity was determined according to the official AOAC 947.05 method (AOAC Association of the Official Analytical Chemists, 2012). A digital pH-meter (720P, ISTEK, Korea) was calibrated with pH 4.0 and pH 7.0 standard solutions. Total fat from kefir beverage was extracted with petroleum ether by the Soxhlet 2000.18 method and quantified gravimetrically (AOAC, 2012).

4.2.4 Milk Fermentation

Ultra high temperature (UHT) whole cow milk (2.95 g of fat per 100 g of sample) was fermented by a starter culture composed of thermophilic yogurt cultures (YF-L903; Chr. Hansen, Valinhos, Brazil) at a final concentration of 1% (v/v) at 40 °C for 8 h. The control fermentation process (C) contained only the starter culture, while the probiotic fermentation process (P) was fermented simultaneously by a consortium of the starter culture and the

Lactococcus lactis subsp. *cremoris* MRS47 strain (1.0×10^8 CFU/mL) at a concentration of 5% (v/v) of the total milk volume. The whole milk prior to inoculation was considered as reference.

The cell viability ($\log_{10}$ cfu/mL) was checked previously for *Lactococcus lactis* ssp. *cremoris* by our research group (LEITE *et al.*, 2012) for 24h of fermentation in milk (aliquots every 6h).

The fermented milk with probiotic bacteria in coculture with yogurt starter has similar acidity, texture and, flavor those of milk fermented with yogurt starter alone. The milk fermented with probiotic strain by itself do not show organoleptic attributes comparable to the milk fermented by the starter culture (control fermented milk), which decrease consumer acceptability (XU *et al.*, 2005). The sensory alterations can be attributed to the lack of essential proteolytic activity in probiotic LAB (OLIVEIRA *el al.*, 2001). For these reasons, in this study, whole milk was not fermented with *Lactococcus lactis* subsp. *cremoris* MRS47 strain by itself.

4.2.5. Derivatization of fatty acids and FAME analysis by gas chromatography

Milk CLA and total lipids were extracted from 5 g aliquots with a mixture of methanol and chloroform 2:1 (v/v) (CONTE-JUNIOR *et al.*, 2007). The extracted FAMEs were subjected to acid methylation using an HCl (2N) solution (10% HCl: 90% organic extract, v/v) at 60 ºC for 20 min (CHIN *et al*., 1992; Kishino *et al.*, 2002).

The chromatographic separation of 1 μL of FAMEs was achieved by gas chromatography as described by Canto *et al.* (2015).

FAME gas-chromatographic peaks were identified by comparing their retention time with data from commercial standards. Heptadecanoic acid (C17:0; Sigma Chemical Co.) was used as an internal standard for quantification.

4.2.6. Nutritional lipid quality indices

Atherogenicity (AI) and thrombogenicity (ThI) indices were calculated as proposed by Ulbricht e Southgate (1991).

The hypocholesterolemic/hypercholesterolemic fatty acid ratio was calculated according to Santos-Silva, Bessa e Santos-Silva (2002), as follows:

$$AI = [(C12:0) + (4 \times C14:0) + (C16:0)] / (\sum MUFA + \sum n6 + \sum n3) \quad (1)$$

ThI = [(C14:0 + C16:0 + C18:0)] / [(0.5 x ∑ MUFA) + (0.5 x ∑ n6) + (3 x ∑ n3) + (∑ n3 / ∑ n6)] (2)

hH = (C18:1cis9 + C18:2n6 + C20:4n6 + C18:3n3 + C20:5n3 + C22:5n3 + C22:6n3) / (C14:0 + C16:0) (3)

4.2.7 Desaturase ability (DA)

The Δ^9 desaturase ability was calculated as the ratio of fatty acids that are products to the ones that are substrates plus products for Δ^9 desaturase, as proposed by Lock e Garnsworthy (2003):

DA = (C14:1n5 + C16:1n7 + C18:1n9 + C18:2n6 + C18:2, *cis*-9, *cis*-12 + C18:3n3 + C18:3n6) / (C14:0 + C16:0 + C18:0 + C14:1n5 + C16:1n7 + C18:1n9 + C18:2n6 + C18:2, *cis*-9, *cis*-12 + C18:3n3 + C18:3n6).

4.2.8. Statistical analyses

All analyses were performed in quadruplicate and results were expressed as mean ± standard deviation (SD). A one-way analysis of variance was used to compare data obtained for the different fermentation processes. When a significant *F* was observed ($p < 0.05$), differences between means were evaluated by Tukey´s multiple comparison test. Two-side *p* values < 0.05 were considered statistically significant. Multiple regression analysis at the 0.05 level of significance was also conducted. When a significant *F* was observed ($p < 0.05$) for multiple regression equation, the independent variables were considered to contribute for CLA variability in samples. The statistical analyses were performed using a commercially available statistical package (Systat software, Chicago, IL, USA).

4.3 RESULTS AND DISCUSSION

4.3.1 Screening of potential probiotic LAB strains isolated from kefir grains for CLA production ability

The spectrophotometric method is a simple and straightforward technique for the screening of CLA producing strains, especially when investigating a large number of strains (BARRET *et al.*, 2007; WANG *et al*., 2007). Therefore, in this work, firstly the LAB strains were selected by spectrophotometric method in MRS broth, followed by gas chromatography (GC) for analysis of fatty acids profile, including CLA-isomers, in dairy matrix.

The first step of study was to screen among the 9 probiotic potential strains that with highest CLA production potential from esterified linoleic acid. Thus, the strain designated as *Lactococcus lactis* subsp. *cremoris* MRS47 presented the highest absorbance value ($p < 0.05$) (Table 4). Therefore, considering the linear relationship between absorbance values at 233 nm and CLA concentration (BARRET *et al.,* 2007; WANG *et al.,* 2007), the data suggest that *Lactococcus lactis* subsp. *cremoris* MRS47 exhibited the highest CLA-producing ability.

The ability of LAB strains in producing CLA from esterified linoleic acid lipid was tested herein by adding sunflower seed oil to the MRS broth. It is relevant because in whole milk the linoleic acid, which is substrate for CLA production, is available in esterified way.

4.3.2 Chemical parameters of unfermented and fermented whole milk

The pH, titratable acidity and fat content of unfermented whole milk (M), whole milk fermented by the starter culture (C), and whole milk fermented by the consortium formed by the starter culture and the *Lactococcus lactis* subsp. *cremoris* MRS47 strain (P) are displayed in Figure 5. As expected, fermentation by either the starter culture (C) or the consortiums (P) resulted in acid production (lower pH) ($p < 0.05$) than the whole milk counterpart, indicating that lactose in milk was metabolized by microorganisms into acidic products during fermentation (YADAV *et al.,* 2007). Furthermore, when solely considering this parameter, it was not possible to distinguish C from P ($p > 0.05$). These results are in accordance to those reported by XU *et al.* (2005).

Titratable acidity (TA) is used for evaluating fermentation progress and quality in the final dairy product (SERAFEIMIDOU *et al.*, 2012). Similar to pH, fermentation also affected ($p < 0.05$) the TA, where whole milk samples exhibited the lowest ($p < 0.05$) values, while no

difference ($p > 0.05$) was observed between C and P. The starter culture is responsible by rapid acidification of femented product, however no difference ($p > 0.05$) of pH and TA between the fermentative processes was observed. These behavior indicates that the starter culture had not inferior competitiveness compared to probiotic LAB (COPPOLA *et al.*, 2000). In addition, there was not significant difference in fat content among samples (Figure 5).

In order to evaluate if the chemical parameters of the fermentative processes are associated with the differences in *cis*-9, *trans*-11-CLA content (Table 5), these data were subjected to a multiple regression analysis. Overall, the multiple regression equation for chemical parameters was not significant (standard error of estimate = 3.316; $R^2 = 0.321$; $p = 0.632$). The partial correlations are shown in Table 6. This is in accordance with Serafeimidou *et al.* (2012) which have not found correlation of pH, titratable acidity and fat with CLA content in yogurt. Similarly, Prandini *et al.* (2001) also reported that there was no significant correlation between CLA and fat content in dairy products. These findings suggest that there should be no association between *cis*-9, *trans*-11-CLA content and the chemical parameters studied herein.

4.3.3 CLA content in fermented whole milk

PUFA, MUFA, SFA, and CLA isomer contents of whole milk (M), whole milk fermented with the starter culture (C), and whole milk fermented with the consortium of the starter culture plus the *Lactococcus lactis* subsp. *cremoris* MRS47 strain (P) are expressed as g/100g total fatty acids (Table 5). Samples containing the probiotic strain (P) exhibited greater ($p < 0.05$) CLA than M and C content after fermentation, indicating that only *Lactococcus lactis* subsp. *cremoris* MRS47 had the ability to produce CLA in whole milk during fermentation, through the lipolysis of milk fat. Only the *cis-9, trans-11*-CLA isomer exhibited changes ($p < 0.05$) during fermentation (Table 5). In addition, *trans-10, cis-12*-CLA and *cis-10, cis-12*-CLA isomers also presented increase during probiotic fermentation when compared to M and C, however this increase was not enough to reach statistical significance ($p < 0.05$) (Table 5). These results are according to the biological CLA production by LAB to be isomer-selective (OGAWA *et al.*, 2005).

The *cis-9, trans-11*-CLA contents in M and C samples were similar to the values reported by Zegarska, Paszczyk e Borejszo (2008) for commercial beverages available at the market; 0.1-2.9 g in whole milk and 0.33-1.27 g in a fermented milk beverage per 100 g of total fatty acids, respectively. Nevertheless, these CLA contents reported in the literature are

relatively lower than those found during the probiotic fermentation process in the present study (6.65g/100g fatty acids) (Table 5).

The probiotic fermentation process (P) enhanced CLA levels by 9.3 times when compared to C, and 6.1 times when compared to M (Table 5). This result is superior to those found in the literature. Likewise, Xu *et al.* (2005) documented a 1.7-fold increase in CLA levels in milk fermented with the probiotic *Propionibacterium freudenreichii* subsp. *Shermanii* 51 in co-culture with yogurt strains when compared to yogurt cultures alone converting hydrolyzed soy oil as the lipid source. In addition, Alkalin *et al.* (2007) reported that yogurt manufactured with probiotic *Lactobacillus acidophilus* or *Bifidobacterium animalis* strains and a yogurt starter culture exhibited 2.71- and 2.90-fold increases in CLA, respectively, when compared to yogurt fermented by the starter culture. In agreement with the present data, Hernández *et al.* (2007) reported a 4.0-fold CLA content increase in dairy products (2g/100g of total fatty acids) manufactured with milk from cows fed a sunflower seed-supplemented diet. Therefore, the screening of *Lactococcus lactis* subsp. *cremoris* MRS47 strain regarding to potential ability for CLA production prior to your use in the milk fermentation process is a clear advantage over the majority of previous studies, and probably favored the greater CLA content reported herein.

Taking into account the relative CLA concentration in fermented dairy products, El-Salam *et al.* (2010) found CLA concentrations of 21.6 mg/100mL of milk fermented with probiotic *Lactococcus lactis lactis* using sesame oil treated by lipases as the lipid source. This value is lower than the CLA concentration found in milk fermented with the probiotic strain demonstrated herein (85mg /100mL milk) (Figure 6). The CLA content in the probiotic fermentation process (6.65g/100g fatty acids) is close to the value found by Bell e Kennelly (2001) (5.63g/100g fatty acids) for milk from cows fed a fat-rich diet.

Based on daily milk consumption data available from European countries (WOLFF; PRECHT, 2002) was estimated that the consumption of probiotic fermented milk produced herein with *Lactococcus lactis* subsp. *cremoris* MRS47 would lead to an intake of 0.95g/day, 0.89g/day and 0.44g/day C18:2, *cis*-9, *trans*-11 for France, German and Spain, respectively. Thus, the daily intake would represent a percentage ranging from 31.6%, in France, to 14.6%, in Spain, of the recommended daily consumption of 3g CLA/day to achieve the maximum health benefits (IP *et al.,* 1994).

4.3.4 Influence of different fermentation processes on the fatty acid profile of whole milk

The probiotic fermented product (P) exhibited SFA content around 8% lower ($p < 0.05$) than control (Table 5). This can be attributed to the higher Δ9-desaturase ability in P, which could have contributed to lower content of palmitic (16:0), oleic (18:0) and tetracosanoic acid (24:0) in milk fermented with *Lactococcus lactis* subsp. *cremoris* MRS47 when compared to control ($p < 0.05$). Similarly e Hernández *et al*. (2007) reported a SFA content reduction of 16% in CLA-rich dairy products. It can be related to CLA production through desaturase activity (LIN, 2006).

Considering PUFA contents, the probiotic fermentation process (P) presented higher ($p < 0.05$) PUFA content than milk and control. This can be attributed to lower MUFA and higher Δ9-DA in probiotic milk when compared to unfermented milk and control, respectively (Table 5). These findings are similar to Hernández *et al*. (2007) who documented a SFA decrease and unsaturated fatty acids (UFA) increase in CLA-rich dairy products when compared to control counterparts. Moreover, the intake of the probiotic sample could potentially reduce the risk of cardiovascular disease events by replacing SFA with PUFA (OHLSSON, 2010). Addicionally, it is known that PUFA/SFA ratios impact the viability of lactic acid bacteria (BÉAL *et al.*, 2001). Therefore, the increased PUFA/SFA ratio in P following fermentation could contribute to increases in the *Lactococcus lactis* subsp. *cremoris* MRS47 strain viability in food matrices. This is a desirable characteristic regarding both technological and nutritive aspects, since probiotic bacteria must be viable to promote the proposed beneficial health effects (SHAH, 2007).

Regarding to MUFA, its decrease after fermentation on both control and probiotic can be attributed to the consumption of oleic acid (18:1n9), and the production of tetracosanoic acid (24:0) during fermentation. It occurs in order to improve control of membrane fluidity during stress conditions, including fermentation. The products from oleic acid (18:1n9) metabolism, which is a MUFA, increase the bacterial membrane rigidity and decrease the permeability to H^+ during fermentation. Similarly, the increase ($p < 0.05$) on the tetraecosanoic acid (24:0) content on both C and P indicates a potential metabolic stress adaptation in order to shift the fatty acid chain length in cellular membrane to appropriate proportions (GUERZONI *et al.,* 2001). Moreover, the oleic acid (18:1n9) also is used by LAB as substrate for CLA production (YANG *et al.*, 2013). It explains why the MUFA content significantly contributes for observed changes in CLA content of samples (Table 6).

The nutritional quality of the lipid fraction in the dairy matrix of the present study was estimated through three indices (AI, ThI and hH) from fatty acid composition data, expressed as a percentage of the identified total fatty acids (Table 5). The increase of SFA and decrease of MUFA and omega-3 (n-3) fatty acids during fermentation led to an increase ($p < 0.05$) in AI and ThI values in both C and P (Table 5). Similarly, Stajić, Živković, Perunović, Šobajić e Vranić (2011) also reported an AI increment in fermented food. Regarding to the ThI, this value in probiotic fermentation process (P) was 12.43% lower ($p < 0.05$) than its C counterpart. This result is probably due to the lower palmitic (16:0) and stearic acid (18:0) contents in milk fermented with *Lactococcus lactis* subsp. *cremoris* MRS47 when compared to milk fermented with starter culture alone (Table 5). The reduction of these saturated fatty acids can be attributed to higher Δ9-DA abilities in probiotic than in control (Table 5). Additionally, no data about ThI in fermented food has been described in the literature. However, the decrease of ThI indices in CLA-rich dairy product compared to those not enriched in CLA has been previously reported in the literature (HERNÁNDEZ *et al.,* 2007). In addition, the fermentation process with *Lactococcus lactis* subsp. *cremoris* MRS47 did not influence the hH indexes because there was not significant diference ($p > 0.05$) between probiotic and control fermentation processes (Table 5). Moreover, hH indexes for fermented dairy products were not found in literature.

The Δ9-DA abilities of the milk fermented with *Lactococcus lactis* subsp. *cremoris* MRS47 (P) presented higher ($p < 0.05$) values than control. As only P showed significant CLA increases during fermentation, the findings in this study are consistent with Lin (2006), who reported that CLA production during fermentation can occur by either isomerase or desaturase enzyme activities expressed in LAB. This is corroborated by significant and high positive partial correlation between Δ9-DA abilities and CLA content (Table 6). The multiple regression equation for sums and ratios of fatty acids as independent variables is highly significant (standard error of estimate: 0.094; $R^2 = 0.999$; $p < 0.001$), so that only MUFA and Δ9-DA show significant partial correlation (Table 6). Therefore, MUFA and Δ9-DA are associated to variability in CLA content.

The isomerase activity is related to biohydrogenation system for detoxification of free unsaturated long-chain fatty acids, such as linoleic acid (KIM; LIU, 2002). On the other hand, the desaturase activity is related to cell membrane protection during stress conditions, including fermentation. The unsaturated fatty acids resultant of desaturase activity increase the bacterial membrane rigidity and decrease the permeability to H^+, Na^+, and potentially to H_2O_2 during stressing conditions (GUERZONI *et al.*, 2001). Therefore, as in this study the

fatty acids are mostly on esterified way well as there is acidification of medium (after fermentation), the desaturase activity should be the pathway mainly implicated in the CLA production by *Lactococcus lactis* subsp. *cremoris* MRS47. The Δ9-desaturase ability has been previously reported in LAB (LIN, 2006), and under the stress conditions provoked by fermentation there is an activation of fatty acid desaturation pathways in order to protect the cell membrane from damage (GUERZONI *et al.,* 2001). Thus, the higher Δ9-DA in P should contribute to increase resistance of the *Lactococcus lactis* subsp. *cremoris* MRS47 strain during fermentation.

4.4 CONCLUSIONS

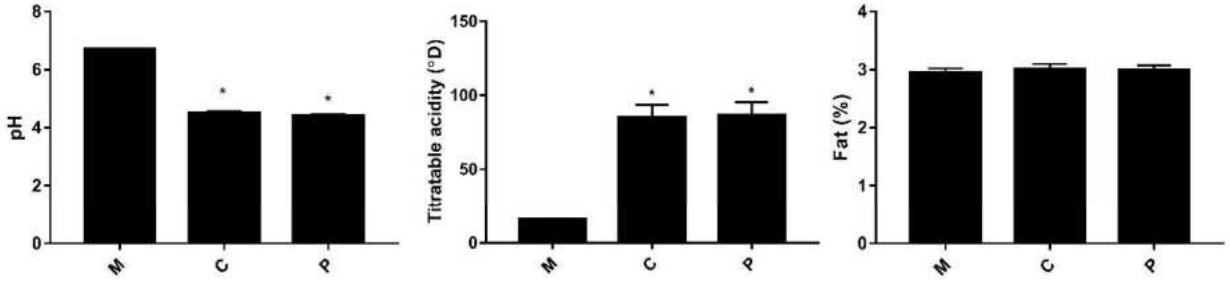
The probiotic *Lactococcus lactis* ssp. *cremoris* MRS 47 strain isolated from Brazilian kefir grains is able to change the fatty acid profile by milk fermentation. The use of sunflower oil as a substrate for CLA production during the screening step contributed for the selection of strains that were also able to produce CLA from milk fat. The probiotic fermentation process reduced SFA and ThI and increased PUFA and PUFA/SFA ratios during fermentation. The higher Δ9-DA ability contributed to improve the nutritional lipid quality in the final probiotic fermented product. Moreover, Δ9-DA ability increment is useful to increase the CLA production in fermented product. The previous selection of potentially probiotic strains regarding CLA production capability is an useful and relevant step to produce CLA-rich fermented milk. This is a previous study, so that further sensory research of this fermented milk will be useful for future industrial application.

4.5 ACKNOWLEDGEMENTS

The authors would like to thank the Fundação de Amparo à Pesquisa do Estado do Rio de Janeiro (process no. E-26/201.185/2014 and E-26/010.001.911/2015, FAPERJ, Brazil) and the Conselho Nacional de Desenvolvimento Científico e Tecnológico (process no. 311361/2013-7, CNPq, Brazil) for the financial support. C.P. Vieira was supported by a CNPq fellowship.

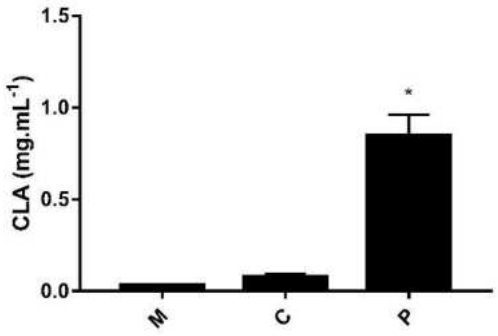
4.6 FIGURES AND TABLES

Figure 5 - Chemical parameters of unfermented whole milk (M) and the beverage fermented by the starter culture in C, while in P whole milk was fermented by a microbial consortium composed of the starter culture and the selected probiotic *Lactococcus lactis* subsp. *cremoris* MRS47 strain at 40 ºC for 8 h. Assays were performed in quadruplicate and the values are reported as means ± SD. * denotes significant difference ($p < 0.05$) between M, C and P.



Source: the author.

Figure 6 - CLA concentration ($mg.mL^{-1}$) of unfermented whole milk (M) and the beverage fermented by the starter culture in C, while in P whole milk was fermented by a microbial consortium composed of the starter culture and the selected probiotic *Lactococcus lactis* subsp. *cremoris* MRS47 strain at 40 ºC for 8 h. Assays were performed in quadruplicate and the values are reported as means ± SD. * denotes significant difference ($p < 0.05$) between M, C and P.



Source: the author.

Table 4 - Screening of potential probiotic lactic acid bacteria strains for the ability to synthesize conjugated linoleic acid (CLA) from sunflower seed oil, evaluated by absorbance at 233nm

Strains	**Absorbance**	
	Mean	**SD**
Lactobacillus casei/paracasei MRS59*	0.021[a]	0.006
Lactobacillus casei/paracasei MR1**	0.014[a]	0.001
Lactococcus lactis subsp. *cremoris* M1711*	0.052[bc]	0.013
Lactococcus lactis subsp. *cremoris* MRS47***	0.150[d]	0.012
Lactococcus lactis subsp. *lactis* MRN3*	0.060[c]	0.008
Leuconostoc mesenteroides GYP5*	0.059[c]	0.008
Leuconostoc mesenteroides MRS53*	0.019[a]	0.008
Leuconostoc mesenteroides GYP12**	0.004[a]	0.006
Leuconostoc mesenteroides GYP7*	0.026[ab]	0.000

Probiotic lactic acid bacteria strains isolated from Brazilian kefir grains in MRS broth containing 1.7% (v/v) of sunflower seed oil micellar solution at 37ºC for 6h.
SD, standard deviation. [a-d] Means with different superscripts are significantly different. ANOVA ($p < 0.05$) with Tukey´s post-hoc test: * $p < 0.05$; ** $p < 0.01$; and *** $p < 0.001$.
Source: the author.

Table 5 - Fatty acid profile (g/100g total fatty acids) in whole milk (**M**), whole milk fermented with starter culture (**C**) and with the microbial consortium between the starter culture and the probiotic *Lactococcus lactis* subsp. *cremoris* MRS47 strain (**P**) at 40 ºC for 8 h.

Fatty acids	**M**		**C**		**P**	
	Mean	**SD**	**Mean**	**SD**	**Mean**	**SD**
Individual fatty acids						
6:0	0.00	0.00	0.83	0.05	1.42	0.95
8:0	0.57	0.00	0.61	0.01	0.54	0.01
10:0	2.08	0.50	2.70	0.07	2.46	0.05
12:0**	2.75[a]	0.63	5.47[b]	0.30	5.06[b]	0.24
13:0	0.14	0.03	0.28	0.05	0.23	0.04
14:0***	11.07[b]	1.59	6.55[a]	0.36	6.05[a]	0.29
14:1n-5	1.15	0.17	1.82	0.33	1.80	0.29
15:0***	1.27[a]	0.12	17.50[b]	0.83	16.09[b]	0.66
15:1n-5	0.34	0.03	1.70	0.12	1.59	0.10
16:0***	34.24[c]	0.81	29.38[b]	0.08	26.34[a]	0.24
16:1n-7	1.83	0.08	1.51	0.01	1.36	0.01
17:1n-7	0.34	0.02	0.18	0.00	0.17	0.00
18:0**	13.88[a]	1.41	18.74[c]	0.98	16.28[b]	0.99
18:1n-9***	26.42[b]	2.23	2.41[a]	0.14	2.08[a]	0.14
18:2n-6t	0.00	0.00	0.13	0.00	0.10	0.02
18:2n-6c	2.08	0.17	1.22	0.00	0.11	0.00
18:3n-6	0.22	0.08	0.26	0.01	0.24	0.01
18:3n-3	0.38	0.02	0.16	0.01	0.14	0.01
18:2;9c11t***	1.09[a]	0.07	0.71[a]	0.10	6.65[b]	0.89
18:2;10t12c	0.00	0.00	0.04	0.07	1.87	0.30
18:2;10c12c	0.00	0.00	0.08	0.13	1.34	1.22
18:2;tt	0.00	0.00	0.00	0.00	0.08	0.11
20:0	0.24	0.05	0.13	0.03	0.08	0.00
20:1n-9	0.11	0.10	0.00	0.00	0.12	0.00
20:2n-6	0.00	0.00	0.33	0.01	0.29	0.01
20:3n-6	0.00	0.00	0.22	0.00	0.19	0.01
20:4n-6	0.00	0.00	0.00	0.00	0.00	0.00
20:3n-3	0.00	0.00	0.00	0.00	0.00	0.00
20:5n-3	0.00	0.00	0.00	0.00	0.00	0.00
21:0	0.00	0.00	0.00	0.00	0.00	0.00
22:0	0.00	0.00	0.00	0.00	0.66	0.01
22:1n-9	0.00	0.00	0.00	0.00	0.00	0.00
22:2n-6	0.00	0.00	0.56	0.48	0.00	0.00
22:6n-3	0.00	0.00	0.00	0.00	0.00	0.00
23:0	0.00	0.00	0.00	0.00	0.00	0.00
24:0***	0.00[a]	0.00	6.58[b]	0.16	5.81[b]	0.19
24:1n-9	0.00	0.00	0.00	0.00	0.00	0.00
Sums of fatty acids						
SFA***	66.05[a]	2.17	88.76[c]	0.36	80.77[b]	1.66
MUFA***	30.19[b]	1.97	7.44[a]	0.02	7.07[a]	0.32
PUFA***	3.76[a]	0.20	3.25[a]	1.29	10.90[b]	1.74
n-3*	0.38[b]	0.02	0.16[a]	0.00	0.09[a]	0.08
n-6	2.30	0.15	2.38	1.38	4.15	0.96
Ratios						
PUFA/SFA*	0.06[a]	0.00	0.04[a]	0.01	0.13[b]	0.02
AI***	2.49[a]	0.39	5.57[b]	0.13	4.93[b]	0.39
ThI***	3.39[a]	0.26	9.41[c]	0.20	8.24[b]	0.42

hH***	0.64[b]	0.09	0.11[a]	0.00	0.07[a]	0.00
Δ9-DA***	0.36[c]	0.02	0.13[a]	0.00	0.20[b]	0.01

SD, standard deviation; SFA, saturated fatty acid; MUFA, monounsaturated fatty acid; PUFA, polyunsaturated fatty acid; AI, atherogenicity index; ThI, thrombogenicity index; hH, hypocholesterolemic/hipercholesterolemic fatty acids; Δ9-DA, Δ9 desaturase ability. [a-c] Means within the same row with different superscripts are significantly different. ANOVA ($p < 0.05$) with Tukey´s post-hoc test: * $p < 0.05$; ** $p < 0.01$; and *** $p < 0.001$. Source: the author.

Table 6 - Partial correlations from multiple regression analysis for CLA as dependent variable in milks from different fermentative processes at 40 ºC for 8 h.

Independent variable	**Statistical parameters**			
	b	**R²**	**t**	**p-value**
Acidity	7.643	0.990	0.635	0.559
pH	7.076	0.990	0.678	0.535
Fat	-0.771	0.385	-0.866	0.435
SFA	-0.129	0.999	-0.236	0.834
MUFA	-3,399	0.998	-10.490	0.008
PUFA	-0.205	0.999	-0.359	0.753
PUFA/SFA	0.108	0.999	0.177	0.875
Δ9-DA	2.744	0.999	6.442	0.023

b, intercept; R^2, squared partial correlation; t, t-statistic; p-value, probability value; SFA, saturated fatty acid; MUFA, monounsaturated fatty acid; PUFA, polyunsaturated fatty acid; Δ9-DA, Δ9 desaturase ability. Source: the author.

4.7 REFERENCES

ALKALIN, A. S. *et al.* Occurrence of conjugated linoleic acid in probiotic yogurts supplemented with fructooligosaccharide. **Internationl Dairy Journal,** v. 17, p. 1089-1095, 2007.

AOAC. **Official methods of analysis of AOAC.** 19. ed. Maryland: Association of the Official Analytical Chemists Press, 2012.

BARRET, E. *et al.* Rapid screening method for analyzing the conjugated linoleic acid production capabilities of bacterial cultures. **Applied and Environmental Microbiology,** v. 73, n. 7, p. 2333-2337, 2007.

BÉAL, C.; FONSECA, F.; CORRIEU, G. Resistance to freezing and frozen storage of Streptococcus thermophilus is related to membrane fatty acid composition. **Journal of Dairy Science,** v. 84, p. 2347-2356, 2001.

BELL, J.A.; KENNELLY, J.J. Conjugated linoleic acid enriched milk: a designer milk with potential. **Advances in Dairy Technology,** v, 13, p. 213-227, 2001.

BOLLA, P. A. *et al.* Kefir-isolated Lactococcus lactis subsp. lactis inhibits the cytotoxic effect of Clostridium difficile in vitro. **The Journal of Dairy Research,** v. 8, n. 1, p. 96-102, 2013.

CANTO, A. C. V. C. S. *et al.* Fatty acid profile and bacteriological quality of caiman meat subject to high hydrostatic pressure. **LWT - Food Science and Technology,** v. 63, p. 872-877, 2015.

CHIN, S. F. *et al.* Dietary sources of conjugated dienoic isomers of linoleic acid, a newly recognized class of anticarcinogens. **Journal of Food Composition and Analysis**, v. 5, p. 185-197, 1992.

CONTE-JUNIOR, C. A. *et al.* Estudio de la producción de ácido linoleico conjugado por cepas de *Lactobacillus* sp. and *Enterococcus* sp. de distintos orígenes. **Revista Complutense de Ciencias Veterinarias,** v.1, p. 482-489, 2007.

COPPOLA, S. *et al*. Microbial succession during ripening of Naples-type salami, a southern Italian fermented sausage. **Meat Science,** v. 56, p. 321-329, 2000.

DE LA TORRE, A. *et al*. Beef conjugated linoleic acid isomers reduce human cancer cell growth even when associated with other beef fatty acids.**The British Journal of Nutrition,** v. 95, n. 2, p. 346-52, 2006.

EL-SALAM, M. H. *et al.* Screening of some potentially probiotic lactic acid bacteria for their ability to synthesis conjugated linoleic acid. **International Journal of Dairy Technology,** v. 63, n.1, p. 62-69, 2010.

GORISSEN, L. *et al.* Bacterial production of conjugated linoleic and linolenic acid in foods: a technological challenge. **Critical Reviews in Food Science and Nutrition,** v, 55, n.11, p. 1561-1574, 2015.

GORISSEN, L. *et al.* Linoleate isomerase activity occurs in lactic acid bactéria strains and is affected by pH and temperature. **Journal of Applied Microbiology,** v. 111, p. 593-606, 2011.

GUERZONI, M. E. *et al.* Alteration in cellular fatty acid composition as a response to salt, acid, oxidative and thermal stresses in *Lactobacillus helveticus*. **Microbiology,** v. 147, p. 2255-2264, 2001.

HERNÁNDEZ, E. R. S. *et al.* Alto contenido de ácido linoleico conjugado (CLA) en leche y productos derivados al incorporar semillas de girasol a la dieta vacuna. Implicaciones sobre El riesgo trombo/aterogênico. **Archivos Latinoamericanos de Nutricion,** v. 57, n. 2, p. 173-178, 2007.

IP, C. *et al.* Conjugated Linoleic Acid Suppresses Mammary Carcinogenesis and Proliferation Activity of the Mammary Gland in the Rat. **Cancer Research,** v. 54, p. 1212–1215, 1994.

KIM, Y. J.; LIU, R. H. Increase of conjugated linoleic acid content in milk by fermentation with lactic acid bacteria. **Journal of Food Science,** v. 67, n. 5, p. 1731-1737, 2002.

KISHINO, S. *et al.* Ricinoleic acid and castor oil as substrates for conjugated linoleic acid production by washed cells of *Lactobacillus plantarum.* **Bioscience, Biotechnology and Biochemistry,** v. 66, p. 2283-2286, 2002.

LEITE, A. M. O. *et al*. Assessment of the microbial diversity of Brazilian kefir grains by PCR-DGGE and pyrosequencing analysis. **Food Microbiology,** v. 31, p. 215-221, 2012.

LEITE, A. M. O. *et al.* Probiotic potential of selected lactic acid bacteria strains isolated from Brazilian kefir grains. **Journal of Dairy Science,** v. 98, p. 3622-3632, 2015.

LIN, T. Y. Conjugated linoleic production by cells and enzyme extract of Lactobacillus delbrueckii ssp. Bulgaricus with additions of different fatty acids. **Food Chemistry,** v. 94, p. 437-441, 2006.

LOCK, A. L.; GARNSWORTHY, P. C. Seasonal variation in milk conjugated linoleic acid and Δ^9–desaturase activity in dairy cattle. **Livestock Production Science,** v. 79, p. 47-59, 2003.

MANZO, N. *et al.* The effects of probiotics and prebiotics on the fatty acid profile and conjugated linoleic acid content of fermented cow milk. **International Journal of Food Sciences and Nutrition,** v. 66, n. 3, p. 254-259, 2015.

OGAWA, J. *et al.* Production of conjugated fatty acids by lactic acid bacteria. **Journal of Bioscience and Bioengineering,** v. 100, n. 4, p. 355-364, 2005.

OHLSSON, L. Dairy products and plasma cholesterol levels. **Food & Nutrition Research,** v. 54, 2010.

OLIVEIRA, M. N. *et al*. Effect of milk supplementation and culture composition on acidification, textural properties and microbiological stability of fermented milks containing probiotic bacteria. **International Dairy Journal,** v. 11, Suppl. 11-12, p. 935-942, 2001.

PENEDO, L. A. Intake of butter naturally enriched with cis9, trans11 conjugated linoleic acid reduces systemic inflammatory mediators in healthy young adults. **Journal of Nutritional Biochemistry**, v. 24, p. 2144-2151, 2013.

PRANDINI, A. *et al.* Survey on the level of onjugated linoleic acid in dairy products. **Italian Journal of Food Science,** v.13, n. 2, p. 243-254, 2001.

SANTOS-SILVA, J. *et al.* Effect of genotype, feeding system and slaughter weight on the quality of light lambs: II. Fat acid composition of meat. **Livestock Production Science**, v. 77, n. 2-3), p. 187-194, 2002.

SERAFEIMIDOU, A. *et al.* Chemical characteristics, fatty acid composition and conjugated linoleic acid (CLA) content of traditional Greek yogurts. **Food Chemistry**, v. 134, p. 1839-1846, 2012.

SHAH, N. P. Functional cultures and health benefits. **International Dairy Journal,** v. 17, p. 1262-1277, 2007.

STAJIĆ, S. *et al.* Cholesterol content and atherogenicity of fermented sausages made of pork meat from various breeds. **Procedia Food Science,** v.1, p. 568-575, 2011.

ULBRICHT, T. L. V.; SOUTHGATE, D. A. T. Coronary heart disease: seven dietary factors. **Lancet**, v. 338, n. 8773, p. 985-992, 1991.

WANG, L.-M. *et al.* Production of conjugated linoleic acid by Propionibacterium Freudenreichii. **Food Chemistry,** 103, 313-318, 2007.

WOLFF, R. L.; PRECHT, D. Reassessment of the contribution of bovine milk fats to the *trans*-18:1 isomeric acid consumption by European populations. Additional data for rumenic (*cis*-9, *trans*-11 18:2) acid. **Lipids,** v. 37, n. 12, p. 1149-1150, 2002.

XU, S. *et al.* Conjugated linoleic acid content and organoleptic attributes of fermented milk products produced with probiotic bacteria. **Journal of Agricultural and Food Chemistry,** v. 53, p. 9064-9072, 2005.

YADAV, H. *et al*. Production of free fatty acids and conjugated linoleic acid in probiotic dahi containing Lactobacillus acidophilus and Lactobacillus casei during fermentation and storage. **International Dairy Journal,** v.17, p. 1006-1010, 2007.

YANG, B. *et al.* Myosin-cross-reactive antigens from four different lactic acid bacteria are fatty acid hydratases. **Biotechnology Letters,** v. 35, n. 1, p. 75-81, 2013.

YANG, B. *et al.* Review of the roles of conjugated linoleic acid in health and disease. **Journal of Functional Foods**, v.15, p. 314-325, 2015.

ZEGARSKA, Z. *et al*. Conjugated linoleic acid (CLA) and *trans* C18:1 and C18:2 isomers in fat of some commercial dairy products. **Polish Journal of Natural Sciences,** v. 23, n. 1, p. 248-256, 2008.

5 CHAPTER II

Title: CLA prevents neuronal loss through inhibiting neuroinflammation mediated by LPS-activated microglia

A foot manuscript to be published at Nature Communications

Qualis: A1
Impact Factor: 11.880

CLA prevents neuronal loss through inhibiting neuroinflammation mediated by LPS-activated micróglia

Carla P. Vieira[a], Estefania P. C. Azevedo[b], Valter V. de Andrade-Neto[c], Bárbara G. Rosa[d], Luzia Sampaio[e], Raquel M. Campos[e], Liana Monteiro[f], Matheus Almeida[g], Lawrence Albuquerque[g], Isabel F. La R. de Freitas[h], Elias B. da S. Junior[h], Livia P. Pinheiro[i], Renata Frauches[j], Priscila F. Silva[l], Debora Foguel[l], Luiz A. Alves[f], Débora D. R. Lima[h], Celio G. F. de Lima[h], Luciana Romão[d], Flavia R. S. Lima[d], Rafael M. Meyer[i], Paula M. de Luca[m], Paulo R. Z. Antas[g], Carlos A. Conte-Junior[a,*]

[a] Food Science Program, Instituto de Química, Universidade Federal do Rio de Janeiro, 21941-909, Rio de Janeiro, Brazil.

[b] Rockefeller University

[c]Trypanosomatid Biochemistry Laboratory, Instituto Oswaldo Cruz, 21040-900, Rio de Janeiro, Brazil.

[d] Glial Cell Biology Laboratory, Instituto de Ciências Biomédicas, Universidade Federal do Rio de Janeiro, 21941-590, Rio de Janeiro, Brazil.

[e] Neurochemistry Laboratory, Instituto de Ciências Biomédicas, Universidade Federal do Rio de Janeiro, 21941-590, Rio de Janeiro, Brazil.

[f] Cellular Communication Laboratory, Instituto Oswaldo Cruz, 21040-900, Rio de Janeiro, Brazil.

[g] Clinical Immunology Laboratory, Instituto Oswaldo Cruz, 21040-900, Rio de Janeiro, Brazil.

[h] Immunomodulation Laboratory, Instituto de Biofísica Carlos Chagas Filho, Universidade Federal do Rio de Janeiro, 21941-590, Rio de Janeiro, Brazil.

[i] Structural Biology Laboratory, Instituto Oswaldo Cruz, 21040-900, Rio de Janeiro, Brazil.

[j] Exercise Science Laboratory, Instituto Biomédico, Universidade Federal Fluminense, 24210-130, Rio de Janeiro, Brazil

[l] Laboratory of Protein Aggregation and Amyloidosis, Instituto de Bioquímica Médica, Universidade Federal do Rio de Janeiro, 21941-590, Rio de Janeiro, Brazil.

[m] Immunoparasitology Laboratory, Instituto Oswaldo Cruz, 21040-900, Rio de Janeiro, Brazil.

*Corresponding author

Professor Carlos Adam Conte-Junior, D.V.M., M.Sc., Ph.D.

Avenida Athos da Silveira Ramos, n. 149, Bloco A, 5° andar, sala 534A e 545, CEP: 21941-909, Rio de Janeiro, RJ, Brazil

Phone: +55-21-3938-7825; E-mail address: conte@iq.ufrj.br (C.A. Conte-Junior).

Abstract

The inhibition of microglia-mediated neuroinflammation has been regarded as a prospective strategy for treating neurodegenerative disorders. Recently, microglial NF-κB was proposed as a pivotal therapeutic target in neurodegenerative disorders, such as Parkinson´s disease. Conjugated linoleic acid (CLA) have been widely described as potent anti-inflammatory modulator of immune response of peripheral macrophages, mainly via inhibition of NF-κB pathway. If CLA also presents similar effect in microglia, it could be a prospective agent for treating these disorders. However, CLA effects on microglia-mediated neuroinflammation are still unknown. Here we investigated *in vitro* anti-neuroinflammatory and neuroprotective effects of CLA on LPS-activated microglia. We demonstrated that CLA downregulates the LPS-induced microglia activation, reducing secretion of NO and IL-1β and increasing IL-4 and IL-10 levels by mice primary microglia and N9 cell line. Although linoleic acid (LA) has exhibited similar effects, only CLA significantly inhibited the LPS-induced arginase activity suppression, which is a M2 phenotype marker in microglia (neuroprotective). This microglial polarization was due to, at least in part, isomer-specific inhibition of LPS-induced NF-κB pathway and iNOS expression, as consequence of enhanced expression of IκBα inhibitor. Co-treatment with CLA and LPS completely prevented cortical neuronal death induced by the endotoxin, again being this neuroprotection also isomer-specific. PPARγ seemed plays a relevant role for CLA action on microglia, and a lesser extent for LA. These data strongly suggest that CLA may be a novel and promising neuroprotective agent via inhibiting microglia-mediated neuroinflammation, in what seems to have isomer-specific particularities.

Keywords: conjugated linoleic acid, bioactive compound, microglial polarization, M2 phenotype, arginase activity, neuroprotection

5.1 INTRODUCTION

Microglia, resident macrophages of the brain, play a pivotal role in innate immunity in the Central Nervous System (CNS) (SHI; HOLTZMAN, 2018). It is well documented that microglia-mediated neuroinflammation is critically involved in the initiation and progression of neurological disorders, such as Parkinson´s and Alzheimer´s disease (GILHUS; DEUSCHL, 2019). When exposed to inflammatory stimuli, such as lipopolysaccharide (LPS), a cascade of intracellular signaling culminates with the activation and translocation of nuclear transcription factor κB (NF-κB) in microglia. NF-κB is translocated from the cytoplasm into the nucleus and plays a crucial role by triggering the transcription of proinflammatory genes (CHE *et al.*, 2018). Consequently, activated microglia produces proinflammatory and cytotoxic factors, such as tumor necrosis factor-α (TNF-α), interleukin-1β (IL-1β) and nitric oxide (NO) (HICKMAN *et al.*, 2018). Accumulation of these factors is deleterious directly to neurons and consequently enhance the activation of microglia, orchestrating a vicious cycle of

inflammation (HENEKA *et al*., 2014). Consistently, activation of microglial NF-κB is related to neuronal loss in Parkinson´s disease (GHOSH *et al.*, 2007) and Alzheimer´s disease patients (CHEN *et al.,* 2012).

Therefore, the inhibition of chronic inflammatory microglial activation may provide therapeutic benefits for neuroinflammatory disorders (LECOURS *et al*., 2018). Furthermore, anti-inflammatory compounds are becoming one of the most promising candidates for Parkinson´s disease (ELKOUZI *et al.*., 2019) and Alzheimer´s disease therapies (SIVANDZADE *et al*., 2019). In addition, recently NF-κB was proposed as a pivotal therapeutic target in those neurodegenerative disorders (SIVANDZADE *et al.*, 2019), including multiple sclerosis (SRINIVASAN; LAHIRI, 2015).

Conjugated linoleic acid (CLA) is a mixture of geometric and positional isomers of C18:2, *cis*-9*, cis*-12 (linoleic acid). The two isomers with most relevant biological activity described are cis-9, trans-11 CLA and trans-10, cis-12 CLA, which are commonly found in beef and dairy product from ruminants (GORISSEN *et al*., 2015), although lower at the recommended levels to achieve health benefits (VIEIRA *et al*., 2017). Anti-inflammatory therapeutics effects of CLA have been extensively described in literature for chronic metabolic disorders in peripheral organs system in animal models and some human research clinical trials with CLA supplementation (FUKE; NORNBERG, 2017; HARTIGH, 2019). Studies have revealed CLA as potent anti-inflammatory immunemodulator of peripheral macrophages (CHINETTI-GBAGUIDI *et al.,* 2015). Consistently, supplementation of CLA in diet induces an increase in IL-10 level, while reducing TNF-α expressed by macrophages in animal models of inflammatory diseases (McCARTHY *et al*., 2013). *In vitro*, CLA increases IL-10 and reduces IL-1β secreted both by mouse (YU *et al*., 2002) and human macrophages (GAETANO *et al*., 2015). And these effects of CLA are thought to be mainly via inhibition of NF-κB proinflammatory signaling pathway in animals (VILLACORTA *et al.*, 2018; BASSAGANYA-RIERA *et al.*, 2004) and human *in vivo* models (PENEDO *et al*., 2013).

In addition, CLA drives macrophage polarization from M1 (proinflammatory) into a M2 phenotype (anti-inflammatory) in *in vivo* animal models (CHINETTI-GBAGUIDI *et al*., 2015) and *in vitro* human models (GAETANO *et al.*, 2015). This would allow the targeting microglia polarization by CLA as a therapeutic avenue in neurodegenerative diseases (JIN *et al.*, 2019). Evidences that embryonic microglia derive from primitive macrophages (FERRERO *et al.*, 2018) suggests that microglia may exhibit a similar response to macrophage when exposed to CLA. Notably, CLA freely pass through the blood-brain barrier, it being actively incorporated and metabolized in the brain (BINYAMIN *et al*., 2019; FA *et*

al., 2005). Therefore, CLA may be a prospective agent for treating of Parkinson´s disease and other neurodegenerative disorders. However, CLA effects on microglia-mediated neuroinflammation are still unknown.

Thus, it is notable that broad activation of microglial NF-κB and subsequent chronic inflammatory process have been suggested to be the predominant mechanisms for degeneration of neurons *in vivo* and *in vitro* (LIU *et al.*, 2016; FRAKES *et al.*, 2014). In this context, our major original hypothesis was that CLA has anti-inflammatory effect on microglial activation inhibiting the NF-κB pathway. The additional hypothesis was that CLA primes microglia from M1 (proinflammatory) to drive into a M2 (anti-inflammatory) phenotype, and that this microglial polarization is neuroprotective. Therefore, the present study aimed to explore whether the CLA-isomers could inhibit neuroinflammation mediated by LPS-activated microglia and thus prevent neuronal loss.

5.2 METHODS

5.2.1 Animals

Neonatal (P0-P3) Swiss mice, both males and females, were chosen for experiments of primary neuronal and microglial cell cultures, which were all carried out in line with the Guide for the Care and Use of Laboratory Animals of the Brazilian National Council of Animal Experimentation (http://www.cobea.org.br/) and the Federal Law 11.794 (October 8, 2008). Experimental approval was obtained from the Ethics Committee for Animal Experimentation (CEUA) of the Federal University of Rio de Janeiro, protocol #DAHEICB027.

5.2.2 Microglial cell line culture

The N9 microglial cell line was kindly provided by Dr. Behnam Badie of University of Wisconsin (USA), and was maintained in Dulbecco´s Modified Eagle Medium (DMEM) supplemented with F12 medium and 10% fetal bovine serum (FBS), at 37 °C and in a controlled atmosphere containing 5% CO_2. N9 microglial cell line was used until 10 passages after thawed vial.

The BV-2 microglial cell line was kindly provided by Dr. Alba Minelli of University of Perugia (Italy) and was maintained in Roswell Park Memorial Institute Medium (RPMI)

supplemented with 10% FBS, at 37 °C and in a controlled atmosphere containing 5% CO_2. The changes of medium were performed every 2-3 days, and the passages of cells were performed after release of cells from bottles with the aid of cell scrapers.

All cell lines were routinely submitted to mycoplasma test using MycoAlert™ Mycoplasma Detection Kit (Lonza Group Ltd. Basel Switzerland).

5.2.3 Primary microglial culture

Primary microglial cell cultures were obtained as described previously (LIMA *et al.*, 2001). Briefly, cortices from neonatal Swiss mice were dissociated, and the resulting cells were plated on poly-L-lysine-coated 75-cm^2 flasks. The cells were maintained in DMEM supplemented with F12 medium (1 : 1 ratio), 10% FBS and 1% penicillin/streptomycin for 2 weeks at 37 °C in a humidified chamber with 5% CO_2. The medium was changed after 1 week and before cell harvesting. After 14 days, microglia were harvested using an orbital shaker. The microglial cultures were assessed by immunocytochemistry using anti-Iba1 (ionizing calcium-binding adaptor molecule 1) antibody, and cells presented more than 99% purity. The cells were counted and plated in 6-, 24- or 96-well plates depending on the assay.

5.2.4 Primary cortical neuron culture

Neuron primary cultures were prepared from the cerebral cortex of E18 mice as previously described (LIMA *et al.*, 2007). Briefly, single cell suspensions were obtained by gently dissociating cells from the cerebral cortex in DMEM/F12 supplemented with the same compounds as in the microglia medium. The resulting neurons were counted and maintained in 96-well plates (previously coated with poly-L-lysine) in Neurobasal medium supplemented with B27 (Gibco) for 24 h and kept at 37 °C in a humidified chamber with 5% CO_2 until use. The neuronal primary cultures were assessed by immunocytochemistry using an anti-III-tubulin antibody and cells presented purity higher than 95%.

5.2.5 Cell viability Assays

Microglial cultures were stimulated or not with LPS (1 µg/mL) and treated with or without CLA (20 to 400 µM) for 48 h. An equimolar blend of CLA (*cis*-9*, trans*-11 and *trans*-10*, cis*-12 CLA) with 99% of purity (Larodan, Solna, Sweden) was employed for all assays.

For the reduction assays (MTT and resazurin), microglia (primary and cell-lines) were seeded in 96-well plates at a density of $5x10^4$ cells per well. For primary cortical neurons ($5x10^4$ cells per well; 96-well plates), the treatments were similar to those employed to microglia, however the concentration of CLA used ranged from 30 µM to 100 µM and the incubation time was of 24h. Linoleic acid, C18:2, *cis*-9*, cis*-12 LA with 98% of purity (Sigma Aldrich, CA, USA) was used as lipid control and cells incubated with 0.04% of Triton X-100 at 37 °C for 5 min were used as death control. For the (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromidefor) MTT reduction assay (Promega), after 48 h of incubation, 5 mg/mL MTT was added and the cells incubated for 2 h at 37 °C in a humidified chamber with 5% CO_2. Purple formazan was solubilized using Dimethylsulphoxide (DMSO) and absorbance measured at 570 nm (AZEVEDO *et al*., 2013). For the resazurin reduction assay, AlamarBlue® was added to the cell cultures to a final concentration of 10% v/v, and the plates were then incubated at 37°C for additional 4 h. The absorbance was measured at excitation/emission of 560/590 nm (REBELLO *et al*., 2019). For both assays, medium alone was considered as 100% viability.

For the Live/Dead assay (Invitrogen, Carlsbad, CA, USA), N9 microglial cell-line were seeded on coverslips in 24-well plates at a density of $1.5x10^4$ cells/well, and the assay was performed according to the manufacturer's instructions; nine images were acquired in each condition, with 2–372 cells/image. The percentage of live cells was calculated relative to the number of control (medium alone-treated) cells.

Apoptosis was measured by the percentage of caspase 3 positive cells by flow cytometry. N9 microglial cell line (10^6 cells per well in 6-well plates) were incubated with LPS (1 µg/mL) and/or CLA at 100 µM for 48h. After incubation, the cells were fixed with 4% paraformaldehyde in phosphate-buffered saline (PBS) for 20 min, and permeabilized with 0.3% Triton X-100 for 5 min. Cells were then blocked with 5% bovine serum albumin (BSA) solution for 30 min and incubated for 3 h with primary antimouse antibody against the active (cleaved) form of caspase 3 (Millipore), 1:400 in 1% BSA solution. Antibody-antigen reaction was revealed by a secondary antibody conjugated to Alexa Fluor 546, 1:1000 (Invitrogen). Acquisition was performed using a fluorescence-activated cell sorting (FACS) Calibur flow cytometer (Becton-Dickinson, USA) and data analysis was performed using WinMDI 2.8 software. A total of 100,000 events were acquired from three independent experiments for each tested condition (ACQUARONE *et al.*, 2015).

5.2.6 Nitric Oxide (NO) production assay from microglia

Microglial cells (primary and cell-lines) cultured in 96-well plates ($5x10^4$ cells seeded per well) were activated or not by LPS (1 µg/mL) and treated with or without CLA (60 to 200 µM) for 48 h. Aliquots of the culture medium were then harvested for assays. For quantification of NO (detected as nitrite) by colorimetric Griess method, 50 µL of cell culture medium and 50 µL of Griess reagent (0.1% N-1-naphthylethylenediamine in 5% phosphoric acid and 1% sulfanilamide in the proportion 1:1) were reacted in a 96-well plate at room temperature for 20 min, and the absorbance was then measured at 540 nm using a microplate reader. Nitrite concentrations were determined by comparison with a standard curve prepared with sodium nitrite (AZEVEDO *et al*., 2013; GENESTRA *et al*., 2003).

For quantification of NO (detected as nitrite) by high-performance liquid chromatography (HPLC) (Tahboub, 2008), 100 µL of culture medium were incubated with 10 µL of freshly prepared 2,3-diaminonaphthalene - DAN (316 µM in 0.62 M HCl) at room temperature for ten minutes followed by addition of 30 µL ultrapure water and 5 µL of 2.8 M NaOH. Then 30 µL of the resulting mixture was injected into the HPLC. The HPLC system (Shimadzu, Kyoto, Japan) consisted of a LC-20AT pump, SPD-M20A diode-array-detector, CTO-20A oven, and SIL-20AC autosampler, all of them connected to a CBM-20A controller. The chromatographic separation was achieved using a Kromasil® C18 column (150 x 4.6 mm i.d., 5 µm) and a mobile phase (flow rate: 1 mL min^{-1}) composed of 15 mM potassium phosphate buffer (pH= 7.5) in 50% of methanol (A) ultrapure water (B) and methanol (C) at following elution condition: 0–3 min 100% A, 3.1–5 min 100% B, 5.1–8 min 100% C, 8.1–10 min 100% B and 10.1–15 min 100% A. The column temperature was maintained at 30 ± 1 ºC. Detection was performed at 369 nm. The nitrite was identified by retention time and by spiking the culture medium with the nitrite standard and quantified by interpolation of peak area in external standard curves (0.5 – 50 µM, $r^2 = 0.996$) using LC Solution software.

5.2.7 Quantification of cytokines secreted by microglia

Microglial cultures (primary and cell-lines) in a 24-well plate ($5x10^5$ cells per well) were stimulated with LPS (1 µg/mL) and treated with or without CLA (60 and 100 µM) for 24h. After, the culture supernatant was collected and used to measure the concentration of TNFα, IL-1β, IL-4 and IL-10 by sandwich enzyme-linked immunosorbent assay (ELISA) kits

(Peprotech, Rock Hill, NJ, USA) according to the manufacturer´s instructions, and the absorbance was then measured at 520 nm using a microplate reader.

5.2.8 Arginase Activity of microglial cultures

Microglia (primary and cell-lines) stimulated or not with 1µg/mL of LPS and treated with or without CLA (60 or 100 µM) for 48h were lysed to determine the arginase activity. After lyse, the arginase activity was determined by spectrophotometric method in accordance with Ferreira *et al.* (2014). The microglia lysates were obtained from $5x10^5$ cells treated with 100 µL of 0.1% Triton X-100 for 30 min, followed by the addition of 100 µL of a buffer containing 25 mM Tris-HCl (pH 7.4) and 10 µL of 10 mM $MnSO_4$. The enzyme was then activated by heating for 10 min at 56°C, and arginine hydrolysis was carried out by incubating 100 µL of the activated lysate with 100 µL of 0.1 M arginine (pH 9.7) at 37°C for 1 h. To stop the reaction, 800 µL of H_2SO_4-H_3PO_4-H_2O (1:3:7 [vol/vol/vol]) and 40 µL of 10% α-isonitrosopropiophenone in 100% methanol were added and the mixture heated to 100°C for 30 min. The activity was determined through urea quantification catalyzed by enzyme from L-arginine hydrolysis measured at 540 nm. Incubation with 20 ng/mL of IL-4 was used as positive control.

5.2.9 Western Blotting Analysis

Expression of total and phosphorylated NF-κB, inducible nitric oxide synthase (iNOS) and IκBα (Inhibitory Subunit of NF-κBα) was determined by Western Blotting Analysis. N9 microglial cell line (10^6) were seeded in 6-well plates and treated with LPS (1µg/mL) and/or CLA (60 or 100 µM) for 30 min. The protein integral fractions were extracted with RIPA buffer and then centrifuged for 10 min at 10,000 rpm at 4°C. The protein content of the soluble fraction was quantified using the Coomassie Plus Protein Assay Reagent Kit, and 15 µg of protein was denatured and separated on 15% sodium dodecyl sulfate-polyacrylamide gel electrophoresis gels and then transferred to poly(vinylidene difluoride) membranes (PVDF). Then, PVDF membranes were blocked for 1h in blocking buffer and incubated overnight at 4 °C with primary antibodies (anti-NF-κB, anti-phospoNF-κB, anti-IκBα and anti-Cyclophylline – 1:500 and iNOS – 1:250). After, the membranes were washed with PBS containing 0.1% Tween-20 and incubated for 45 min with anti-rabbit IgG-Dylight700. Imunoreactive protein bands were developed by using enhanced chemiluminescence (ECL)

kits. The membranes were then washed in PBS and immunoreactive protein bands were quantified through densitometric analysis by densitometry using the imageJ software . The expression levels were analyzed relative to control (vehicle alone; 100%). As intern control it was used cyclophylin (AZEVEDO *et al.*, 2013; Chen *et al.*, 2004).

5.2.10 Statistical Analysis

All statistical analysis was performed with GraphPad Prism (v7.00). Results are expressed as mean ± SD. Grubbs's test ($p < 0.05$) was used for outlier analysis. Data were checked for normality using the Shapiro-Wilk normality test. When normality was ensured, statistical analysis between multiple groups was assessed using either one- or two-way ANOVA followed by Tukey´s post hoc test for multiple comparison with the use of Geisser-Greenhouse test for correction of desequal variability of differences. For multiple groups with non-normal data, Kruskal-Wallis test followed by Dunn´s post hoc test was employed. Statistical analysis between two groups was assessed accordingly with two-tailed parametric or nonparametric tests (unpaired t-test with Welch's correction or Mann–Whitney test, respectively). See figure legends for more details. Although testing has ensured data normality (NO secretion and arginase activity), Spearman's correlation was employed between the variables due to better fit of data than Pearson´s correlation test. Spearman´s rank correlation coefficient and significance are reported in figures. Values of $P \leq 0.05$ were considered to be statistically significant (shown in Figures as $*P \leq 0.05$, $**P \leq 0.01$ and $***P \leq 0.001$).

5.3 RESULTS AND DISCUSSION

5.4 FATTY ACIDS-MEDIATED SUPPRESSION OF ACTIVATION OF BOTH N9 AND BV2 MICROGLIAL CELL LINES

5.4.1 Viability Assays

The effects of various concentrations of the CLA and LA (ranging from 20 to 400 μM) on the viability of N9 and BV2 cell lines were determined by MTT and resazurin reduction assays, respectively, and illustrated in Figure 7. The cell viability of the positive control (cells treated with vehicle alone) was used as reference for comparison with other treatments. The

fatty acids were not cytotoxic to both cell lines at concentrations up to 100 μM. However, at 200 μM and 400 μM CLA was cytotoxic to N9 cell line (Figure 7 A1-A23), while LA was cytotoxic to BV2 cell line at 200 μM in one approach, and 400 μM by other assesment (Figure 7 B1-B2). However, interestingly the cytotoxic effect at 200 μM was observed only for cells treated with fatty acid alone (A22 and B1) or with fatty acid added before LPS (A23 and B2). Thus, LPS seems to reduce the cytotoxicity of fatty acids. This can be attributed to fact that LPS down-regulates the expression of PPARγ (peroxisome proliferator-activated receptor-gamma) in rat primary microglia cultures (WARDEN *et al*., 2016).

The nuclear receptor PPARγ is anti-inflammatory in a cell-based system and in animal models of endotoxemia (ZHOU *et al*., 2008). On the other hand, CLA have been extensively described as strong binder of PPARγ in murine macrophages both *in vitro* and *in vivo* models (BASSAGANYA-RIERA *et al*., 2012; EWASCHUK *et al*., 2006). LA also is reported as natural binder of PPARγ, although it are relatively poor activator of PPARγ when compared to both its oxidative derivatives in microglia (BERNARDO; MINGHETTI, 2006) and to CLA in macrophages (MARTIN, 2010; NAGY *et al*., 1998). Therefore, the downregulation of nuclear receptor of LA and those supposedly of CLA in microglia may reduce the fatty acids effects in cells previously treated with LPS. Consistent with these findings, no cytotoxic effect was reported for BV2 cells treated simultaneously with LPS and CLA at 100 μM (NALLATHAMBY *et al*., 2016) and up to 200 μM for 24 h (Lowry, 2014). CLA was reported not significantly change the *in vitro* viability of murine macrophage cell-lines treated simultaneously with LPS and CLA up to 200 μM for 18 h (CHENG *et al*., 2004). Despite of this, at 200 μM morphological changes, such as accumulation of cytotoxic granules in cytoplasm, were observed in cells herein, even if previously treated with LPS (Figure 7). Hence, in all subsequent assays, concentrations from 200 μM of fatty acids were omitted.

The difference in cytotoxicity at 200 and 400 μM according to the cell line and fatty acid used may be explained by the fact that the cytotoxicity varies according to the type of fatty acid, and even between isomers of given a fatty acid, and also according to the sensitivity of each cell type (BRINKMANN *et al*., 2013). Cytotoxicity is generated mainly by lipid peroxidation induced by fatty acid in the threshold concentration, leading to oxidative stress. Lipoxins, resolvins and protectins resulting from peroxidation have progressive inhibitory actions (POLAVARAPU *et al*., 2014).

In order to corroborate the results above obtained for reduction assays, other assays with more sensitivity were also employed; N9 cell line treated with fatty acids and LPS was assayed by live / dead and flow cytometry, as showed in Figure 8. The results from reduction

assays were supported here, because concentration up to 100 µM of CLA did not show cytotoxicity under any condition tested. In addition, the images ratified that there was no cell morphological change up to this concentration (Figure 8 A1-12 and B1-B6).

5.4.2 LPS-induced NO and cytokine profiles

The effects of treatments with fatty acids on NO secretion by N9 and BV2 cell lines are reported in Figure 9. No fatty acid changed the NO levels when added alone to cell cultures, suggesting that the fatty acids do not alter the NO baseline levels in both cell lines. In contrast, as expected, the addition of LPS significantly stimulated both N9 and BV2 to secrete NO (AZEVEDO *et al*., 2013). These results are consistent with the LPS upregulating the inducible nitric oxide synthase (iNOS) isoform, leading the microglia to produce NO at enhanced rates in animal *in vitro* and *in vivo* models (POSSEL *et al*., 2000; ZHANG *et al*., 2012). However, when the fatty acids were present, they significantly reduced NO secretion by the LPS-stimulated N9 cell-line, no matter LPS scheme (Figure 9A and B). LA caused a more dramatic reduction (up to 75%), while CLA reduced up to 26.72% of NO when compared to the positive control in N9 cell line. As a whole, NO reductions were more expressive when fatty acids were added before the inflammatory stimulus (LPS), which indicates that they can be even more promising for preventive effects (Figure 9).

Regard to BV2 cell line, the effects of both fatty acids on NO secretion by LPS-stimulated microglia were less dramatic when compared to N9 cells (Figure 9C and D). CLA did not present any significative effect, while LA reduced up to 40% the NO levels released by BV2 cells. Therefore, LA presented pronounced effects when compared to CLA in both microglial cell-lines studied herein. Consistent with these findings, LA has been reported to reduce the NO levels released at 24h by LPS-stimulated BV2 cell line; at 100 µM, it reduced about 50% (NALLATHAMBYET *et al.*, 2016; LOWRY, 2014) and at 250 µM, 75% (LOWRY, 2014). Also, CLA is reported to reduce the NO secretion by macrophages in inflammatory disease´s animal models both *in vitro* (BRUEN *et al.*, 2016) and *ex vivo* (YANG; COOK, 2003).

In order to again corroborate the results obtained above for NO quantification by Griess method with a more sensitive assay, N9 cell line treated with CLA and LPS was assayed by high performance liquid chromatography - HPLC (Figure 10). The results obtained by Griess method were validated by HPLC, with no significant change in NO baseline levels (Figure 10 A), and significant reduction of NO in LPS-induced CLA-treated

cells at 100 uM (Figure 10 B). The Figure 10 C shows the LPS-induced peak of nitrite, being this peak markedly reversed after treatment with CLA (Figure 10 D).

Cytokines were quantified in both N9 (Figure 11) and BV2 (Figure 12) cell lines after treatments with the fatty acids. For proinflammatory cytokines in N9 cell line, CLA at 60 μM reduced the IL-1β secretion, while LA at 100 μM was able of reduced it compared to baseline (Figure 11 A). Similar behavior was observed in BV2 cell-line, where LA reduced IL-1β levels for both concentrations tested, while CLA reduced only at 100 μM in non-stimulated cells (Figure 12 A). Regarding TNF-α, none of the fatty acids changed the baseline level of this cytokine for N9 cell-line (Figure 11 C). On the other hand, both fatty acids at 60 μM increased the TNF-α levels, while at 100 μM, both reduced this cytokine in BV2 cells (Figure 12 C).

Microglial activation occurred through LPS treatment as observed by the increase of TNF-α in BV2 cell-line (Figures 11 D and 12 D). We no have found statistical significance in N9 cells, despite the increase in the level of TNF-α in about 2.90 fold. The lack of statistical significance for LPS-induced TNF-α in N9 cells may be attributed to its marked high levels (up to 7.06 fold) by LPS plus fatty acids at 100 μM (Figure 11 D). LPS-stimulated microglial activation also increased significantly the IL-1β secretion (2.14 fold) by N9 cells (Figure 11 B), the same was not true for the BV2 cell-line (Figure 12 B). Similarly, Ackerman *et al.* (2015) reported that LPS-treated BV-2 cells did not show the robust induction of IL-1β . Moreover, addition of ATP during treatment with LPS in BV2 cell-line has been reported induce cleavage of pro-IL-1β to mature IL-1β (LEE *et al.*, 2016). It is important to highlight that ATP was not used herein and then no additional conjectures could be made.

The LPS-mediated activation of NF-κB pathway is a well-known mechanism that culminates with triggering the transcription of proinflammatory genes (CHE *et al.*, 2018). Thus, regarding LPS-stimulated N9 cells, both fatty acids tested at 60 μM reduced IL-1β secretion to baseline level, when added 1h before LPS stimulus. However, only the CLA showed a reduction effect of this cytokine also when added 3h after the LPS (Figure 11 B). In BV2 cells, as LPS did not stimulate the IL-1β secretion, all treatments with fatty acid and LPS, even those with LPS alone, had IL-1β levels below baseline, excepting CLA at 100 μM (Figure 12 A). In this concentration, the IL-1β level was similar to that of the baseline.

Regarding TNF-α, both fatty acids at 100 μM increased it when previously added to LPS in N9 cell line, however, only CLA leaded to this increase when added later to endotoxin, also in the concentration of 100 μM (Figure 11 D). On the other hand, for BV2 cell line, only LA (at 100 μM) increased TNF-α levels both before and after LPS addition, while

CLA had opposite effect, reducing TNF-α to baseline level when added both before and after LPS (Figure 12 D). The different effects observed for a same fatty acid between cell lines may be explained by the fact that immortalized cell-lines, to be immortalized, are genetically modified with the insertion of viral oncogenes, such as *v-myc* or *v-mil* of the avian retrovirus MH2 in N9 cell-line. Oncogenes from J2 virus, turn on, were inserted to obtain BV2 line. These genetic modifications leads to phenotypic differences (STANSLEY; POST; HENSLEY, 2012).

Additionally, the LA and CLA effects on cytokines profile are controversial in scientific literature. LA has been reported as inflammatory pathway activator in microglia that is observed through upregulation in pro-inflammatory cytokine expression (IL-1β and TNF-α) (DESALE; CHINNATHAMBI, 2020). Similar effect to enhance of TNF-α by CLA also was reported in murine macrophages cell line (DARMANI *et al.*, 1995). However, there are reports on LA effects (at 30 μM) reducing gene transcription of IL-1β and TNF-α by BV2 cell-line activated by palmitic acid, which is described to mimic LPS-induced inflammatory responses (TU *et al*., 2019). In addition, LA metabolites has been showed to act as inhibitor of LPS-induced inflammation in microglia both *in vitro* (IKEGUCHI *et al*., 2018) and *in vivo* animal models (MIYAMOTO *et al*., 2015). Regarding CLA, it reduced LPS-stimulated TNF-α production in murine macrophage cell line; on the other hand, mice submitted to intraperitoneal LPS-injection and CLA-supplemented diet during 6 weeks, did not show significant difference compared to control group (corn oil-supplemented diet) when peritoneal resident macrophages were assayed *ex vivo* for TNF-α production (KANG *et al*., 2007). However, a meta-analysis on controlled human research clinical trials showed that there was no significant heterogeneity for the impact of CLA supplementation on TNF-α, so that CLA increases the blood TNF-α level (HAGHIGHATDOOST; GH, 2018). Interestyling, the increase of TNF-α by CLA was described to be mediated by a PPARγ-dependent pathway in porcine peripheral blood polymorphonuclear cells (PBMCs) *in vitro* (KANG; LEE; YANG, 2007).

The differences of effects of fatty acids on cytokines profile among different studies may be attributed to, at least in part, concentration of fatty acids, because recent findings suggest that LA is involved in both pro- and anti-inflammatory signaling pathways, and being the predominant effect influenced by concentration range employed (FRITSCHE, 2008). In adittion, treatment with CLA exhibited anti-inflammatory effect on LPS-induced PBMCs, while it showed proinflammatory effect in non-stimulated cells. This highlights that CLA may elicit different actions depending on the environmental conditions, thus requiring further

mechanistic investigations (VILAMIDOU; HONTECILLAS; BASSAGANYA-RIERA, 2015).

Regard to anti-inflammatory cytokines, both fatty acids at 60 µM increased IL-4 levels, while only CLA also increased it in concentration of 100 µM for N9 cell line (Figure 11 E). For BV2 cell line, CLA at 60 µM increased IL-4 levels, while LA at 100 µM had opposite effect (Figure 12 E). For IL-10, none fatty acid changed its levels in baseline in N9 cells (Figure 11 G). When the cells were LPS-induced, CLA at 100 µM increased IL-4 both when added before as after LPS, while LA increased it only when added after LPS in N9 cells (Figure 11 F). For BV2 cells, LA at 60 µM increased IL-4 levels only when added before LPS, while both fatty acids at 100 µM increased this cytokine for same condition (Figure 12 F). For IL-10, both fatty acids at 100 µM increased it; however, CLA had this effect when added before LPS, while LA did it when added after LPS in N9 cells (Figure 11 H).

Reports of effects of both fatty acids on anti-inflammatory cytokines are scarce, even for macrophage. Because promising therapeutic strategies should inhibiting the canonical proinflammatory pathway, but maintaining the non-canonical anti-inflammatory pathway of NF-κB, in order to promote the profile of regeneration and neuroprotection in the microglia, such study is also important (SANCHEZ-GUAJARDO *et al*., 2013). Mice fed with CLA-supplemented diet had increased IL-10 levels by bone marrow-derived macrophages in an *in vivo* model of atherosclerosis (MCCARTHY *et al.*, 2013). In addition, CLA was described to increase the expression of IL-10 mediated by the ERK signaling pathway in dendritic cells (LOSCHER *et al*., 2005). IL-4 and IL-10 are M2 phenotype-related cytokines in microglia (GU *et al*., 2018). Therefore, the increase of theses cytokines by both fatty acids suggests that they may be driving microglia from M1 to M2 phenotype.

5.4.3 Arginase activity in N9 and BV2 cell lines

The arginase activity was measured in both cell lines treated with LPS and fatty acids for 48 h, and the results are showed in Figure 9. IL-4 was used as positive control of the method. For N9 cells, CLA alone did not show tendency to change the arginase activity (Figure 9 A), while LA, in both concentrations tested, reduced this activity when compared to non-treated control (Figure 9 B). In addition, CLA alone did not show tendency to change the arginase activity in BV2 cells, similar to that observed in N9 cells (Figure 9 C), while LA at 60 µM seemed increased this activity (Figure 9 D). LPS induced arginase activity suppression in relation to baseline in both studied lines (Figure 9). In addition, both fatty acids when

added before LPS inhibited the LPS-induced suppression, presenting activity values near to the baseline, while when the fatty acids were added after LPS, they potentiated the suppression effect by LPS in N9 cells (Figures 9 A and B). For BV2 cells, LA had similar behavior to that observed in N9 cells, although less pronounced (Figure 9 D). On the other hand, CLA did not show clear tendency on arginase activity in BV2 cells treated with LPS, presenting weak effect on arginase activity in this cell line (Figure 9 C).

However, these differences were not sufficient to reach statistical significance in both cell-lines (Figure 9). Even so, significant inverse correlation between arginase activity and NO levels ($r \geq - 0.717$; $p \leq 0.037$) for cells treated with fatty acids corroborated the their tendency to influence the arginase activity both in N9 and BV2 cells. Arginase is an enzyme that competes with iNOS for the L-arginine substrate, which is used by iNOS for NO production (LISI *et al.*, 2016). Moreover, arginase is an anti-inflammatory M2 activation state maker in microglia (SUBRAMANIAM; FEDEROFF, 2017). Thus, although the differences in the arginase activity were not statistically significant herein, they indicate that both fatty acids tend to skew the microglia to M2 phenotype when added before LPS stimulus. Microglial M2 activation phenotype has been reported to favor neuroprotection (CHERRY; OLSCHOWKA; O´BANION, 2014). Consistent with the tendency observed herein, LA was reported to increase the M2 maker, CD36 expression in THP-1 human monocytic cell-line after 24 h treatment (VALLVE *et al.*, 2002). Similarly, CLA affected the *in-vitro* macrophage polarization by reducing the CD68 expression in M1 cells, and increasing both M2-phenotypes associated CD163 and CD206 in human macrophages (de GAETANO *et al.*, 2015). In addition, CLA *in vivo* supplementation induced the M2 phenotype in macrophages via increased IL-10 production in mice (McCARTHYET *et al.*, 2013a; McCARTHY *et al.*, 2013b).

5.5 CLA-MEDIATED SUPPRESSION OF ACTIVATION OF PRIMARY MICROGLIA

5.5.1 Viability Assays

Primary microglia treated with CLA, LA and LPS was assayed for viability after 48 h of incubation, and results are exhibited in Figure 13. CLA was innocuous for all concentrations tested (60 to 200 μM), both when added alone (Figure 13 A) and simultaneously with LPS (Figure 13 B). On the other hand, LA was cytotoxic in a dose-dependent manner, leading to significant viability loss (from 21.96 to 91.67 %) when

compared to baseline even at the lowest concentration tested (60 μM) (Figure 13 B). Most studies evaluated the LA effect on microglial cell line, however, there are only few reports of the effects of LA on primary microglia; in addition, the few studies did not carry out a viability assay (OH *et al*., 2009; MALDONADO-RUIZ *et al*., 2019), which makes impossible to compare our result with other studies. Furthermore, primary microglia unlike cell lines, did not present reduced cytotoxicity by previous treatment with LPS (Figure 13 B). As LPS downregulates PPARγ (WARDEN *et al*., 2016), the findings indicate that there should be a weak bind of LA to PPARγ in primary microglia, so that reduction of PPARγ was any able to mitigate cytotoxic effects of LA on primary microglia. This result corroborates reports about LA to be a poor agonist of PPARγ in microglia when compared to other polyunsaturated fatty acids (BERNARDO; MINGHETTI, 2006).

5.5.2 Effect of CLA on LPS-induced NO and on the cytokine profile in primary microglia

The levels of secretion of NO and cytokines by murine primary microglia treated with CLA and LPS are presented in the Figures 13 and 14. CLA alone did not change the NO baseline levels secreted by primary microglia for any CLA concentration tested herein (Figure 13 C). This CLA behavior on NO levels mirrors those observed previously for N9 and BV2 cell lines (Figure 9). LPS induced NO secretion, increasing in 2.25 fold when compared to baseline (Figure 13 D). Primary microglia pre-treated with CLA reduced secretion of NO from 49.54 % to 53.64 %, while cells post-treated presented NO reduction ranging from 26.43 % to 43.86 % (Figure 13 D). Therefore, CLA showed a more dramatic effect of inhibition of NO release when added previously to LPS, which corroborates CLA as a preventive more than therapeutic agent, as also observed for cell lines herein (Figure 9 A). However, for both conditions, CLA reduced NO to baseline, except when at 200 μM in post-treated cells. In addition, the effects of CLA on NO reduction were more pronounced in primary microglia than N9 cells (up to 26.72 %), while had no significant effect in BV2 cells (Figures 9A and C). Even with the similarity to primary microglia, the cell lines do contain oncogenes that render them in some ways different from primary microglia, such as increased proliferation and adhesion, and increased variance of morphologies (HORVATH *et al.*, 2008). In addition, BV2 cell line appears to be a trade-off of magnitude of stimulatory response compared to primary microglia (STANSLEY; POST; HENSLEY, 2012), and the upregulation of LPS-

induced genes in the BV2 was far less pronounced than in primary microglia (HENN *et al.*, 2009).

Regarding that the CLA effects in cell lines and its effect on NO secretion in primary microglia were more promising when the cells were pre-treated with CLA, the cytokines quantification and arginase activity assays in primary microglia were performed only with CLA being added before LPS. The cytokines profile for primary microglia treated with CLA and LPS for 24 h are reported in Figure 14. CLA alone reduced in a dose-dependent manner the baseline of IL-1β (up to 92.76 %) for both concentrations tested (Figure 14 A). On the other hand, the TNFα baseline was not changed by CLA (Figure 14 C). In addition, CLA at 100 μM increased 3.08 fold the IL-4 levels compared to baseline (Figure 14 E).

Again as expect, treatment with LPS, turn on, induced increase of secretion of IL-1β and TNFα in 2.31 and 32.28 fold, respectively, compared to baseline (Figure 14 B and D). In this scenario, CLA reduced LPS-induced IL-1β release dose-dependent manner, decreasing its values up to 32.41% (Figure 14 B). On the other hand, CLA had opposite effect on TNFα, increasing it up to 4.14 fold (Figure 14 D). Regard to IL-4, CLA at 60 μM increased it in 3.57 fold when compared to cell treated with LPS (Figure 14 F). The increase of IL-4 levels, which is a characteristic cytokine of M2 microglial activation (SUBRAMANIAM; FEDEROFF, 2017), indicates that CLA may be driving primary microglia from M1 into M2 phenotype, disregard the enhanced TNFα release.

The cytokines behavior in CLA-treated primary culture was similar to those observed in N9 cell line for all cytokines studied both baseline and LPS-induced (Figure 11). On the other hand, the cytokines behavior in primary microglia was similar at a lesser extent to BV2 cells (Figure 12), since unlike primary microglia, CLA increased IL-1β and reduced TNFα in cells treated with LPS, besides reduce baseline TNFα when at 100 μM in BV2 cells (Figure 12). Indeed, the validity of BV2-cells as a sufficient substitute for primary microglia has been debated (STANSLEY; POST; HENSLEY, 2012). Consistently, transcriptome sequencing revealed that LPS-triggered transcriptional responses in microglia BV2-cell lines were poorly representative of primary microglia (DAS *et al.*, 2016). In addition, the expression of PPAR-γ in primary microglia may explain the discrepancy with studies on BV-2 cells, with lack of PPARγ expression in BV2-cells and, thus, they may not be fully representative of primary microglia (BERNARDO; MINGHETTI, 2006). This is one more evidence that PPARγ pathway should play a key role for CLA action on microglia, and that this receptor is less relevant for LA effect, since CLA did not present significant effect on some relevant

inflammatory markers, as NO and arginase activity in BV2 cells, while LA maintained significant effects, although to a lesser extent.

In line with these findings, CLA is reported to regulate activation of PPARγ by modulation of phosphorylation performed through ERK 1/2 signaling in human *in vitro* macrophages, while LA did not present a significant effect on PPARγ phosphorylation (STACHOWSKA *et al*., 2011). It is important to highlight that PPAR-γ agonists are currently gaining increasing attention as promising disease-modifying drugs in the Parkinson´s disease, selectively targeting the expression of neurotoxic factors in reactive microglia (CARTA; PISANU, 2012).

5.5.3 Arginase activity in primary microglia

The arginase activity was obtained after treatment of primary microglia with CLA and LPS for 48 h and are reported in Figure 15. As both microglial cell-lines presented more promising results for arginase activity in a preventive manner, the its activity was tested in primary microglia adding CLA before LPS. Similar to observed for N9 and BV2 cell-lines, CLA alone did not affect the baseline level of activity of enzyme compared to non-treated control (Figure 15 A). In addition, LPS suppressed the activity of enzyme in primary microglia as observed previously for both microglial lines, however the effect was more dramatic in primary microglia (reduction of 55.16 %), presenting thus statistic significance ($p \leq 0.05$). This is consistent with LPS-induced M1 polarization in microglia with significant reduction of arginase expression (WEN *et al.*, 2017). However, CLA at 100 µM completely inhibited LPS-induced suppression, presenting arginase activity similar to baseline. Such effect, however, was not observed for CLA at 60 µM. Therefore, the tendencies observed for effects of CLA plus LPS on arginase in the N9 cell-line were corroborated by primary microglia, which presented significant inhibition of LPS-induced arginase suppression. Consistently, a more strongest correlation between arginase activity and NO levels (Figure 15 B) was observed for primary microglia ($r = - 0.900$; $p = 0.043$) compared to cell lines.

5.6 EFFECTS OF FATTY ACIDS ON EXPRESSION OF M1 ACTIVATION MARKERS USING N9 CELL LINE AS REPRESENTATIVE OF PRIMARY MICROGLIA

Amongst the microglia cell-lines studied herein, N9 was the one that presented similar behavior to primary microglia regarding the effect on NO secretion, cytokine profiles and

arginase activity. Thus, N9 cells were used for studying of signaling pathway of fatty acids in microglia. Representative immunoblots and analyses of expression of NF-κB phosphorylade, IκBα and iNOS proteins are reported in Figure 16. CLA alone exclusively increased IκBα expression (up to 4.28 fold) in both concentrations tested, while LA did no present effect (Figure 16 A). For NF-κB phosphorylade, the changes observed were no significant (Figure 16 C). NF-κB is expressed and plays an important role in the activation of the M1 microglial phenotype; activation of the NF-κB pathway lead to expression of various innate immune and inflammatory factors (CHE *et al.*, 2018). On the other hand, several inhibitors of NF-κB have been identified, including the isoform IκBα, which block the transcriptional activity of p65 subunit of NF-κB, thus inhibiting the transcription of pro-inflammatory genes (FERREIRO; KOMIVES, 2010). Therefore, in a isomer-specific manner, CLA lead to enhancement of IκBα inhibitor compared to baseline. However, this increase in inhibitor was not sufficient to lead to significant changes in expression of NF-κB phosphorylade in cells without inflammatory challenge (Figure 16 C).

When inflammatory stimulus occurs, phosphorylation of domain of NF-κB-bound IκBα is performed, leading to degradation of inhibitor and consequent translocation of NF-κB to the nucleus, activating the transcription of pro-inflammatory genes (FERREIRO; KOMIVES, 2010). Coherently, the treatment with LPS increased NF-κB phosphorylade in 3.88 fold (Figure 16 D). In addition, CLA increased the IκBα expression (up to 2.90 fold), and this increase of inhibitor was accompanied by the suppression of LPS-induced NF-κB expression (up to 51.80 %) in both CLA concentrations tested (Figure 16 D). In line with these findings, CLA was reported to reduce the activation of NF-κB *in vitro* in dendritic cells (LOSCHER *et al.*, 2005) and macrophages (CHENG *et al.*, 2004).

The effect of inhibition of NF-κB herein was isomer-specific, indicating that the increase of IκBα inhibitor caused by CLA, but not LA, was relevant to suppress the LPS-induced NF-κB activation. This finding also indicates that the anti-inflammatory effects of LA may be exerted through a signaling pathway other than NF-κB, while NF-κB pathway showed to be relevant for the CLA effects observed. It is consistent with the fatty acids behavior in murine *in vitro* macrophages previously reported by Li *et al.* (2005); CLA significantly decreased NF-κB activation, and this effect was attributed, at least partially, to the inhibition of IκBα degradation, whereas LA had no inhibitory effects on its degradation. In addition, the findings herein support CLA as driving the microglial activation state from M1 into M2, in what seems be mediated via inhibiting pro-inflammatory NF-κB pathway.

On the other hand, the reports of effects of LA on NF-κB pathway in microglia are controversial. Tu *et al.* (2019) reported significant increase of IκBα inhibitor by LA (at 30μM) in BV2-cells treated with palmitic acid, which mimic LPS-induced proinflammatory challenge. On the other hand, similar to reported herein, Ikeguchi *et al.* (2018) found that LA metabolites were no able to inhibit NF-κB activation or increase the IκBα expression in BV2 cells. In addition, they found that the anti-inflammatory effects of LA metabolites may be exercised in another pathway, through inhibition of ERK phosphorylation induced by LPS. Different results for same cell line among different laboratories were attributed to genetic drift of the cell line or possibly technical differences in the experimental preparation (STANSLEY; POST; HENSLEY, 2012).

Regard to iNOS, both fatty acids at both concentrations tested increased its baseline expression up to 3.72 fold (Figure 16 E). Interestingly, this increase in iNOS expression was not accompanied by elevation of NO secretion (Figure 9). The treatment with LPS stimulated enhanced expression of iNOS (6.54 fold), which can be attributed to NF-κB activation, which induces iNOS expression (ZHANG *et al*., 2012). This expression was reduced to baseline levels by CLA, leading to inhibition up to 54.05 %. Similar effect of CLA on iNOS in LPS-induced murine *in vitro* macrophages was reported (IMAKIRI; SAMPSON; ALLEN, 2002).

On the other hand, LA showed only a tendency to reduce iNOS expression herein (Figure 16 F). Nevertheless, significant reduction of expression of LPS-induced iNOS by LA has been reported in BV2-cell line (LOWRY, 2014; NALLATHAMBY *et al*., 2016; IKEGUCHI *et al.*, 2018). Again, however, the reduction of iNOS was not accompanied by NO proportional reduction herein, since LA did not inhibit significantly iNOS, however, it reduced nitrite release levels more pronouncedly than CLA (Figure 9). These results may seem contradictory; however, regulation of iNOS activity is a factor need to be considered when interpreting the result that LA treatment reduced nitrite levels without reducing iNOS protein expression. When activated with LPS, murine macrophages with genic deletion for essential enzyme in the biosynthesis of tetrahydrobiopterin (BH4), a required cofactor for iNOS activation, induced iNOS expression in an indistinguishable fashion from wild-type controls, but produced no detectable NO (McNEILL *et al.*, 2015). Therefore, further studies should assess the regulator factors of activation of iNOS induced/inhibited by LA and CLA in microglia to address this research gap.

5.7 CLA PROTECTS CORTICAL NEURON FROM LPS-INDUCED NEUROTOXICITY

Results of viability for primary cortical neurons treated with fatty acids and LPS for 24 h are shown in Figure 17. Fatty acids alone had no cytotoxic effect on primary neurons in concentration ranged tested (30 to 100 μM). The addition of LPS in neurotoxic concentration (100,000 ng / mL) led to neuronal death (46.7% viability reduction). LPS was reported to induce mitochondrial fragmentation in neurons, which lead to neuronal death in mice *in vivo* model (HARLAND *et al*., 2020). Co-treatment with CLA and LPS completely prevented endotoxin-induced neuronal death: CLA increased neuronal viability from 53.31% (neuronal death induced by LPS) to 100.91%. It suggests that CLA has neuroprotective effect on LPS-induced direct neuronal injury. This is in line with the *in vitro* protection of cortical neurons by the CLA against glutamate-mediated excitoxicity previously reported by Hunt *et al.* (2010). In this study, CLA increased the neuronal expression of Bcl-2, which reduced the dissipation of the mitochondrial membrane potential, suggesting a stabilizing function of CLA over the mitochondrial function (HUNT *et al.*, 2010). This effect was not observed for LA, which indicates that neuroprotection from direct neuronal injury by LPS is an specific-isomer property. Indeed, metabolites from LA, but not LA, have been reported a strong neuronal protector from oxidative stress, through inhibiting apoptosis (YAGUCHI; FUJIKAWA; NISHIZAKI, 2010).

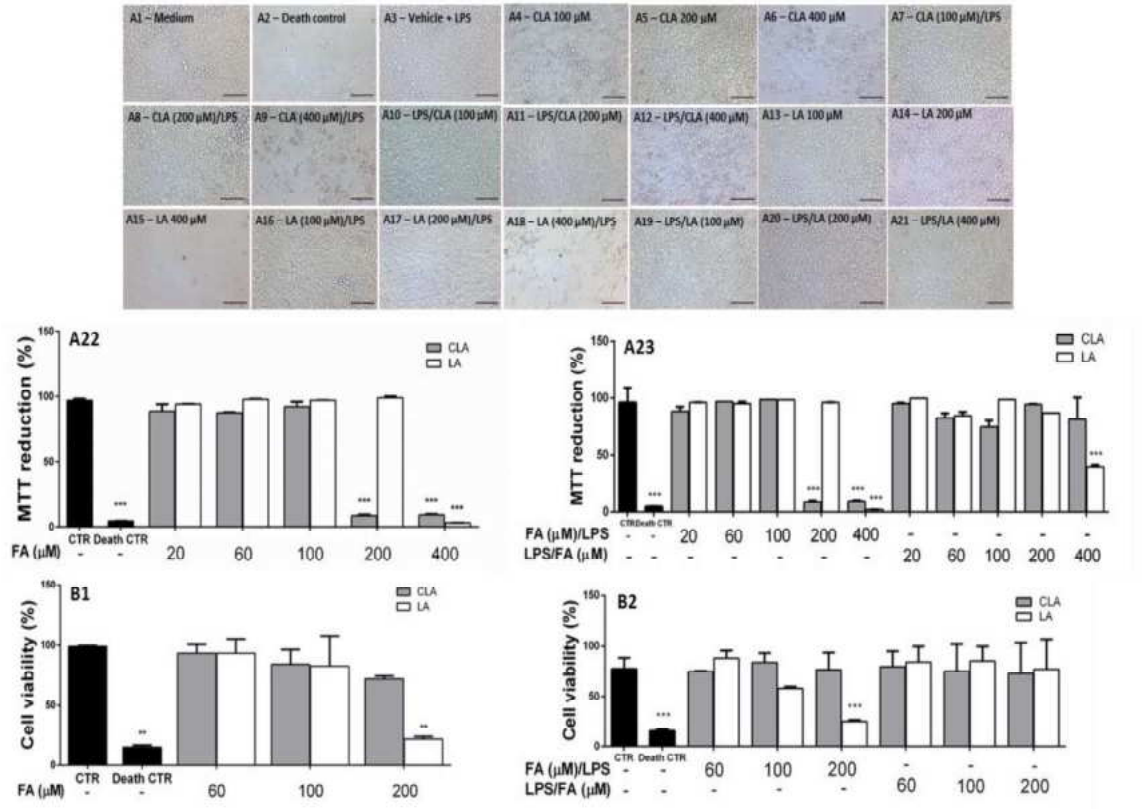
5.8 CONCLUSION

Taken together, the results obtained demonstrated that CLA mitigated the microglial activation by LPS, reducing the secretion of NO and IL-1β pro-inflammatory cytokine and increasing IL-4 and IL-10 anti-inflammatory cytokines by primary microglia culture and N9 cell line. LA showed similar suppressive effects on microglial activation in N9 cells. However, only CLA had significant effect on arginase activity. The treatment with CLA completely prevented the LPS-induced suppression of arginase activity in primary microglia, also the arginase activity strongly correlated with the NO levels both in primary microglia and N9 cells treated with CLA. This suggested that CLA drove microglia from M1 (proinflammatory) to M2 (anti-inflammatory) phenotype. The microglia polarization was resultant, at least in part, of inhibition of NF-κB proinflammatory pathway as well as reduction of iNOS expression through enhanced expression of IκBα inhibitor in LPS-

activated microglia. The inhibitory effect on microglial NF-κB and iNOS was isomer-specific, because LA did not present such effect when microglia was activated. Evidences suggested that PPARγ plays a relevant role for CLA action on microglia, and a lesser extent for LA. Co-treatment with CLA and LPS completely prevented cortical neuronal death induced by the endotoxin, being this neuroprotection also isomer-specific. Thus, CLA has potential as anti-inflammatory and neuroprotective candidates against microglia-mediated neuroinflammatory disorders, in what seems to have isomer-specific particularities.

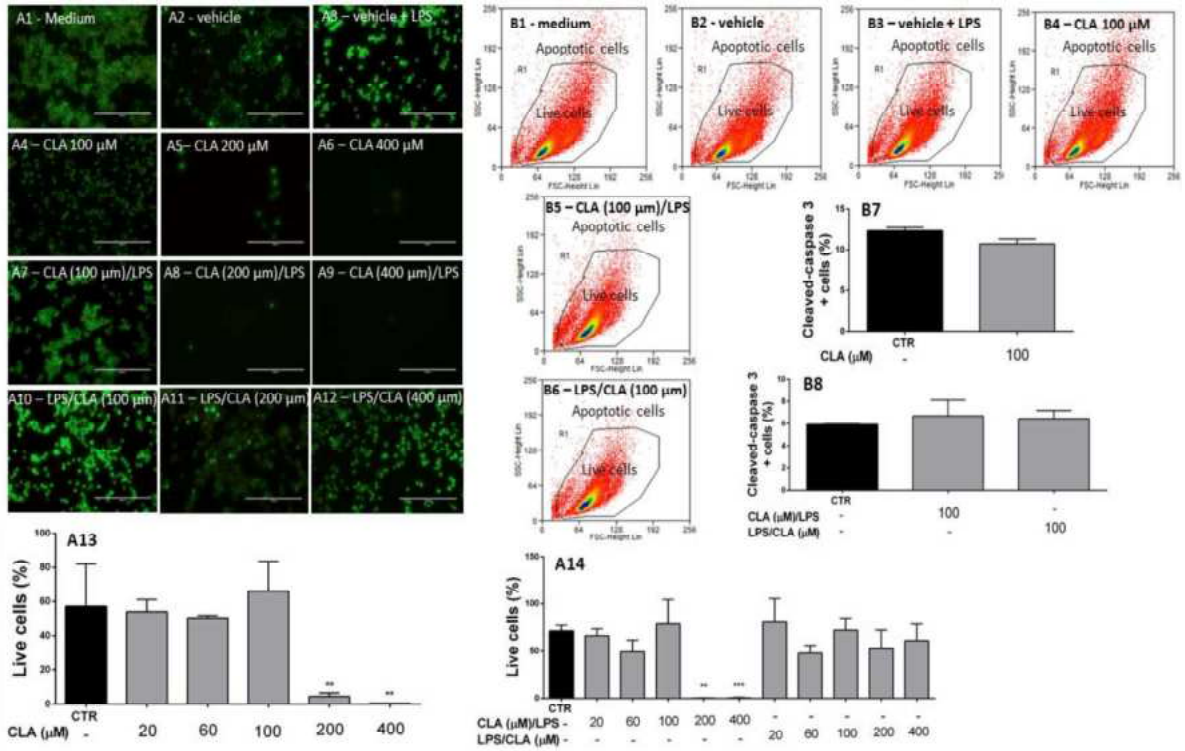
5.9 FIGURES AND TABLES

Figure 7 - (A) Evaluation of cytotoxicity of fatty acids (FA) in N9 cell line.



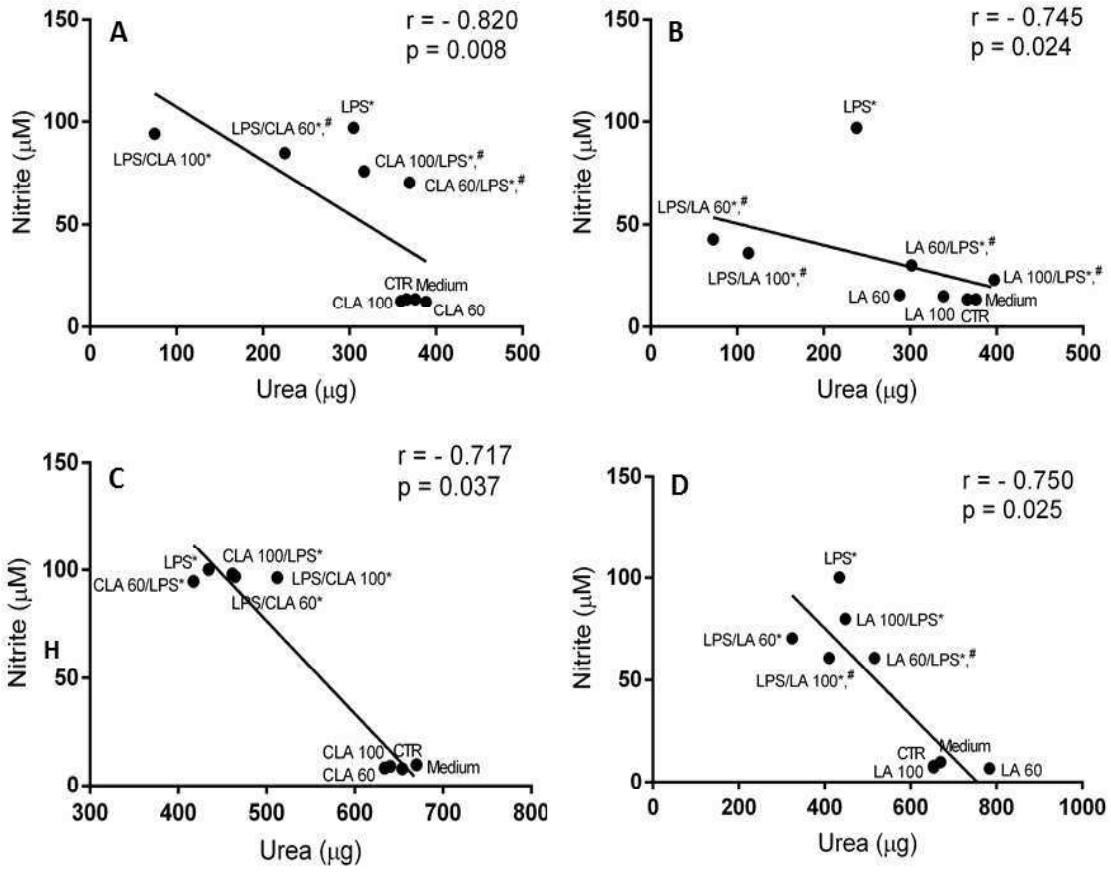
Phase-contrast micrographs showing the general morphology of controls **(A1-3)** and wells treated with conjugated linoleic acid – CLA **(A4-12)** or linoleic acid – LA **(A13-21)**. CLA or LA ranging from 20 to 400 µM were incubated with N9 cells for 48h and the cell death was determined by MTT assay: Cells incubated with vehicle (DMSO) were used as positive control for those treated with FA alone **(A22)**, while cells incubated with vehicle plus LPS were used as positive control for cells treated simultaneously with FA and LPS **(A23)**. **(B)** Viability assay to assess the cytotoxicity of FA in BV2 cell line. Percentage of resazurin reduction by viable cells treated with FA alone **(B1)** or with LPS plus FA **(B2)** for 48h, in concentration ranging from 60 to 200 µM. B1 and B2 show the percentage (%) of live cells compared with medium alone (considered as 100% viability; not shown) and cells incubated with 0.04% of Triton X-100 at 37 °C for 5 min were used as negative control of viability **(A22-23 and B1-2)**. Bars represent the mean ± SD of three independent experiments. **p < 0.01, and ***p < 0.001, compared to the control (CTR). Scale bars are 100 µm. Source: the author.

Figure 8 - (A) Cell viability was assessed in a Live/Dead approach of showed N9 cell line in different concentrations of conjugated linoleic acid (CLA)



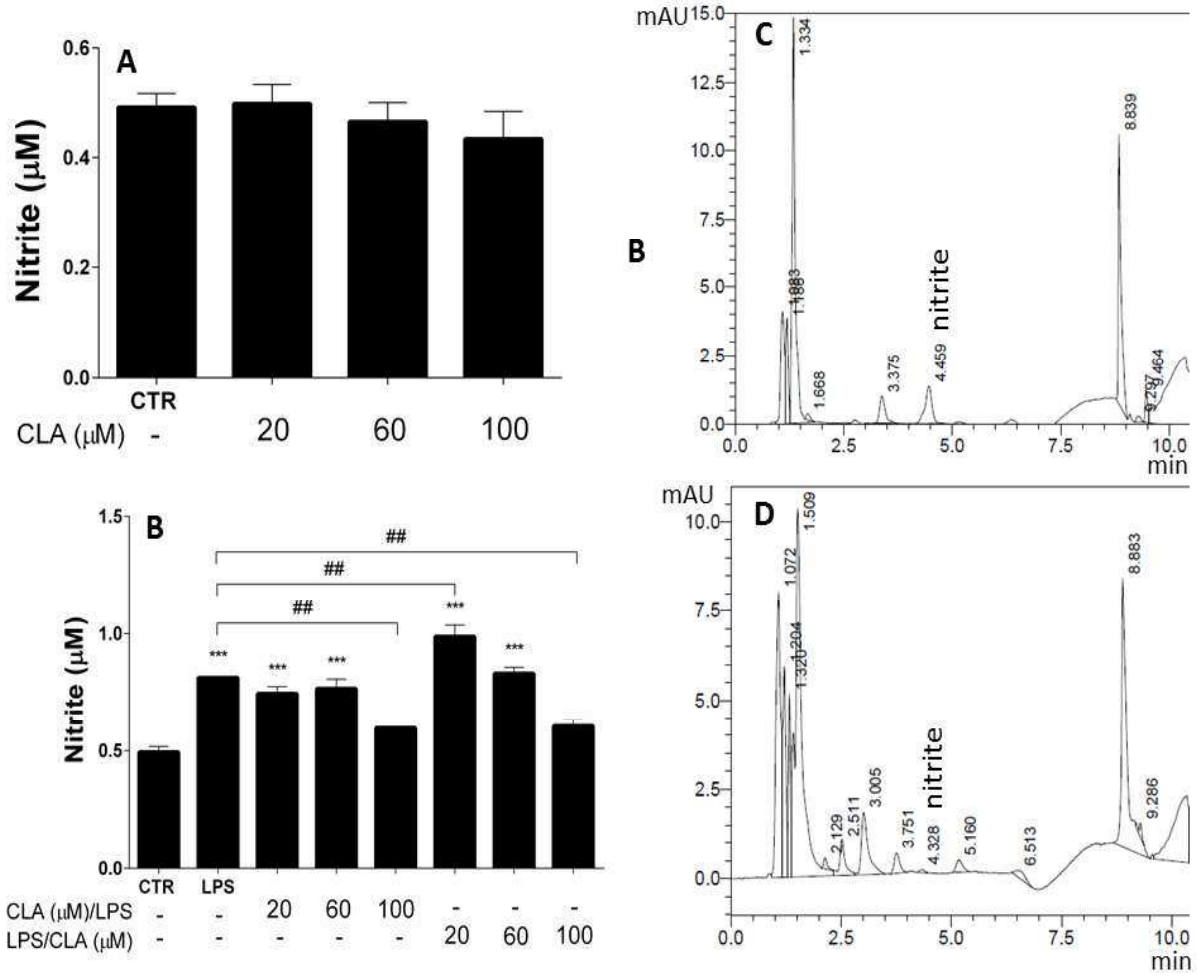
Cell viability was assessed in a Live/Dead approach of showed N9 cell line in different concentrations of conjugated linoleic acid (CLA). N9 cells were incubated with CLA (20 to 400 µM) for 48h. Live cells stained in green and dead ones in red **(A1-14)**. Vehicle at 0.05% **(A13)** and vehicle plus LPS at 1µg/mL **(A14)** were incubated with N9 cells as controls. **A13-14** show percentage (%) of live cells considering medium alone as 100% viability (not shown). Scale bars are 50 µM for all images. **(B)** Apoptosis analysis of N9 cell line after incubation with CLA at 100 µM for 48h. Flow cytometric detection of caspase activity in N9 cells using antibody against the active (cleaved) form of caspase 3 **(B1-8)**. Also as controls, vehicle at 0.05% **(B7)** and vehicle plus LPS at 1µg/mL **(B8)** were directly incubated with N9 cells. Bars represent the mean ± SD of three independent experiments. $^{**}p < 0.01$, and $^{***}p < 0.001$, compared to the control (CTR).
Source: the author.

Figure 9 - Arginase activity levels inversely correlate with nitrite conversion both for N9 cell line **(A-B)** as for BV2 cell line **(C-D)** treated with conjugated linoleic acid (CLA) or linoleic acid (LA) for 48h



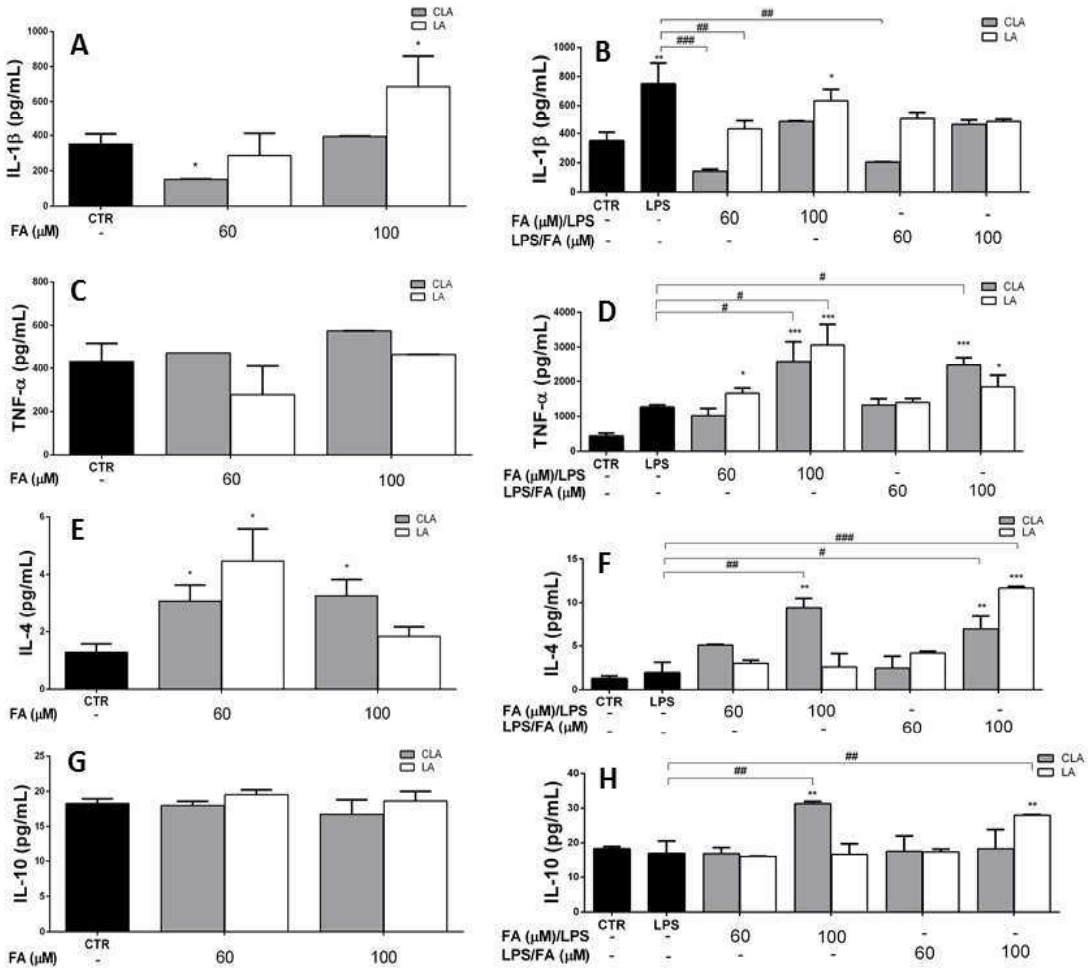
Arginase activity levels inversely correlate with nitrite conversion both for N9 cell line **(A-B)** as for BV2 cell line **(C-D)** treated with conjugated linoleic acid (CLA) or linoleic acid (LA) for 48h. The arginase activity was measured through urea formation catalyzed by enzyme. NO was measured as nitrite using Griess reagents in culture supernatants. As control, vehicle at 0.05% was also incubated with the cells. Additionally, for nitrite levels, cells treated with LPS at 1 μg/mL were used as positive control. The cells were both pre-treated with fatty acid - 1h before LPS - (CLA/LPS or LA/LPS) and pos-treated with fatty acid - 3h after LPS – (LPS/CLA or LPS/LA). Spearman´s correlation coefficient and *p*-values are as indicated. *represents the comparison between control (CTR) and treatments for nitrite levels and $^{\#}$represents the comparison between LPS and treatments for nitrite levels. Bars represent the mean ± SD of three independent experiments. *p and $^{\#}p < 0.05$.
Source: the author.

Figure 10 - Conjugated linoleic acid (CLA) at 100 µM prevent LPS-induced nitric oxide secretion by N9 cell line



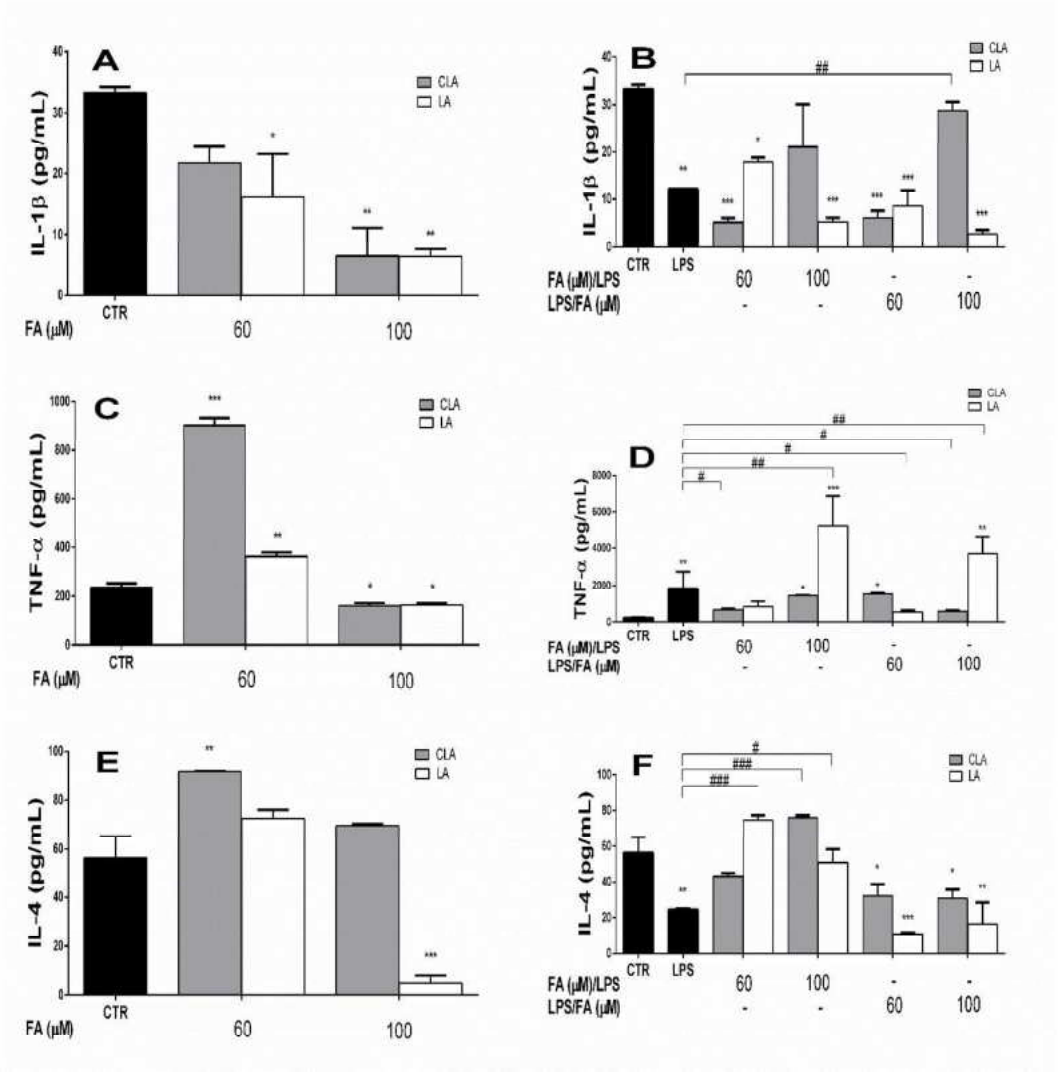
Conjugated linoleic acid (CLA) at 100 µM prevent LPS-induced nitric oxide secretion by N9 cell line, which was treated with CLA alone **(A)** or incubated simultaneously with CLA and LPS **(B)**. Preincubation (1h before LPS) or post incubation (3h after LPS) with CLA was performed for 48h. After, supernatant was measure by High Performance Liquid Chromatography – HPLC (**C and D**). Exposition to LPS (1 µg/mL) activated N9 cells, increasing the nitric oxide secretion **(C)**, while post incubation with CLA at 100 µM prevent this release **(D)**. *represents the comparison between control (CTR) and treatments and $^{\#}$represents the comparison between LPS and treatments. Bars represent the mean ± SD of three independent experiments. ***$p < 0.001$; $^{\#\#}p < 0.01$.
Source: the author.

Figure 11 - Evaluation of pro-inflammatory (IL-1β and TNF-α) and anti-inflammatory (IL-4 and IL-10) cytokine profile respective to N9 cell line treated with conjugated linoleic acid (CLA) or linoleic acid (LA) at 60 or 100 µM after 24h (A-H)



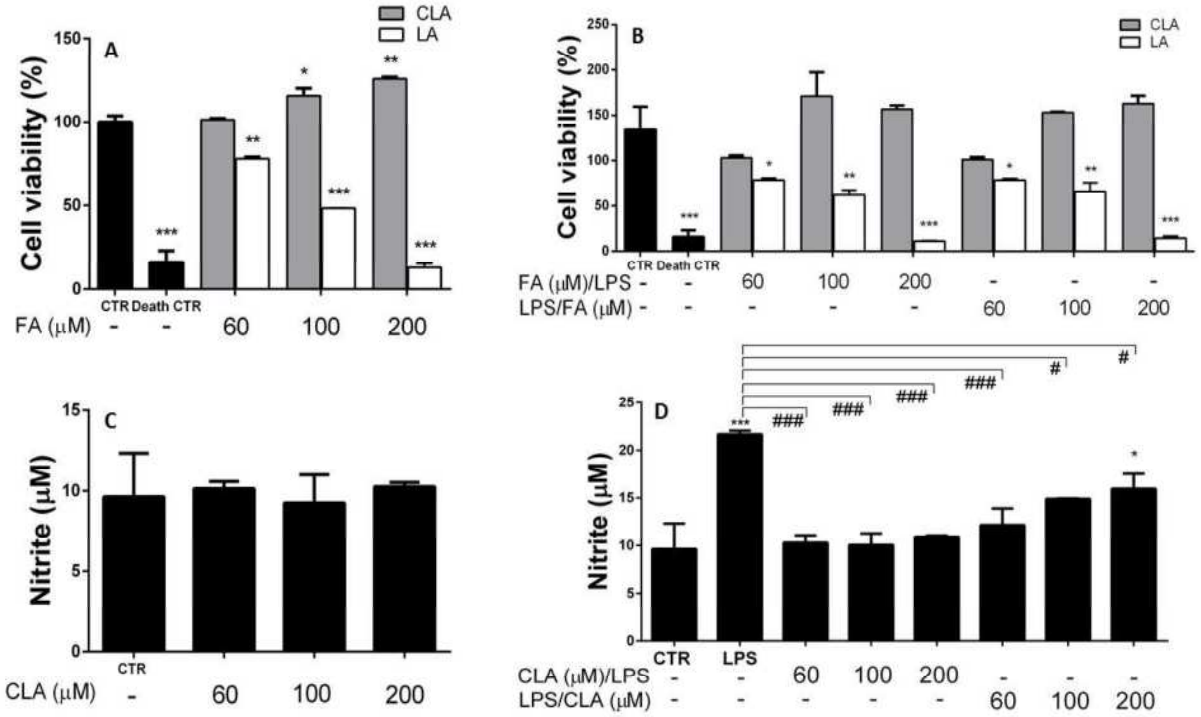
Evaluation of pro-inflammatory (IL-1β and TNF-α) and anti-inflammatory (IL-4 and IL-10) cytokine profile respective to N9 cell line treated with conjugated linoleic acid (CLA) or linoleic acid (LA) at 60 or 100 µM after 24h **(A-H)**. As control, vehicle at 0.05% was also incubated with N9 cells. As positive control, cells were treated with LPS at 1 µg/mL. The cells were treated with FA alone **(A, C, E, and G)** or FA plus LPS **(B, D, F, and H)**. The cells were both pre-treated with FA - 1h before LPS - (FA/LPS) and pos-treated with FA - 3h after LPS – (LPS/FA). *represents the comparison between control (CTR) and treatments and $^{\#}$represents the comparison between LPS and treatments. Bars represent the mean ± SD of three independent experiments. *$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$; $^{\#}p < 0.05$, $^{\#\#}p < 0.01$, and $^{\#\#\#}p < 0.001$.
Source: the author.

Figure 12 - Evaluation of pro-inflammatory (IL-1β and TNF-α) and anti-inflammatory (IL-4 and IL-10) cytokine profile respective to BV2 cell line treated with conjugated linoleic acid (CLA) or linoleic acid (LA) at 60 or 100 µM after 24h (A-H)



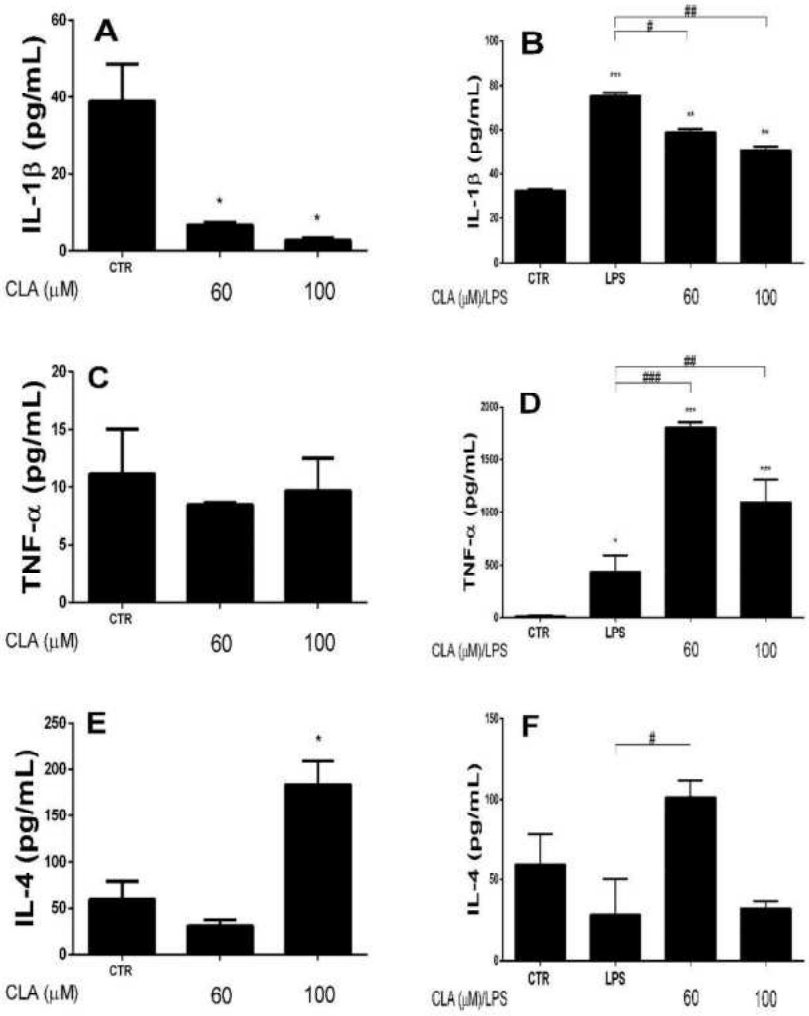
Evaluation of pro-inflammatory (IL-1β and TNF-α) and anti-inflammatory (IL-4 and IL-10) cytokine profile respective to BV2 cell line treated with conjugated linoleic acid (CLA) or linoleic acid (LA) at 60 or 100 µM after 24h **(A-H)**. As control, vehicle at 0.05% was also incubated with BV2 cells. As positive control, cells were treated with LPS at 1 µg/mL. The cells were treated with FA alone **(A, C, E, and G)** or FA plus LPS **(B, D, F, and H)**. The cells were both pre-treated with FA - 1h before LPS - (FA/LPS) and pos-treated with FA - 3h after LPS – (LPS/FA). *represents the comparison between control (CTR) and treatments and $^{\#}$represents the comparison between LPS and treatments. Bars represent the mean ± SD of three independent experiments. *$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$; $^{\#}p < 0.05$, $^{\#\#}p < 0.01$, and $^{\#\#\#}p < 0.001$.
Source: the author.

Figure 13 - Conjugated linoleic acid (CLA) is innocuous and prevents the nitric oxide secretion by primary microglia cultures



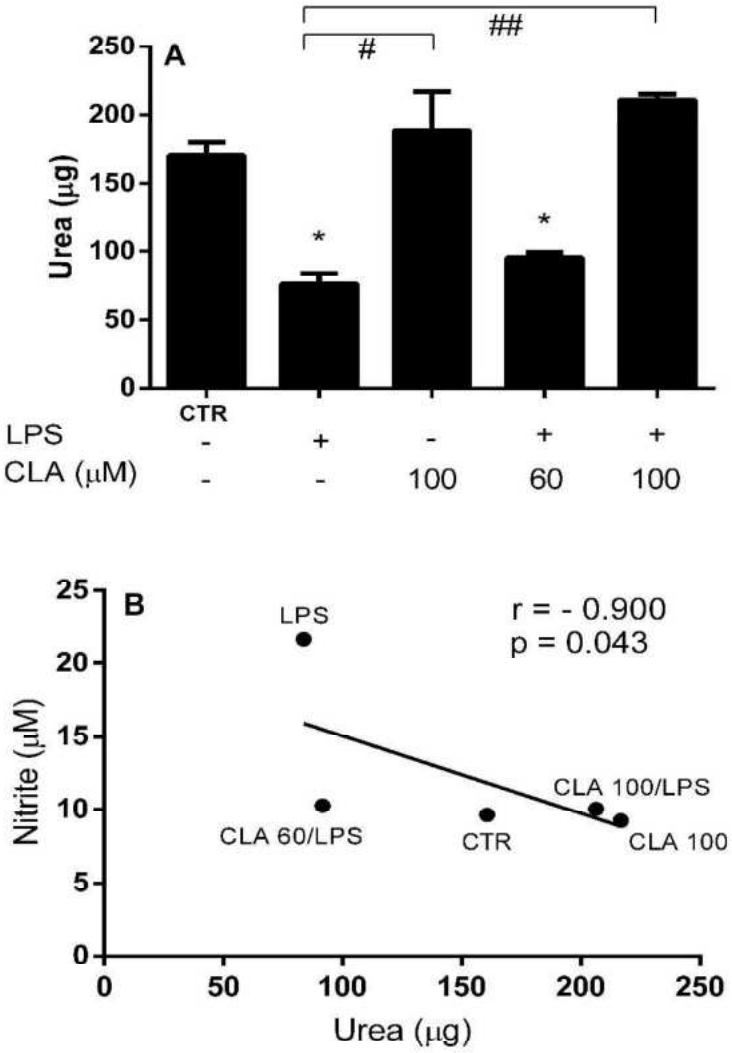
Conjugated linoleic acid (CLA) is innocuous and prevents the nitric oxide secretion by primary microglia cultures. Cell viability to fatty acids (FA) was assessed using resazurin reduction assay **(A-B)**. Primary microglia were treated with FA for 48h. CLA or linoleic acid (LA) were incubated alone **(A)** or simultaneously with LPS **(B)**. Preincubation (FA/LPS) or post incubation (LPS/FA) with FA was performed. Microglia incubated with vehicle was used as control for microglia treated with fatty acid alone **(A)**, while microglia incubated with vehicle plus LPS was used as control for cells treated simultaneously with FA and LPS **(B)**. **A-D** show the percentage (%) of live cells compared with medium alone (considered as 100% viability; not shown) and microglia incubated with 0.04% of Triton X-100 at 37 °C for 5 min was used as negative control of viability. Nitric oxide secretion was measured by nitrite quantification in supernatant of primary microglia cultures using Griess reagents after 48h of incubation with CLA **(C-D)**. As positive control, cells were treated with LPS at 1 μg/mL. *represents the comparison between control (CTR) and treatments and $^{\#}$represents the comparison between LPS and treatments. Bars represent the mean ± SD of three independent experiments. *$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$; $^{\#}p < 0.05$, and $^{\#\#\#}p < 0.001$.
Source: the author.

Figure 14 - Evaluation of pro-inflammatory (IL-1β and TNF-α) and anti-inflammatory (IL-4 and IL-10) cytokine profile respective to mice primary microglia pre-treated with conjugated linoleic acid (CLA) at 60 or 100 µM after 24h (A-F)



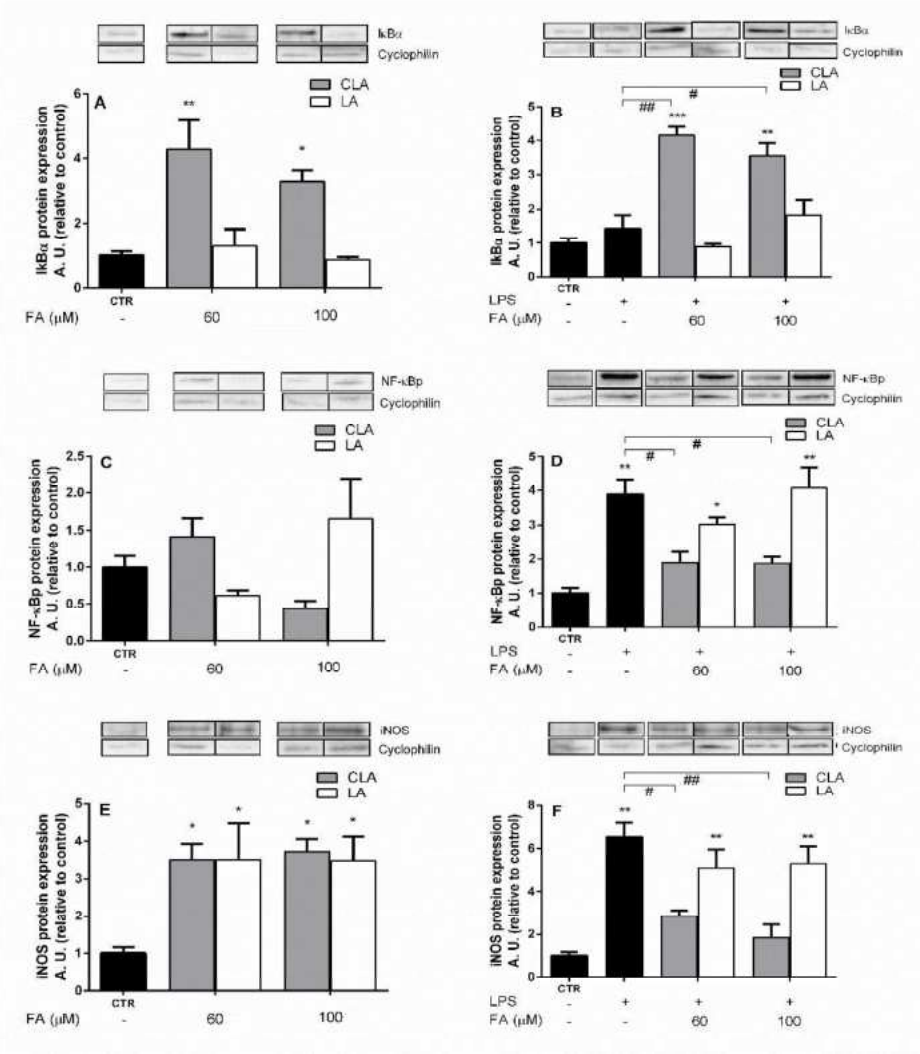
Evaluation of pro-inflammatory (IL-1β and TNF-α) and anti-inflammatory (IL-4 and IL-10) cytokine profile respective to mice primary microglia pre-treated with conjugated linoleic acid (CLA) at 60 or 100 µM after 24h **(A-F)**. As control, vehicle at 0.05% was also incubated with BV2 cells. As positive control, cells were treated with LPS at 1 µg/mL. The cells were treated with CLA alone **(A, C, and E)** or CLA plus LPS **(B, D, and F)**. The cells were both pre-treated with FA - 1h before LPS - (FA/LPS) and pos-treated with FA - 3h after LPS – (LPS/FA). *represents the comparison between control (CTR) and treatments and $^{\#}$represents the comparison between LPS and treatments. Bars represent the mean ± SD of three independent experiments. $^{*}p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$; $^{\#}p < 0.05$, $^{\#\#}p < 0.01$, and $^{\#\#\#}p < 0.001$.
Source: the author.

Figure 15 - Arginase activity was measured through urea formation catalyzed by enzyme in mice primary microglia pre-treated with CLA



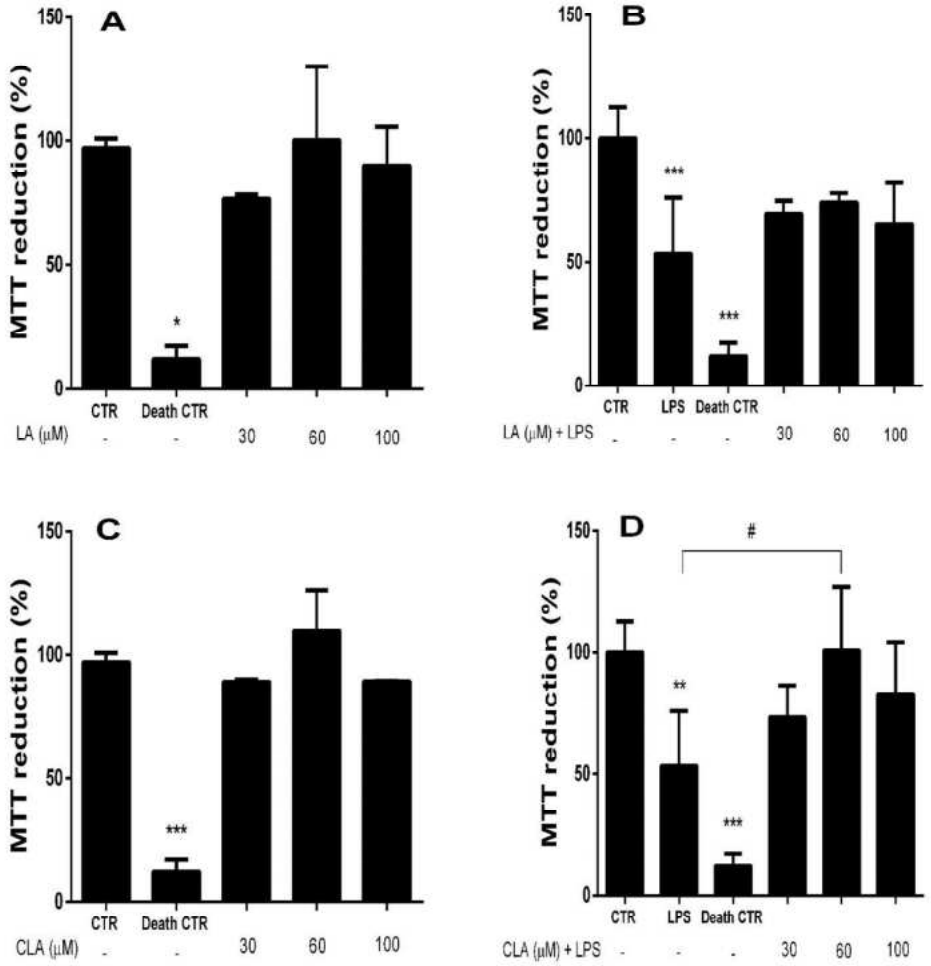
Arginase activity was measured through urea formation catalyzed by enzyme in mice primary microglia pre-treated with CLA. As control, vehicle at 0.05% was also incubated with the cells (CTR) **(A)** Arginase activity levels inversely correlate with nitrite conversion in primary microglia treated with conjugated linoleic acid (CLA) for 48h. CLA was added 1h before LPS (CLA/LPS). NO was measured as nitrite using Griess reagents in culture supernatants. Additionally, for nitrite levels, cells treated with LPS at 1 µg/mL were used as positive control **(B).** Spearman´s correlation coefficient and *p*-values are as indicated for $p < 0.05$ significance level. Bars represent the mean ± SE of three independent experiments. *$p < 0.05$; $^{\#}p < 0.05$, and $^{\#\#}p < 0.01$.
Source: the author.

Figure 16 - Effects of CLA and LA on expression of inflammatory markers in LPS-stimulated N9 microglial cell line for 30 min



Effects of CLA and LA on expression of inflammatory markers in LPS-stimulated N9 microglial cell line for 30 min. Representative immunoblots and protein analyses in cell lysates from N9 cells: IκBα-cyclophilin ratio (A-B); NF-κBp-cyclophilin ratio (C-D); iNOS-cyclophilin ratio (E-F); IκBα, inhibitory subunit of NF-κBα; NF-κBp, phospho-nuclear factor kappa B; iNOS, inducible nitric oxide synthase; FA, fatty acid; CLA, conjugated linoleic acid; LA, linoleic acid. *represents the comparison between control (CTR) and treatments and $^{\#}$represents the comparison between LPS and treatments. Bars represent the mean ± SE of three independent experiments. $^{*}p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$; $^{\#}p < 0.05$, $^{\#\#}p < 0.01$, and $^{\#\#\#}p < 0.001$. The results in A-F are shown in relation to those obtained for the control group, which was normalized to 1.
Source: the author.

Figure 17 - Conjugated linoleic acid (CLA) is innocuous and prevents the LPS-induced neuron death



Conjugated linoleic acid (CLA) is innocuous and prevents the LPS-induced neuron death. Cell viability to fatty acids (FA) was assessed using MTT reduction assay **(A-D)**. Primary cortical neuron were treated with FA for 24h. CLA or linoleic acid (LA) were incubated alone **(A and C)** or simultaneously with LPS **(B and D)**. Co-treatment with FA and LPS was performed. Neuron incubated with vehicle was used as control for neuron treated with fatty acid alone (**A and C**), while neuron incubated with vehicle plus LPS was used as control for cells treated simultaneously with FA and LPS (**B and D**). **A-D** show the percentage (%) of live cells compared with medium alone (considered as 100% viability; not shown) and neuron incubated with 0.04% of Triton X-100 at 37 °C for 5 min was used as negative control of viability. Bars represent the mean ± SD of three independent experiments. *$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$.
Source: the author.

Table 7 - Summary of results without LPS.

	N9 Cells		BV2 Cells		Primary Microglia		Primary Neuron	
Parameters	**CLA**	**LA**	**CLA**	**LA**	**CLA**	**LA**	**CLA**	**LA**
Cell viability	200-400 µM - up to ↓ 90 % **LIVE/DEAD** 200-400 µM up to ↓ 100 %	400 µM - ↓ 95 %	NS	200 µM - ↓ 75 %	100-200 µM - up to ↑ 26 %	60-200 µM up to ↓ 87 %	-	
Neural viability	-	-	-	-	-	-	NS	NS
NO release	NS	NS	NS	NS	NS	-	-	
IL- 4	60-100 µM – up to ↑ 116 %	60 µM – ↑ 216 %	60 µM - ↑ 39 %	100 µM – ↓ 92 %	100 µM – ↑ 180 %	-	-	
IL-10	NS	NS	-	-	-	-	-	
IL-1 β	60 µM - ↓ 63 %	100 µM – ↑ 107 %	100 µM –↓ 62 %	60 - 100 µM – ↓ 76 %	NS	-		
TNF- α	NS	NS	60 µM – ↑ 270 % 100 µM – ↓ 24 %	60 µM – ↑ 58 % 100 µM – ↓ 28 %	NS	-	-	
Arginase activity	NS	NS	NS	NS	100 µM – up to ↑ 181 %	-	-	
Activation markers IκBα							-	
	60-100 µM up to ↑ 400 %	NS	-	-	-	-	-	

NF-κBα	NS	NS	-	-	-	-	-
iNOS	60 -100 µM – ↑ 320 %	60 -100 µM – ↑ 350 %	-	-	-	-	-

Source: the author.

Table 8 - Summary of results with LPS.

	N9 Cells		BV2 Cells		Primary Microglia		Primary Neuron	
Parameters	**CLA**	**LA**	**CLA**	**LA**	**CLA**	**LA**	**CLA**	**LA**
Viability	**CLA/LPS:** 200-400 µM – up to ↓ 91 %	**LA/LPS:** 400 µM – ↓ 99 %	**CLA/LPS:** NS	**LA/LPS:** 200 µM – ↓ 77 %	**CLA/LPS:** NS	**LA/LPS:** 60 – 200 µM up to ↓ 95 %	-	
	CLA/LPS LIVE/DEAD: 200-400 µM up to ↓ 100 %						-	
	LPS/CLA: NS	**LPS/LA:** 400 µM – ↓ 64 %	**LPS/CLA:** NS	**LPS/LA:** NS	**LPS/CLA:** NS	**LPS/LA:** 100 – 200 µM up to ↓ 94 %	-	
	LPS/CLA LIVE/DEAD: NS						-	
Neural viability	-	-	-	-	-	-	**CLA/LPS: 60** µM - ↑ 67 %	**LA/LPS:** NS
NO release	**CLA/LPS:** 100 µM – ↓ 21 %	**LA/LPS:** 60 – 100 µM - up to ↓ 69 %	**CLA/LPS:** NS	**LA/LPS**: 60 – 100 µM – up to ↓ 39 %	**CLA/LPS:** 60-200 µM – up to ↓ 46 %	-		

	LPS/CLA: 20 µM – ↑ 34 % 100 µM - ↓ 21 %	**LPS/LA:** 60-100 µM – up to ↓ 60 %	**LPS/CLA:** NS	**LPS/LA:** 60 – 100 µM – up to ↓ 35 %	**LPS/CLA:** 60-200 µM – up to ↓ 36 %	-	
IL- 4	**CLA/LPS:** 100 µM – ↑ 267 %	**LA/LPS:** NS	**CLA/LPS:** 100 µM – ↑ 212 %	**LA/LPS:** 60 – 100 µM – up to ↑ 212 %	**CLA/LPS:** 60 µM -↑ 110 %		
	LPS/CLA: 100 µM – ↑ 267 %	**LPS/LA:** 100 µM- ↑ 300 %	**LPS/CLA:** NS	**LPS/LA:** NS	-	-	
IL-10	**CLA/LPS:** 100 µM – ↑ 52 %	**LA/LPS:** NS	-	-	-	-	
	LPS/CLA: NS	**LPS/LA:** 100 µM - ↑ 40 %	-	-	-	-	
IL-1 β	**CLA/LPS:** 60 µM – ↓ 80 %	**LA/LPS:** 60 µM – ↓ 51 %	**CLA/LPS:** NS	**LA/LPS:** NS	**CLA/LPS:** 60 - 100 µM – up to ↓ 33 %		
	LPS/CLA: 60 µM – up to ↓ 78 %	**LPS/LA:** NS	**LPS/CLA:** 100 µM – ↑ 138 %	**LPS/LA:** NS	-	-	
TNF-ALFA	**CLA/LPS:** 100 µM – ↑ 138 %	**LA/LPS:**100 µM – ↑ 185 %	**CLA/LPS:** 60 µM - ↓ 75 %	**LA/LPS:** 100 µM – ↑ 150 %	**CLA/LPS:** 60 - 100 µM – up to ↑ 200 %	-	
	LPS/CLA: 100 µM – ↑ 108 %	**LPS/LA:** 60 µM – ↓ 75 % 100 µM ↑ 79 %	**LPS/CLA:** 100 µM – ↓ 75 %	**LPS/LA:** 60 µM - ↓ 75 %	-	-	

Arginase activity	NS	NS	NS	NS	**CLA/LPS:** 100 µM – ↑ 175 %	-	
Activation markers IκBα	**CLA/LPS:** 60 - 100 µM – up to ↑ 150%	NS	-	-	-	-	
NF-κBα	**CLA/LPS:** 60 - 100 µM – up to ↓ 54%	NS	-	-	-	-	
iNOS	**CLA/LPS:** 60 – 100 µM – up to ↓ 70 %	**LA/LPS:** 60 – 100 µM – up to ↓ 19 %	-	-	-	-	

Legend: NS: Not significant

Source: the author

5.10 REFERENCES

ACKERMAN, K. *et al.* BV-2 Microglial cells used in a model of neuroinflammation. **FASEB J**., v. 29, n. 1**,** p. 608.2, 2015.

ACQUARONE, M. *et al.* Mitomycin-treated undifferentiated embryonic stem cells as a safe and effective therapeutic strategy in a mouse model of Parkinson's disease. **Front. Cell. Neurosci.**, v. 9, n. 97, p. 1-11, 2015.

AZEVEDO, E. P. *et al.* Activated microglia mediate synapse loss and short-term memory deficits in a mouse model of transthyretin-related oculoleptomeningeal amyloidosis. **Cell Death Dis.**, v. 4, n. 9, p. e789, 2013.

BASSAGANYA-RIERA, J. *et al*. Activation of PPAR γ and δ by conjugated linoleic acid mediates protection from experimental inflammatory bowel disease. **Gastroenterology**., v. 127, n. 3, p. 777-791, 2004.

BASSAGANYA-RIERA, J. *et al.* Probiotic bacteria produce conjugated linoleic acid locally in the gut that targets macrophage PPARgamma to suppress colitis. **PloSOne 7,** p. e31238, 2012.

BERNARDO, A.; MINGHETTI, L. **PPAR-γ agonists as regulators of microglial activation and brain.**

BINYAMIN, O. *et al.* Brain targeting of 9c,11t-Conjugated Linoleic Acid, a natural calpain inhibitor, preserves memory and reduces Aβ and P25 accumulation in 5XFAD mice. **Sci. Rep**., v. 9, n. 18437, p. 1-12, 2019.

BRINKMANN, C. R. *et al.* The cytotoxicity of fatty acid/α-lactalbumin complexes depends on the amount and type of fatty acid. **Eur. J. Lipid. Sci. Tech.**, v. 115, n. 6, p. 591-600, 2013).

BRUEN, R. *et al.* Atheroprotective effects of conjugated linoleic acid. **Br. J. Clin. Pharmacol**., v. 83, n. 1, p. 46-53, 2016.

CHE, Y. *et al.* Taurine protects dopaminergic neurons in a mouse Parkinson's disease model through inhibition of microglial M1 polarization. **Cell Death Dis**., v. 9, n, 435, p. 1-13, 2018.

CHEN, C.-H. *et al.* Increased NF-kB signalling up-regulates BACE1 expression and its therapeutic potential in Alzheimer's disease. **Int. J. Neuropsychopharmacol.,** v. 15, p. 77-90, 2012.

CHENG, W-L. *et al*. Contribution of conjugated linoleic acid to the suppression of inflammatory responses through the regulation of the NF-kB pathway. **J. Agr. Food Chem.**, v. 52, p. 71-7, 2004.

CHERRY, J. D.; OLSCHOWKA, J. A.; O'BANION, M. K. Neuroinflammation and M2 microglia: the good, the bad, and the inflamed. **J Neuroinflammation**., v. 11, n. 98**,** p. 1-15,

2014.

CHINETTI-GBAGUIDI, G. *et al*. Macrophage subsets in atherosclerosis. **Nat. Rev. Cardiol.** v. 12, n. 1, p. 10-17, 2015.

DARMANI, H. *et al*. Macrophage activation by lipopolysaccharide, interferon-γ and interleukin-4: effect of fatty acid metabolism. **Mediators Inflamm.**, v. 4, n. 1, p. 25-30, 1995.

DAS, A. *et al.* Transcriptome sequencing reveals that LPS-triggered transcriptional responses in established microglia BV2 cell lines are poorly representative of primary microglia. **J. Neuroinflammation,** v. 13, n. 182**,** p. 1-18, 2016.

De GAETANO M. *et al.* Conjugated linoleic acid induces an atheroprotective macrophage MPhi2 phenotype and limits foam cell formation. **J. Inflamm.**, v, 12, n. 15, p. 1-14, 2015.

DESALE, S. E.; CHINNATHAMBI, S. Role of dietary fatty acids in microglial polarization in Alzheimer's disease. **J. Neuroinflammation.,** v. 17, n. 93, p. 1-14, 2020.

ELKOUZI, A. *et al.* Emerging therapies in Parkinson disease - repurposed drugs and new approaches. **Nat. Rev. Neurol.**, v, 15, n. 4, p. 204-223, 2019.

EWASCHUK, J. B. *et al.* Bioproduction of conjugated linoleic acid by probiotic bactéria occurs in vitro and in vivo in mice. **J. Nutr.**, v. 136, p. 1483-1487, 2006.

FA, M. *et al*. Incorporation and metabolism of c9,t11 and t10,c12 conjugated linoleic acid (CLA) isomers in rat brain. **Biochim. Biophys. Acta 1736,** p. 61-66, 2005.

FERREIRA, C. *et al.* Resveratrol is active against *Leishmania amazonensis*: *in vitro* effect of its association with amphotericin B. **Antimicrob. Agents Chemother.**, v. 58, n. 10, p. 6197-6208, 2014.

FERREIRO, D. U.; KOMIVES, E. A. Molecular Mechanisms of System Control of NF-κB Signaling by IκBα. **Biochemistry**., v. 49, n. 8, p. 1560-1567, 2010.

FERRERO, G. *et al.* Embryonic Microglia Derive from Primitive Macrophages and Are Replaced by cmyb-Dependent Definitive Microglia in Zebrafish. **Cell Rep**., v. 24, n. 1, p. 130-141, 2018.

FRAKES, A. E. *et al*. Microglia Induce Motor Neuron Death via the Classical NF-κB Pathway in Amyotrophic Lateral Sclerosis. **Neuron.**, v. 81, n. 5, p. 1009-1023, 2014.

FRITSCHE, K. L. Too Much Linoleic Acid Promotes Inflammation-Doesn't It? **Prostaglandins leukot essent fatty acids***.,* v. 79, n. 3-5, p. 173-175, 2008.

FUKE, G.; NORNBERG, J. L. Systematic evaluation on the effectiveness of conjugated linoleic acid in human health. **Crit. Rev. Food Sci.,** v. 57, n. 1, p. 1-7, 2017.

GAETANO, M. D. *et al*. M1- and M2-Type Macrophage Responses Are Predictive of Adverse Outcomes in Human Atherosclerosis. **Front. Immunol.,** p. **7,** n. 275, 2016.

GENESTRA, M. Effect of l-Arginine analogs and a calcium chelator on nitric oxide (NO˙) production by Leishmania sp. **J. Enzyme Inhib. Med. Chem.**, v. 18, n. 5, p. 445-452, 2003.

GILHUS, N. E.; DEUSCHL, G. Neuroinflammation — a common thread in neurological disorders. **Nat. Rev. Neurol.,** v. 15, n. 8, ´p. 429-430, 2019.

GORISSEN, L. *et al*. Bacterial production of conjugated linoleic and linolenic acid in foods: a technological challenge. **Crit. Rev. Food Sci.,** v. 55, p. 1561-1574, 2015.

GOSH, A. *et al.* Selective inhibition of NF-κB activation prevents dopaminergic neuronal loss in a mouse model of Parkinson´s disease. **PNAS,** v. 104, n. 47, p. 18754-18759, 2007.

GU, M. *et al.* Endogenous omega (n)-3 fatty acids in fat-1 mice attenuated depression-like behavior, imbalance between microglial m1 and m2 phenotypes, and dysfunction of neurotrophins induced by lipopolysaccharide administration. **Nutrients,** v. 10, n. 10, p. 1-17, 2018.

HAGHIGHATDOOST, F.; GH., B. F. N. M. Effect of conjugated linoleic acid on blood inflammatory markers: a systematic review and meta-analysis on randomized controlled trials. **Eur. J. Clin. Nutr***., v.* 72, n. 8, p. 1071-1082, 2018.

HARLAND, M. *et al.* Neuronal Mitochondria Modulation of LPS-Induced *Neurobiol.* **Dis***.,* v. 40, n. 8, p. 1756-1765, 2020.

HARTIGH, L. J. D. Conjugated Linoleic Acid Effects on Cancer, Obesity, and Atherosclerosis: A Review of Pre-Clinical and Human Trials with Current Perspectives. **Nutrients**., v. 11, n. 2, p. 1-29, 2019.

HENEKA, M. T.; KUMMER, M. P.; LATZ, E. Innate immune activation in neurodegenerative disease. **Nat. Rev. Immnunol**., v. 14**,** p. 463-477, 2014.

HENN A *et al.* The suitability of BV2 cells as alternative model system for primary microglia cultures or for animal experiments examining brain inflammation. **ALTEX,** v. 26, p. 83-94, 2009.

HICKMAN, S. *et al.* Microglia in neurodegeneration. **Nat. Neurosci**., v. 21, n. 10, p. 1359-1369, 2018.

HORVATH, R. *et al.* Differential migration, LPS-induced cytokine, chemokine, and NO expression in immortalized BV-2 and HAPI cell lines and primary microglial cultures. **J. Neurochem***., v.* 107, p. 557-569, 2008.

HUNT, W. T. *et al.* Protection of cortical neurons from excitotoxicity by conjugated linoleic acid. **J. Neurochem.**, v. 115, p. 123-130, 2010.

IKEGUCHI, S. *et al.* Inhibitory effect of the gut microbial linoleic acid metabolites, 10-oxo-trans-11-octadecenoic acid and 10-hydroxy-cis-12- octadecenoic acid, on BV-2 microglial cell activation. **J. Pharmacol. Sci.***,* v. 138, n. 1, p. 9-15, 2018.

INFLAMMATION. **Curr. Pharm. Des.**, v, 12, p. 93-109, 2006.

IWAKIRI, Y.; SAMPSON, D. A.; ALLEN, K. G. D. suppression of cyclooxygenase-2 and inducible nitric oxide synthase expression by conjugated linoleic acid in murine macrophages. Prostaglandins Leukot. Essent. **Fatty Acids**., v. 67, n. 6, p. 435-443, 2002.

JIN, X. *et al.* Natural products as a potential modulator of microglial polarization in neurodegenerative diseases. **Pharmacol. Res.**, v, 145, p. 104253, 2019.

KANG, J-H. *et al.* Trans-10, cis-12-conjugated linoleic acid increases phagocytosis of porcine peripheral blood polymorphonuclear cells in vitro. **Br. J. Nutr**., v. 97, n. 1, p. 117-125, 2007.

LECOURS, C. *et al.* Microglial Implication in Parkinson's Disease: Loss of Beneficial Physiological Roles or Gain of Inflammatory Functions? **Front. Cell. Neurosci.**, v. 12, n. 282, p. 1-8, 2018.

LEE, C-M. *et al.* Benzyl isothiocyanate inhibits inflammasome activation in e. coli lps-stimulated bv2 cells. **Int. J. Mol. Med**., v. 38, n. 3**,** p. 912-918, 2016.

LIMA, F. R. *et al*. Cellular prion protein expression in astrocytes modulates neuronal survival and differentiation. **J. Neurochem.,** v. 103, p. 2164 -2176, 2007.

LIMA, F. R. *et al.* Regulation of microglial development: a novel role for thyroid hormone. **J. Neurosci.**, v. 21, p. 2028–2038, 2001.

LISI, L. *et al* Antiretrovirals inhibit arginase in human microglia. **J. Neurochem**., v. 136, n. 2, ′p. 363-372, 2016.

LIU *et al*. Chicoric acid supplementation prevents systemic inflammation-induced memory impairment and amyloidogenesis via inhibition of NF-κB. **FASEB J**., v. 31, n.4, p. 1494-1507, 2016.

LOSCHER, C. E. *et al.* Conjugated Linoleic Acid Suppresses NF-_B Activation and IL-12 Production in Dendritic Cells through ERK-Mediated IL-10 Induction. **J. Immunol. Res**., v. 175, n. 8, p. 4990-4998, 2005.

LOWRY, J. R. **The effects of dietary fatty acids on microglial immune responses.** Thesis for the degree of Master of Science in the college of graduate studies. The University of British Columbia, Okanagan, Canada, 2014.

MALDONADO-RUIZ, R. *et al*. Priming of hypothalamic ghrelin signaling and microglia activation exacerbate feeding in rats' offspring following maternal overnutrition. **Nutrients,** v. 11, n. 6, p**.** 1-17, 2019.

MARTIN, H. Role of PPAR-gamma in inflammation. Prospects for therapeutic intervention by food components. Mutat. Res*.,* v. 690, p. 57-63, 2010.

MCCARTHY C. *et al.* IL-10 mediates the immunoregulatory response in conjugated linoleic acid-induced regression of atherosclerosis. FASEB J., v. 27, p. 499–510, 2013a.

MCCARTHY C. *et al.* Macrophage PPAR gamma co-activator-1 alpha participates in repressing foam cell formation and atherosclerosis in response to conjugated linoleic acid. **EMBO Mol. Med**., v. 5, p. 1443-1457, 2013.

MCCARTHY, C. *et al.* IL-10 mediates the immunoregulatory response in conjugated linoleic acid-induced regression of atherosclerosis. **FASEB J**., v. 27, p. 499-510, 2013.

MCCARTHY, C. *et al.* IL-10 mediates the immunoregulatory response in conjugated linoleic acid-induced regression of atherosclerosis. **FASEB J**., v. 27, n. 2, p. 499-510, 2013.

MCNEILL, E. *et al.* Regulation of iNOS Function and Cellular Redox State by Macrophage Gch1 Reveals Specific Requirements for Tetrahydrobiopterin in NRF2 Activation. **Free Radic. Biol. Med**., v. 79, p. 206-216, 2015.

MIYAMOTO, J. *et al.* A gut microbial metabolite of linoleic acid, 10-Hydroxy-cis-12-octadecenoic acid, ameliorates intestinal epithelial barrier impairment partially via GPR40-MEK-ERK pathway. **J. Biol. Chem**., v. 290, n. 5, p. 2902e2918, 2015.

NAGY, L. *et al.* Oxidized ldl regulates macrophage gene expression through ligand activation of pparγ. **Cell 93,** p. 229-240, 1998.

NALLATHAMBY, N. *et al.* Identification and in vitro evaluation of lipids from sclerotia of lignosus rhinocerotis for antioxidant and anti-neuroinflammatory activities. **Nat. Prod. Commun**., v. 11, n. 10, p. 1485-1490, 2016.

OH, Y. T. *et al.* Oleic acid reduces lipopolysaccharide-induced expression of iNOS and COX-2 in BV2 murine microglial cells: Possible involvement of reactive oxygen species, p38 MAPK, and IKK/NF-B signaling pathways. Neurosci. Lett., v. 464, p. 93-97, 2009.

PENEDO, L. A. *et al.* Intake of butter naturally enriched with cis9, trans11 conjugated linoleic acid reduces systemic inflammatory mediators in healthy young adults. **J. Nutr. Biochem**., v. 24, n. 12, p. 2144-2151, 2013.

POLAVARAPU, S. *et al.* Effect of polyunsaturated fatty acids and their metabolites on bleomycin-induced cytotoxic action on human neuroblastoma cells *in vitro.* **PlosOne,** 2014.

POSSEL, H. Selective upregulation of inducible nitric oxide synthase (inos) by lipopolysaccharide (LPS) and cytokines in microglia: in vitro and in vivo studies. ***Glia* 32,** p. 51-59, 2000.

REBELLO, K. M. *et al.* Miltefosine-Lopinavir Combination Therapy Against Leishmania infantum Infection: In vitro and in vivo Approaches. **Front. Cell. Infect. Microbiol.**, v. 9, p. 229, 2019.

SANCHEZ-GUAJARDO, V. *et al.* Neuroimmunological processes in Parkinson´s disease and their relation to α-sinuclein: microglia as the referee between neuronal processes and peripheral immunity. **ASN Neuro**, v. 5, n. 2, p. 113-139, 2013.

SHI, Y.; HOLTZMAN, D. M. Interplay between innate immunity and Alzheimer disease: APOE and TREM2 in the spotlight. **Nat. Rev. Immunol**., v. 18, p. 759-772, 2018.

SIVANDZADE, F. *et al.* NRF2 and NF-κB interplay in cerebrovascular and neurodegenerative disorders: Molecular mechanisms and possible therapeutic approaches. **Redox Biol**., v. 21, p. 101059, 2019.

SRINIVASAN, M.; LAHIRI, D. K. Significance of NF-κB as a pivotal therapeutic target in the neurodegenerative pathologies of Alzheimer's disease and multiple sclerosis. **Exper. Opin. Ther. Targets**, v. 19, n. 4, p. 471-487, 2015.

STACHOWSKA, E. Conjugated linoleic acid regulates phosphorylation of PPARγ by modulation of ERK 1/2 and p38 signaling in human macrophages/fatty acid-laden macrophages. **J. Agric. Food Chem**., v, 59, n. 21, p. 11846-11852, 2011.

STANSLEY, B. J. *et al.* A comparative review of cell culture systems for the study of microglial biology in Alzheimer's disease. **J. Neuroinflammation**, v. 9, n. 115, p. 1-8, 2012.

SUBRAMANIAM, S. R.; FEDEROFF, H. J. Targeting microglial activation states as a therapeutic avenue in Parkinson´s disease. **Front. Aging Neurosci**., v. 9, n, 176, p. 1-18, 2017.

TAHBOUB, Y. R. Determination of nitrite and nitrate in cell culture medium by reversed-phase high-performance liquid cromatography with simultaneous UV-VIS and fluorescence detection. **Jordan J. Chem.**, v. 3, n.1, p. 69-75, 2008.

TU, T. H. *et al.* Linoleic acid rescues microglia inflammation triggered by saturated fatty acid. Biochem. **Biophys. Res. Commun**., v, 513, n.1, p. 201-206, 2019.

VALLVE, J-C. *et al*. Unsaturated fatty acids and their oxidation products stimulate CD36 gene expression in human macrophages. **Atherosclerosis.**, v. 164, p. 45-56, 2002.

VIEIRA, C. P. *et al*. Lactococcus lactis ssp. cremoris MRS47, a potential probiotic strain isolated from kefir grains, increases cis-9, trans-11-CLA and PUFA contents in fermented milk. **J. Funct**. **Foods**., v. 31, ´p. 172-178, 2017.

VILAMIDOU, HONTECILLAS.; BASSAGANYA-RIERA. Modulation of inflammation and immunity by dietary conjugated linoleic acid. **Eur. J. Pharmacol.**, v. 785, p. 87-89, 2015.

VILLACORTA, L. *et al.* In situ generation, metabolism and immunomodulatory signaling actions of nitro-conjugated linoleic acid in a murine model of inflammation. **Redox Biol**., v. 15, p. 522-531, 2018.

WARDEN, A. *et al.* Localization of PPAR isotypes in the adult mouse and human brain. **Sci. Rep**., v. 6, n. 27618, p. 1-15, 2016.

WEN, X. *et al.* Astaxanthin acts via LRP-1 to inhibit inflammation and reverse lipopolysaccharide-induced M1/M2 polarization of microglial cells. **Oncotarget**., v. 8, n. 41, p. 69370–69385, 2017.

YAGUCHI, T.; FUJIKAWA, H.; NISHIZAKI, T. Linoleic Acid Derivative DCP-LA Protects Neurons From Oxidative Stress-Induced Apoptosis by Inhibiting caspase-3/-9 Activation.

Neurochem. Res., v. 35, n. 5, p. 712-717, 2010.

YANG, M.; COOK, M. E. Dietary Conjugated Linoleic Acid Decreased Cachexia, Macrophage Tumor Necrosis Factor-α Production, and Modifies Splenocyte Cytokines Production. **Exp. Biol. Med**., v. 228, n. 1, p. 51-58, 2003.

YU, Y. *et al.* Conjugated linoleic acid decreases production of pro-inflammatory products in macrophages: evidence for a PPAR gamma-dependent mechanism. **Biochim. Biophys. Acta**., v. 1581, n. 3, p. 89-99, 2002.

ZHANG, G. *et al.* LPS-induced iNOS expression in N9 microglial cells is suppressed by geniposide via ERK, p38 and nuclear factor-κB signaling pathways. **Int. J. Mol. Med**., v. 30, n. 3**,** p. 561-568, 2012.

ZHOU, M. *et al.* Endotoxin Downregulates Peroxisome Proliferator-Activated Receptor-Gamma via the Increase in TNF-alpha Release. **Am. J. Physiol. Regul. Integr. Comp. Physiol**., v. 294, n. 1, p. R84-92, 2008.

6 FINAL CONSIDERATIONS

This doctorate thesis is composed of two original manuscripts, which represents a breakthrough of research field of elaboration of functional dairy product and evaluation of its bioactive effect on neuron-inflammation. Together, the results obtained allowed to conclude that previous selection of *Lactococcus lactis* ssp. *cremoris* MRS 47 strain by its ability to produce CLA from LA in esterified way (sunflower oil) was a relevant step to increase the CLA production from milk fat during fermentation. So, this screening is a clear advantage over the majority of previous studies (ALKALIN *et al.*., 2007; EL-SALAM *et al.*, 2010; HERNANDEZ *et al*., 2007; XU *et al.*, 2005).

The fermentation with the screened LAB strain also positively changed the fatty acids profile, reducing total SFA and increasing PUFA and PUFA/SFA ratios in foodstuff. The Δ9-DA ability increment by probiotic fermentation correlated with increase of CLA production and contributed to improve the nutritional lipid quality, reducing the thrombogenicity index in fermented milk. Regarding to functional effect of CLA-enriched milk previously manufactured, CLA mitigated the microglial activation by LPS, reducing both the secretion of NO and the levels of IL-1β pro-inflammatory cytokine and concurrently increasing IL-4 and IL-10 anti-inflammatory cytokines by the primary microglia culture and N9 cell line. LA showed similar suppressive effects on microglial activation in N9 cells. However, only CLA had significant effect on arginase activity. The treatment with CLA completely prevented the suppression of arginase activity by LPS in primary microglia, besides the arginase activity has correlated with the NO levels both in primary microglia and N9 cells treated with CLA. This suggested that CLA drove microglia from M1 (proinflammatory) to M2 (anti-inflammatory) phenotype.

The microglia polarization was resultant, at least in part, of inhibition of NF-κB proinflammatory pathway as well as reduction of iNOS expression through increased expression of IκBα inhibitor in LPS-activated microglia. The inhibitory effect on microglial NF-κB and iNOS was specific-isomer, because LA did not present such effect when microglia was activated. Evidences suggested PPARγ as a relevant receptor for CLA action on microglia and a lesser extent for LA. Co-treatment with CLA and LPS completely prevented cortical neuronal death induced by the endotoxin, being this neuroprotection also isomer-specific. Thus, CLA has potential as anti-inflammatory and neuroprotective candidates against microglia-mediated neuroinflammatory disorders, in what seems to have isomer-

specific particularities. This is the first report in the literature demonstrating the beneficial effect of CLA on microglia-mediated neuroinflammatory disease model.

7 GENERAL REFERENCES

ABEL, S.; ARENDSE, L.; GELDERBLOM, W. Conjugated linoleic acid isomers as anticancer bioactive compounds. **South African Journal of Clinical Nutrition,** v. 27, n. 3, 2014.

ADDIS, M. *et al.* Milk and cheese fatty acid composition in sheep fed Mediterranean forages with reference to conjugated linoleic acid cis-9, trans-11. **Journal of Dairy Science,** v. 88, p. 3443-3454, 2005.

ALDAI, N. *et al.* Length of concentrate finishing affects the fatty acid composition of grass-fed and genetically lean beef: an emphasis on trans-18:1 and conjugated linoleic acid profiles. **Animal,** v. 5, n. 10, p. 1643-1652, 2011.

ALTEI, W. F. Effect of cis-9, trans-11 Conjugated Linoleic Acid (CLA) on the Metabolism Profile of Breast Cancer Cells Determined by H HR-MAS NMR Spectroscopy. **Journal of the Brazilian Chemical Society,** v. 30, n. 1, p. 3-7, 2019.

ANDRADE, J. C. *et al.* (2012). Production of conjugated linoleic acid by food-grade bacteria: A review. **International Journal of Dairy Technology.,** v. 65, n. 4, p. 467-481, 2012.

ARIAS, N. The combination of resveratrol and conjugated linoleic acid attenuates the individual effects of these molecules on triacylglycerol metabolism in adipose tissue. **European Journal of Nutrition,** v. 53, n. 2, p. 575-582, 2014.

ATKINSON, R. L. Conjugated linoleic acid for altering body composition and treating obesity. In YURAWEEZ, M.; MOSSOBA, M.; KRAMER, J., PARIZA, M.; NELSON, G. *Advances in conjugated linoleic acid research*.v. 1, pp. 348-353. Champaign, IL: AOCS Press, 1999.

AVILEZ, J. P. R.; MEYER, J. R. Conjugated linoleic acid transfer (CLA) from raw milk to powdered milk and condensed milk. **Revista Chilena de Nutrición,** v. 41, n. 4, p. 420-424, 2014.

BADIANI, A. *et al.* Selected nutrient contents, fatty acid composition, including conjugated linoleic acid, and retention values in separable lean from lamb rib loins as affected by external fat and cooking method. **Journal of Agricultural and Food Chemistry,** v. 52, p. 5187-5194, 2004.

BASSAGANYA-RIERA, J.; HONTECILLAS, R. Dietary conjugated linoleic acid and n-3 polyunsaturated fatty acids in inflammatory bowel disease. **Current Opinion in Clinical Nutrition & Metabolic Care,** v. 13, p. 569–573, 2010.

BASSAGANYA-RIERA, J. *et al.* Conjugated linoleic acid modulates imune responses in patients with mild to moderately active Crohn's disease. **Clinical Nutrition,** v. 31, p.721–727, 2012b

BASSAGANYA-RIERA, J. *et al.* Dietary conjugated linoleic acid modulates phenotype and effector functions of porcine CD8(+) lymphocytes. **Journal of Nutrition,** v. 131, ´p. 2370–2377, 2001.

BASSAGANYA-RIERA, J. *et al.* Conjugated linoleic acid ameliorates viral infectivity in a pig model of virally induced immunosuppression. **Journal of Nutrition,** v. 133, p. 3204-3214, 2003.

BASSAGANYA-RIERA, J. *et al*. Probiotic bacteria produce conjugated linoleic acid locally in the gut that targets macrophage PPARgamma to suppress colitis. **PloSOne,** v. 7, 2012a.

BASSAGANYA-RIERA, J. *et al.* Immunoregulatory mechanisms underlying prevention of colitis-associated colorectal cancer by probiotic bacteria. **PlosOne,** v. 7, n. 4, 2012b.

BASSAGANYA-RIERA, J. *et al*. Immunoregulatory mechanisms underlying prevention of colitis-associated colorectal cancer by probiotic bacteria. **PloSone,** v. 7, 2012c.

BAUMAN DE.; LOCK AL. Conjugated Linoleic Acid: Biosynthesis and Nutritional Significance. In FOX, P.F.; McSWEENEY, P.L.H. **Advanced Dairy Chemistry**., v. 2, 2006. pp. 93-136.

BEGUM, P. S. *et al.* Probiotics as functional foods: potential effects on human health and its impact on neurological diseases. **International Journal of Nutrition, Pharmacology, Neurological Diseases,** v. 7, n. 2, p. 23-33, 2017.

BENJAMIN, S.; SPENER, F. Conjugated linoleic acids as functional food: an insight into their health benefits. **Nutrition & Metabolism,** v. 6, n. 36, p. 1-13, 2009.

BEPPU, F. Potent inhibitory effect of trans 9, trans 11 isomer of conjugated linoleic acid on the growth of human colon cancer cells. **The Journal of Nutritional Biochemistry,** v. 7, p. 830-836, 2006.

BETTERO, V. P. Use of protected fat sources to reduce fatty acid biohydrogenation and improve abomasal flow in dry dairy cows. **Animal Feed Science and Technology**., p. 224, 30-38, 2017.

BEUTHER, D. A.; SUTHERLAND, E. R. Overweight, obesity, and incident asthma:a meta-analysis of prospective epidemiologic studies. **American Journal of Respiratory and Critical Care Medicine,** v. 175, p. 661-666, 2007.

BIAŁEK, A.; TOKARZ, A.; ZAGRODZKI, P. Conjugated linoleic acids in diet of female rats inhibit the breast cancer formation in their offspring. **Journal of Food and Nutrition Research,** v. 53, n.1, p. 39-50, 2014.

BINYAMIN, O. Brain targeting of 9c,11t-Conjugated Linoleic Acid, a natural calpain inhibitor, preserves memory and reduces Aβ and P25 accumulation in 5XFAD mice. **Scientific Reports,** v. 9, p. 1-12, 2019.

BLANKSON, H. *et al.* Conjugated linoleic acid reduces body fat mass in overweight and obese humans. **Journal of Nutrition,** v. 130, n. 12, p. 2943-2948, 2000.

BODAS, R. Comparison of the fatty acid profiles in cheeses from ewes fed diets supplemented with different plant oils. **Journal of Agricultural and Food Chemistry**., v. 58, p. 10493-10502, 2000.

BRACHO, S.; BERIAIN, M. J.; SMITH, G. C. Occurrence of conjugated linoleic acid in longissimus dorsi muscle of water buffalo (Bubalus bubalis) and zebu-type cattle raised under savannah conditions. **Meat Science,** v, 69, p. 93-100, 2005.

BRUEN, R.; FITZSIMONS, S.; BELTON, O. Atheroprotective effects of conjugated linoleic acid. **British journal of clinical pharmacology**, v. 83, n. 1, p. 46-53, 2007.

BRZOZOWSKA, A. M. Metabolism of fatty acids in tissues and organs of the ruminants - a review. **Animal Science Papers & Reports,** v. 34, n. 3, p. 211-219, 2016.

BU, D. P. Effectiveness of oils rich in linoleic and linolenic acids to enhance conjugated linoleic acid in milk from dairy cows. **Journal of Dairy Science,** v. 90, p. 998-1007, 2007.

BUCCIONI, A. Lipid metabolism in the rumen: New insights on lipolysis and biohydrogenation with an emphasis on the role of endogenous plant factors. **Animal Feed Science and Technology,** v.174, n. 1-2, p. 1-25, 2012.

CABIDDU, A. Effect of corn and beet pulp based concentrates on sheep milk and cheese fatty acid composition when fed Mediterranean fresh forages with particular reference to conjugated linoleic acid cis-9, trans-11. Animal Feed Science and Technology, v. 31, p. 292-311, 2006.

CAMILLERI, M. GI clinical research 2002-2003: the year in review. **Clinical, Gastroenterology and Hepatology,** v. 1, p. 415-42, 2003.

CAMPANA, R.; VAN HEMERT, S.; BAFFONE, W. Strain-specific probiotic properties of lactic acid bacteria and their interference with human intestinal pathogens invasion. *Gut Pathogens,* 9(12), 1-12, 2017.

CARNEIRO, R. P. **Desenvolvimento de uma cultura iniciadora para produção de kefir.** Electronic MSc Dissertation Series. Faculdade de Farmácia da Universidade Federal de Minas Gerais, Brasil, 2010.

CASTILLO, J.; OLIVERA, M.; CARULLA, J. Description of the biochemistry mechanism of polyunsaturated fatty acid ruminal biohydrogenation: a review. **Revista U.D.C.A. Actualidad & Divulgación Científica,** v. 6, n. 2, p. 459-468, 2013.

CHAPLIN, A. *et al.* Conjugated Linoleic Acid Supplementation under a High-Fat Diet Modulates Stomach Protein Expression and Intestinal Microbiota in Adult Mice. **PlosOne,** v. 10, n. 4, 2015.

CHIN, S. F. Dietary sources of conjugated dienoic isomers of linoleic acid, a newly recognized class of anticarcinogens. **Journal of Food Composition and Analysis,** v. 5, p.185-197, 1992.

CHIN, S. F. Dietary sources of conjugated dienoic isomers of linoleic acid, a newly recognized class of anticarcinogens. **Journal of Food Composition and Analysis,** v. 5, p. 185–197, 1992.

CONTE-JUNIOR, C. A. Estudio de la producción de acido linoleico conjugado por cepas probióticas de Lactobacillus sp y Enterococcus sp. de distintos orígenes. **Revista Complutense de Ciências Veterinárias**., v. 2, p. 482-489, 2007.

COOPER, M. H. Conjugated linoleic acid isomers have no effect on atherosclerosis and adverse effects on lipoprotein and liver lipid metabolism in apoE−/− mice fed a high-cholesterol diet. **Atherosclerosis,** v. 200, n. 2, p. 294-302, 2008.

DE RENOBALES, M. Part-time grazing improves sheep milk production and its nutritional characteristics. **Food Chemistry,** v. 130, n. 1, p. 90-96, 2012.

DHIMAN, T.R.; NAM, S.H.; URE, A.L. Factors affecting conjugated linoleic acid content in milk and meat. **Critical Reviews in Food Science and Nutrition,** v. 45, p. 463-482, 2007.

DILZER, A.; PARK, Y. Implication of Conjugated Linoleic Acid (CLA) in Human Health. **Critical Reviews in Food Science and Nutrition,** v. 52, n. 6, p. 488-513, 2012.

DOMINGO, J. J. S. Review of the role of probiotics in gastrointestinal diseases in adults. **Gastroenterología y Hepatología,** v. 40, n. 6, p. 417-429, 2017.

DRUART, C. Ability of the gut microbiota to produce PUFA-derived bacterial metabolites: proof of concept in germ-free versus conventionalized mice. **Molecular Nutrition & Food Research***,* v. 59, n.8, p. 1603-1613, 2015.

DRUART, C. Role of the lower and upper intestine in the production and absorption of gut microbiota-derived PUFA metabolites. PlosOne, v. 9, n. 1, 2014.

DUFEY, P. A. Fleisch ist eine CLA-Nahrungsquelle. **Agrarforschung***,* v. 6, p. 177-180, 1999.

EFSA Panel on Dietetic Products NaAN. Scientific opinion on the safety of conjugated linoleic acid (CLA)-rich oil' (Clarinol®) as a novel food ingredient. **European Food Safety Authority Journal,** v. 8, p. 1-41, 2010.

EFTEKHARI, M. H. *et al.* The effect of conjugated linoleic acids and omega-3 fatty acids supplementation on lipid profile in atherosclerosis. **Adanced Biomedical Research,** v. 3, n.15, p. 1-13, 2014.

EVANS, N. P. Conjugated linoleic acid ameliorates inflammation-induced colo rectal cancer in mice through activation of PPAR-gamma. **Journal of Nutrition,** v. 140, n. 3, p. 515-521, 2010.

EWASCHUK, J. B. Bioproduction of conjugated linoleic acid by probiotic bactéria occurs *in vitro* and *in vivo* in mice. **Journal of Nutrition,** v. 136, p. 1483-1487, 2006.

FAN, J. *et al.* Rabbit models for the study of human atherosclerosis: From pathophysiological mechanisms to translational medicine. **Pharmacology & Therapeutics,** v. 146, p. 104-119, 2015.

FAO/WHO. Probiotic in foods. Health and nutritional properties and guidelines for evaluation. **FAO Food and Nutrition Paper**, v. 85, 2006.

FARAMARZI, E. Effect of conjugated linoleic acid supplementation on quality of life in rectal cancer patients undergoing preoperative chemoradiotherapy. **Pakistan Journal of Medical Sciences**., v. 33, n. 2, p. 383-388, 2017.

FERNANDEZ, R.; RODRIGUEZ, G. Effect of dietary and animal factors on milk fatty acids composition of grazing dairy cows: a review. **Iranian Journal of Applied Animal Science**., v. 2, p. 97-109, 2012.

FERREIRA, E. M. *et al.* Perfil de ácidos graxos do leite de ovelhas recebendo óleo de soja e óleo de peixe como fontes de gordura suplementar. **Anais...** 48ª Reunião Anual da Sociedade Brasileira de Zootecnia. Sociedade Brasileira de Zootecnia, Belém, 2011.

FRITSCHE, J.; STEINHARDT, H. Amounts of conjugated linoleic acid (CLA) in German foods and evaluation of daily intake. **Zeitschrift für Lebensmittel-Untersuchung und - Forschung A – Food Research and Technology**., v. 206, p. 77-82, 1998.

FUENTES, P. H. A. **Avaliação da qualidade de óleos de soja, canola, milho e girassol durante o armazenamento**. Dissertação de Mestrado do Programa de Pós-Graduação em Ciência dos Alimentos da Universidade Federal de Santa Catarina, Brasil, 2011.

Gama, M. A. S. Conjugated linoleic acid-enriched butter improved memory and up-regulated phospholipase A2 encoding-genes in rat brain tissue. **Journal of Neural Transmission**., v. 122, p. 1371-1380, 2015.

GARCIA, C. *et al.* Synthesis of the suspected trans-11, cis-13 conjugated linoleic acid isomer in ruminant mammary tissue by FADS3-catalyzed Δ13-desaturation of vaccenic acid. **Journal of Dairy Science**., v. 100, n. 1, p. 783-796, 2017.

GAULLIER, J. M. Clinical trial results support a preference for using CLA preparations enriched with two isomers rather than four isomers in human studies. **Lipids**., v. 37, p. 1019-1025, 2002.

GAULLIER, J. M. Conjugated linoleic acid supplementation for 1 y reduces body fat mass in healthy overweight humans. **The American Journal of Clinical Nutrition**., v. 79, n. 6), p. 1118-1125, 2004.

GEDDES, R. I. Conjugated Linoleic Acid Administration Induces Amnesia in Male Sprague Dawley Rats and Exacerbates Recovery from Functional Deficits Induced by a Controlled Cortical Impact Injury. **PlosOne**., v. 12, n. 1, 2017.

GNÄDIG, S. *et al.* Influence of manufacturing conditions on the conjugated linoleic acid content and the isomer composition in ripened French Emmental cheese. **Journal of Dairy Research**., v. 71, p. 1-5, 2004.

GOMES, A. C. *et al.* Gut microbiota, probiotics and diabetes. **Nutrition Journal**., v. 13, n.60, p. 1-13, 2014.

GÓMEZ-CORTÉS, P. *et al.* Effect of supplementation of grazing dairy ewes with a cereal concentrate on animal performance and milk fatty acid profile. **Journal of Dairy Science,** v. 92, n. 8, p. 3964-3972, 2009.

GONG, M. *et al.* Production of conjugated fatty acids: A review of recent advances. **Biotechnology advances**., v. 37, n. 8, p. 107454, 2019.

GORISSEN, L. *et al*. Bacterial production of conjugated linoleic and linolenic acid in foods: a technological challenge. **Critical Reviews in Food Science and Nutrition**., v. 55, p. 1561-1674, 2015.

GUTIÉRREZ, L. F. Conjugated linoleic acid in milk and fermented milks: variation and effects of the technological processes. **Vitae**., v. 23, n. 2, p. 134-145, 2016.

HAMET, M. F.; PIERMARIA, J. A.; ABRAHAM, A. G. Selection of EPS-producing Lactobacillus strains isolated from kefir grains and rheological characterization of the fermented milks. **LWT-Food Science and Technology**., v. 6, n. 1, p. 129-135, 2015.

HARTIGH, L. J. Conjugated Linoleic Acid Effects on Cancer, Obesity, and Atherosclerosis: A Review of Pre-Clinical and Human Trials with Current Perspectives. **Nutrients**., v. 11, n. 2, p. 1-29, 2019.

HONTECILLAS, R.; BASSAGANYA-RIERA, J. Peroxisome proliferator-activated receptor gamma is required for regulatory CD4+ T cell-mediated protection against colitis. **The Journal of Immunology**., v. 178, p. 2940-2949, 2007.

HONTECILLAS, R. *et al.* 2002. Nutritional regulation of porcine bacterial-induced colitis by conjugated linoleic acid. **Journal of Nutrition**., v. 132, p. 2019–2027, 2002.

HOSSEINI, E. S. *et al.* Conjugated linoleic acid production from various substrates by probiotic Lactobacillus plantarum. **Annals of Microbiology**., v. 65, p. 27-32, 2015.

HUNT, W. T. *et al.* Protection of cortical neurons from excitotoxicity by conjugated linoleic acid. **Journal of Neurochemistry**., v. 115, p. 123-130, 2010.

JACOBS, A. A. A. *et al.* Effects of feeding rapeseed oil, soybean oil, or linseed oil on stearoyl-CoA desaturase expression in the mammary gland of dairy cows. Journal of Dairy Science, v. 94, n. 2, p. 874-887, 2011.

JAUDSZUS, A. *et al.* Cis-9,trans-11-conjugated linoleic acid inhibits allergic sensitization and airway inflammation via a PPARgamma-related mechanism in mice. **Journal of Nutrition**., v. 138, p. 1336-1342, 2008.

JÉSSICA, L. O. B. *et al*. Conjugated linoleic acid (CLA) concentration and fatty acid composition of Brazilian fermented dairy products. **Journal of Nutrition & Food Sciences,** v. 5, n. 5, p. 1-3, 2015.

JIANG, C.; TING, A.T.; SEED, B. PPAR-gamma agonists inhibit production of monocyte inflammatory cytokines. **Nature**., v. 391, p. 82-86, 1998.

JIN, Y. C. *et al*. Conjugated linoleic acid synthesis-related protein proteasome subunit α 5 (PSMA5) is increased by vaccenic acid treatment in goat mammary tissue. **Journal of Dairy Science**., v. 95, n. 8, p. 4286-4297, 2012.

KAKISU, E. *et al.* Physicochemical, microbiological and sensory profiles of fermented milk containing probiotic strains isolated from kefir. **Journal of Dairy Research**., v. 78, n. 4, p. 456-463, 2011.

KANTER, J. E. *et al.* Conjugated linoleic acid-driven weight loss is protective against atherosclerosis in mice and is associated with alternative macrophage enrichment in perivascular adipose tissue. **Nutrients**., v.10, p. 1416, 2018.

KATHIRVELAN, C.; BANUPRIYA, S.; TYAGI, A. K. Nutritional and biotechnological means of enhancing nutraceutical value of milk through conjugated linoleic acid (CLA) content and its anticarcinogenic potency. **Indian Journal of Science**., v. 15, n. 46, p. 69-74, 2015.

KENNEDY, A. *et al.* Antiobesity mechanisms of action of conjugated linoleic acid. **The Journal of Nutritional Biochemistry**., v. 21, n. 3, p. 171-179, 2010.

KENNEDY, A. *et al.* Antiobesity mechanisms of action of conjugated linoleic acid. **Journal of Nutritional Biochemistry**., v. 21, p. 171-179, 2010.

KHANAL, R. C.; OLSON, K. C. Factors affecting conjugated linoleic acid (CLA) content in milk, meat, and egg: a review. **Pakistan Journal of Nutrition**., v. 3, n. 2, p. 82-98.

KHOSRAVI-DARANI, K. *et al*. Bioproduction of conjugated linoleic acid in yogurt by probiotic bacteria. **International Journal of Biotechnology for Wellness Industries**., v. 3, n. 2, p. 62-68, 2014.

KIM, J. H. *et al.* Conjugated linoleic acid: potential health benefits as a functional food ingredient. **Annual Review of Food Science and Technology**., v. 7, p. 221-244, 2016.

KIM, B. *et al.* The effects of conjugated linoleic acid (CLA) on metabolic syndrome patients: A systematic review and meta-analysis. **Journal of Functional Foods**., v. 25, p. 588-598, 2016b.

KIM, D. I. *et al.* Trans-10, cis-12-conjugated linoleic acid modulates NF- κB activation and TNF-α production in porcine peripheral blood mononuclear cells via a PPARγ-dependent pathway. **The British Journal of Nutrition**., v. 105, n. 9, p. 1329-1336, 2011.

KIM, D. I. *et al.* Trans-10,cis-12-conjugated linoleic acid modulates NF-kappaB activation and TNF-alpha production in porcine peripheral blood mononuclear cells via a PPARgamma-dependent pathway. **Britsh Journal of Nutrition**., v, 105, p. 1329-1336, 2011.

KIZILŞAHIN, S.; NALBANTSOY, A.; YAVAŞOĞLU, N. U. K. In vitro synergistic efficacy of conjugated linoleic acid, oleic acid, safflower oil and taxol cytotoxicity on PC3 cells. **Formerly Natural Product Letters**., v. 29, n. 4, p. 378-382, 2014.

KOBA, K.; YANAGITA, T. Health benefits of conjugated linoleic acid (CLA). **Obesity Research & Clinical Practice**., v. 8, n. 6, p. 525-532, 2014.

KRITCHEVSKY, D. *et al.* Conjugated linoleic acid isomer effects in atherosclerosis: Growth and regression of lesions. **Lipids**., v. 39, p. 611-616, 2004.

KUCUK, O.; HESS, B. W.; RULE, D.C. Soybean oil supplementation of a high-concentrate diet does not affect site and extent of organic matter, starch, neutral detergent fiber, or nitrogen digestion, but influences both ruminal metabolism and intestinal flow of fatty acids in limit-fed lambs. **Journal of Animal Science**., v. 82, p. 2985-2994, 2004.

KUMAR, K. S. *et al.* Colon cancer prevention through probiotics: An overview. **Cancer Science & Therapy**., v. 7, n. 2, p. 81-92, 2015.

KUUGBEE, E. D. *et al.* Structural change in microbiota by a probiotic cocktail enhances the gut barrier and reduces cancer via TLR2 signaling in a rat model of colon cancer. **Digestive Diseases and Sciences**., v. 61, p. 2908-2920, 2016.

LAITINEN, T. T. *et al.* Lifetime measures of ideal cardiovascular health and their association with subclinical atherosclerosis: The Cardiovascular Risk in Young Finns Study. **International Journal of Cardiology**., v. 185, p. 186-191, 2015.

LAKATOS, P. L. Recent trends in the epidemiology of inflammatory bowel diseases: up or down? **World Journal of Gastroenterology**., v. 12, p. 6102-6108, 2006.

LAWSON, R. E.; MOSS, A. R.; GIVENS, D. I. The role of dairy products in supplying conjugated linoleic acid to man's diet: a review. **Nutrition Research Reviews**., v. 14, p. 153-172, 2001.

LEE, E. *et al.* Effect of Conjugated Linoleic Acid, μ-Calpain Inhibitor, on Pathogenesis of Alzheimer's Disease. **Biochimica et Biophysica Acta**., v. 1831, n. 4, p. 709-718, 2012.

LEE, K. N.; KRITCHEVSKY, D.; PARIZA, M.W. Conjugated linoleic acid and atherosclerosis in rabbits. **Atherosclerosis**., v. 108, p. 19-25, 1994.

LEE, Y. Effect of pH on conjugated linoleic acid (CLA) formation of linolenic acid biohydrogenation by ruminal microorganisms. **Journal of Microbiology**., v. 51, p. 471-476, 2013.

LEITE, A. M. O. *et al.* Microbiological, technological and therapeutic properties of kefir: a natural probiotic beverage. **Brazilian Journal of Microbiology**., v. 44, n. 2, p. 341-349, 2013.

LIM, J-N. *et al.* Trans-11 18:1 vaccenic acid (TVA) has a direct anti-carcinogenic effect on MCF-7 human mammary adenocarcinoma cells. **Nutrients**., v. 6, p. 627-636, 2014.

LOSCHER, C. E. *et al.* Conjugated linoleic acid suppresses NF-kappaB Activation and IL-12 production in dendritic cells through ERK-mediated IL-10 induction. **Journal of Immunlogy,** v. 175, p. 4990-4998, 2005.

LOURENÇO, M.; RAMOS-MORALES, E.; WALLACE, R. J. The role of microbes in rumen lipolysis and biohydrogenation and their manipulation. **Animal**., v. 4, n. 7, p. 1008-1023, 2010.

LUNA, P. *et al.* Conjugated linoleic acid content and isomer distribution during ripening in three varieties of cheeses protected with designation of origin. **Food Chemistry***.,* v. 103, p. 1465-1472, 2007.

LYNCH, J. M. *et al.* Flavor and Stability of Pasteurized Milk with Elevated Levels of Conjugated Linoleic Acid and Vaccenic Acid. **Journal of Dairy Science**., v. 88, p. 489-498, 2005.

MA, L. *et al.* Short communication: Effect of trans-10, cis-12 conjugated linoleic acid on activation of lipogenic transcription factors in bovine mammary epithelial cells. **Journal of Dairy Science***.,* v. 97, n. 8, p. 5001-5006, 2014.

MCCARTHY, C. *et al.* IL-10 mediates the immunoregulatory response in conjugated linoleic acid-induced regression of atherosclerosis. **The FASEB Journal**., v. 27, n. 2, p. 499-510, 2013.

MACONI, G. *et al.* Prevalence, detection rate and outcome of Cytomegalovirus infection in ulcerative colitis patients requiring colonic resection. **Digestive and Liver Disease**., v. 37, p. 418-423, 2005.

MACREDMOND, R.; DORSCHEID, D. R. Conjugated linoleic acid (CLA): is it time to supplement asthma therapy? **Pulmonary Pharmacology & Therapeutics**., v. 24, p. 540-548, 2011.

MAIA, M. R. G. *et al.* Toxicity of unsaturated fatty acids to the biohydrogenating ruminal bacterium, Butyrivibrio fibrisolvens. **BMC Microbiology**., v. 10, n. 52, p. 1-10, 2010.

MALLIA, S. *et al.* Determination of storage stability of butter enriched with unsaturated fatty acids/conjugated linoleic acids (UFA/CLA) using instrumental and sensory methods. **International Dairy Journal**., v. 18, n. 10-11, p. 983-993, 2008.

MARANESI, M. *et al.* Effect of microwave cooking or broiling on selected nutrient contents, fatty acid patterns and true retention values in separable lean from lamb rib-loins, with emphasis on conjugated linoleic acid. **Food Chemistry**., v. 90, n. 1-2, p. 207-218, 2005.

MARQUES, T. M. *et al.* Dietary trans-10, cis-12-conjugated linoleic acid alters fatty acid metabolism and microbiota composition in mice. **British Journal of Nutrition**., v. 113, n. 5, p. 728-738, 2015.

MARTINEZ, K. *et al.* Trans-10, cis-12 conjugated linoleic acid instigates inflammation in human adipocytes compared with preadipocytes. **Journal of Biological Chemistry**., v. 285, n. 23, p. 17701-17712, 2010.

MARTÍNEZ-MONTEAGUDO, S. I. *et al.* Oxidative stability of ultra high temperature milk enriched in conjugated linoleic acid and trans-vaccenic acid. **International Dairy Journal**., v. 43, p. 70-77, 2015.

MARTINS, S. V. *et al.* Contents of conjugated linoleic acid isomers in ruminant-derived foods and estimation of their contribution to daily intake in Portugal. **British Journal of Nutrition**., v. 98, n. 6, p. 1206-1213, 2007.

MCGOWAN, M. M. *et al.* A proof of principle clinical trial to determine whether conjugated linoleic acid modulates the lipogenic pathway in human breast cancer tissue. **Breast Cancer Research and Treatment**., v. 138, p. 175-83, 2013.

MEIR, K. S.; LEITERSDORF, E. Atherosclerosis in the apolipoprotein-E-deficient mouse: A decade of progress. **Arteriosclerosis, Thrombosis, and Vascular Biology**., v. 24, p. 1006-1014, 2004.

MIERLITA, D.; DARABAN, S.; LUP, F. Effects of breed on milk fatty acid profile in dairy ewes, with particular reference to cis-9, trans-11 conjugated linoleic acid. **South African Journal Of Animal Science**., v. 41, n. 3, p. 223-231, 2011.

MIRI, V. H.; EBRAHIMI, S. H.; TYAGI, A. K. The effect of cumin (Cuminum cyminum) seed extract on the inhibition of PUFA biohydrogenation in the rumen of lactating goats via changes in the activity of rumen bacteria and linoleate isomerase enzyme. **Small Ruminant Research**., v. 125, p. 56-63, 2015.

MITCHELL, P. L. *et al.* t-10, c-12 CLA Dietary Supplementation Inhibits Atherosclerotic Lesion Development Despite Adverse Cardiovascular and Hepatic Metabolic Marker Profiles. **PlosOne**., v. 7, 2012.

MITCHELL, P. L. *et al.* Effect of conjugated linoleic acid isomers on lipoproteins and atherosclerosis in the Syrian Golden hamster. **Biochimica et Biophysica Acta (BBA) - Molecular and Cell Biology of Lipids**., v. 1734, n. 3, p. 269-276, 2005.

MITCHELL, P. L.; MCLEOD, R. S. Conjugated linoleic acid and atherosclerosis: studies in animal models. **Biochemistry and Cell Biology**., v. 86, p. 293-301, 2008.

MOHAMMADZADEH, M. *et al.* Effect of conjugated linoleic acid supplementation on inflammatory factors and matrix metalloproteinase enzymes in rectal cancer patients undergoing chemoradiotherapy. **Integrative Cancer Therapies**., v. 12, p. 496-502, 2013.

MOLONEY, F. *et al.* 2004). Conjugated linoleic acid supplementation, insulin sensitivity, and lipoprotein metabolism in patients with type 2 diabetes mellitus. **The American Journal of Clinical Nutrition**., v. 80, p. 887-895, 2004.

MONACO, A. *et al.* Conjugated linoleic acid prevents age-dependent neurodegeneration in a mouse model of neuropsychiatric lupus via the activation of an adaptive response. **Journal of Lipid Research**., v. 59, n. 1, p. 48-57, 2019.

MONDRAGÓN, M. G. C. Conjugated linoleic acid (cla) intake, a mini review. **Annals. Food Science and Technology**., v. 7, n. 1, p. 150-154, 2016.

MOZZON, M. *et al*. Effect of dietary fish oil supplements on levels of n-3 polyunsaturated fatty acids, trans acids and conjugated linoleic acid in ewe milk. **Food Technology and Biotechnology**., v .40, p. 213-219, 2002.

MUNGURE, T. E. *et al.* Effect of extended storage on the quality and stability of conjugated linoleic acid (CLA) in wet and dry-aged, frozen-thawed beef. **Annals…** 63rd International Congress of Meat Science and Technology, p, 152-153, 2017.

MURUZ, H.; ÇETINKAYA, N. The effect of dairy cow feeding regime on functional milk production. **International Advanced Researches and Engineering Journal**., v .3, n. 1, p. 1-6, 2019.

NAKANISHI, T. *et al.*. Dietary Conjugated linoleic acid reduces cerebral prostaglandin E(2) in mice. **Neuroscience Letters**., v. 341, n. 2, p. 135-138, 2003.

NAVARRO, V., Macarulla, M. T., Fernández-Quintela, A., Rodríguez, V. M., Simón, E., & Portillo, M. P. (2007). Effects of trans-10, cis-12 conjugated linoleic acid on cholesterol metabolism in hypercholesterolaemic hamsters. **European Journal of Nutrition**., v. 46, p. 213-219.

NIEZGODA, N. *et al.* Production of concentrates of CLA obtained from sunflower and safflower and their application to the lipase-catalyzed acidolysis of egg yolk phosphatidylcholine. **European Journal of Lipid Science and Technology**., v. 118, n. 10, 1566-1578, 2016.

NIEZGODA, N. *et al.* Synthesis and evaluation of cytotoxic activity of conjugated linoleic acid derivatives (esters, alcohols and their acetates) towards cancer cell lines. **European Journal of Lipid Science and Technology**., v. 119, n. 10, p. 1-25, 2017.

NUDDA, A. *et al.* Feeding strategies to design the fatty acid profile of sheep milk and cheese. **Revista Brasileira de Zootecnia**., v. 43, n. 8, p. 445-456, 2014.

NUDDA, A. *et al.* Comparison of conjugated linoleic acid (CLA) content in milk of ewes and goats with the same dietary regimen. **Italian Journal of Animal Science**., v. 2, n. 1, p. 515-517, 2003.

O'SHEA, M.; BASSAGANYA-RIERA, J.; MOHEDE, I. C. M. Immunomodulatory properties of conjugated linoleic acid. **The American Journal of Clinical Nutrition**., v. 79, p. 1199S-1206S, 2004.

OCHOA, J. J. *et al.* Conjugated linoleic acids (CLAs) decrease prostate câncer cell proliferation: diferente molecular mechanisms for cis-9, trans-11and trans-10,cis-12 isomers. **Carcinogenesis**., v. 25, p. 1185-1191, 2004.

OH, J-J. *et al.* Trans vaccenic acid (trans-11 18:1), a precursor of cis-9, trans-11-conjugated linoleic acid, exerts a direct anti-carcinogenic function in t47d breast carcinoma cells. **Food Science and Biotechnology**., v. 23, n. 2, p. 641-646, 2014.

OLESZCZUK, J. *et al.* Biological effects of conjugated linoleic acids supplementation. **Polish Journal of Veterinary Sciences**., v. 15, p. 403-408, 2012.

OLESZCZUK, J. *et al.* Biological effects of conjugated linoleic acids supplementation. **Polish Journal of Veterinary Sciences**., v. 15, p. 403-408, 2012.

PECOVÁ, L. *et al.* Fatty acids stability in goat yoghurt. **Ciência Rural**., v. 49, n.7, p. 1-10, 2019.

PESTANA, J. M. *et al.* Content and distribution of conjugated linoleic acid isomers in bovine milk, cheese and butter from Azores. **Dairy Science & Technology**., v. 89, p. 193-200, 2009.

PETERSON, K. M. *et al.* Effects of dietary supplementation with conjugated linoleic acid on experimental human rhinovirus infection and ill-ness. **Antiviral Therapy**., v. 14, p. 33-43, 2009.

PFEUFFER, M. *et al.* CLA does not impair endothelial function and decreases body weight as compared with safflower oil in overweight and obese male subjects. **Journal of the American College of Nutrition**., v. 30, p. 19-28, 2011.

PINI, M. *et al.* Adipose tissue adaptive response to trans-10, cis-12–conjugated linoleic acid engages alternatively activated M2 macrophages. **The Faseb Journal**., v. 30, n. 1, p. 241-251, 2015.

PLESSAS, S. *et al.* Microbiological exploration of different types of kefir grains. **Fermentation**., v. 3, n. 1, p. 1-10, 2017.

PLOURDE, M. *et al.* Conjugated linoleic acids: why the discrepancy between animal and human studies? **Nutrition Reviews**., v. 66, n. 7, p. 415-421, 2008.

POLYUNSATURATED fatty acids in inflammatory bowel disease. **Opinion in Clinical Nutrition & Metabolic Care**., v. 13, p. 569-573, 2013.

PREMA, D. *et al.* Rapid determination of total conjugated linoleic acid concentrations in beef by ^{1}H NMR spectroscopy. **Journal of Food Composition and Analysis**., v. 41, p. 54-57, 2015.

QI, R. *et al.* Supplementation with conjugated linoeic acid decreases pig back fat deposition by inducing adipocyte apoptosis. **BMC Veterinary Research**., v. 10, n. 141, p. 1-8, 2014.

QUEIROZ, M. P. *et al.* Effect of conjugated linoleic acid on memory and reflex maturation in rats treated during early life. **Frontiers in Neuroscience**., v. 13, n. 370, p. 1-12, 2019a.

QUEIROZ, M. P. *et al.* Maternal suppplementation with conjugated linoleic acid reduce anxiety and lipid peroxidation in the offspring brain. **Journal of Affective Disorders**., v. 243, p. 75-82, 2019b.

RACINE, N. M. *et al.* Effect of conjugated linoleic acid on body fat accretion in overweight or obese children. **American Journal of Clinical Nutrition,** v. 91, n. 5, p. 1157-1164, 2010.

RAES, K. *et al.* Meat quality, fatty acid composition and flavour analysis in Belgian retail beef. **Meat Science**., v. 65, p. 1237-1246, 2001.

RAHMAN, S. M. *et al*. Effects of Conjugated Linoleic Acid on Serum Leptin Concentration, Body-Fat Accumulation, and Beta-Oxidation of Fatty Acid in OLETF Rats. **Nutrition**., v. 17, p.5, p. 385-390, 2001.

RIBEIRO, S. C. *et al.* Conjugated linoleic acid production and probiotic assessment of Lactobacillus plantarum isolated from Pico cheese. **LWT – Food Science and Technology.,** v. 90, p. 403-411, 2018.

RISÉRUS, U.; BERGLUND, L.; VESSBY, B. Conjugated linoleic acid (CLA) reduced abdominal adipose tissue in obese middle-aged men with signs of the metabolic syndrome: A randomized controlled trial. **International Journal of Obesity and Related Metabolic Disorders**., v. 25, p. 8, p. 1129-1135, 2001.

RITCHIE, M. L.; ROMANUK, T. N. A meta-analysis of probiotic efficacy for gastrointestinal diseases. **PlosOne**., v. 7, n. 4, 2012.

ROSA, D. D. *et al.* Milk kefir: nutritional, microbiological and health benefits. **Nutrition Research Reviews**., v. 30, n. 1, p. 82-96, 2017.

ROSBERG-CODY, E. *et al.* Recombinant lactobacilli expressing linoleic acid isomerase can modulate the fatty acid composition of host adipose tissue in mice. **Microbiology-SGM**., v. 157, p. 609-615, 2011.

RUIZ, J. P. A.; ALONZO, M. W.; PERTÍÑEZ, M. D. Conjugated linoleic acid of dairy foods is affected by cows' feeding system and processing of milk. **Scientia Agricola**., v. 73, n. 2, p. 103-108, 2016.

RULE, D. C. *et al.* Comparison of muscle fatty acid profiles and cholesterol concentrations of bison, beef cattle, elk, and chicken. **Journal of Animal Science**., v.80, p. 1202-1211, 2002.

SABA, F. Downregulation of inflammatory markers by conjugated linoleic acid isomers in human cultured astrocytes. **Nutritional Neuroscience**., v. 22, n. 3, p. 207-214, 2019.

SAMAH, S. *et al.* Probiotics for the management of type 2 diabetes mellitus: A systematic review and meta-analysis. **Diabetes Research and Clinical Practice**., v. 118, p. 172-182, 2016.

SALSINHA, A. S. *et al.* Microbial production of conjugated linoleic acid and conjugated linolenic acid relies on a multienzymatic system. **Microbiology and Molecular Biology Reviews**., v. 82, n. 4, 2018.

SCRASCIA, M. *et al.* Susceptibility to rifaximin of Vibriocholerae strains from diferente geographical areas. **Journal of Antimicrobial Chemoterapy**., v. 52, p. 303-305, 2003.

SERAFEIMIDOU, A. *et al.* Chemical characteristics, fatty acid composition and conjugated linoleic acid (CLA) content of traditional Greek yogurts. **Food Chemistry,** v. 134, p. 1839-1846, 2012.

SHANTHA, N. C.; CRUM, A. D.; DECKER, E.A. Evaluation of conjugated linoleic acid concentrations in cooked beef. **Journal of Agricultural and Food Chemistry**., v. 42, p. 1757–1760, 1994.

SHINGFIELD, K. J.; WALLACE, R. J. Synthesis of Conjugated Linoleic Acid in Ruminants and Humans. **Conjugated Linoleic Acids and Conjugated Vegetable Oils**., Chapter 1, pp. 1-65, 2014.

SIKORSKI, A. M.; HEBERT, N.; SWAIN, R. A. Conjugated linoleic acid (CLA) inhibits new vessel growth in the mammalian brain. **Brain Research**., v. 1213, p. 35-40, 2008.

SILVA-RAMÍREZ, A. S. *et al.* Synthesis of conjugated linoleic acid by microwave-assisted alkali isomerization using propylene glycol as solvent. **European Journal of Lipid Science and Technology**., v. 119, n.4, p. 1600079, 2016.

SIMON, E. *et al.* Trans-10, cis-12 conjugated linoleic acid prevents adiposity but not insulin resistance induced by an atherogenic diet in hamsters. **Journal of Nutrition Biochemistry**., v. 17, n. 2, p. 126-131, 2006.

SNAEBJORNSSON, M. T.; JANAKI-RAMAN, S.; SCHULZE, A. Greasing the wheels of the cancer machine: the role of lipid metabolism in cancer. **Cell metabolismo**., v. 31, n. 1, p. 62-76, 2020.

SIURANA, A.; CALSAMIGLIA, S. A metaanalysis of feeding strategies to increase the content of conjugated linoleic acid (CLA) in dairy cattle milk and the impact on daily human consumption. **Animal Feed Science and Technology**., v. 217, p. 13-26, 2016.

SOARES, J. K. B. *et al.* Anxiety behavior is reduced, and physical growth is improved in the progeny of rat dams that consumed lipids from goat milk: An elevated plus maze analysis. **Neuroscience Letters**., v. 552, p. 25-29, 2013.

SOARES, J. K. B. *et al.* Conjugated linoleic acid in the maternal diet differentially enhances growth and cortical spreading depression in the rat progeny. **Biochimica et Biophysica Acta**., v. 1820, p. 1490-1495, 2012.

SOFI, F. *et al*. Effects of a dairy product (pecorino cheese). naturally rich in cis-9, trans-11 conjugated linoleic acid on lipid, inflammatory and haemorheological variables: A dietary intervention study. **Nutrition, Metabolism & Cardiovascular Diseases**., v. 20, p. 117-124, 2010.

STANTON, C. *et al.* Animal feeding strategies for conjugated linoleic acid enrichment of milk. In: Adlof, R.O., Christie, W.W., Sébédio, J.L., editors. **Advances in Conjugated Linoleic Acid Research**., v. 2, pp. 123-145, 2003.

STAWARSKA, A. The effect of Conjugated Linoleic Acids (CLA) supplementation on the activity of enzymes participating in the formation of arachidonic acid in liver microsomes of rats-Probable mechanism of CLA anticancer activity. **Nutrition and câncer**., v. 67, n. 1, p. 145-155, 2015.

SULLIVAN, M. P. *et al.* The gerbil, hamster, and guinea pig as rodent models for hyperlipidemia. **Laboratory Animal Science**., v. 43, p. 575-578, 1993.

TAYLOR, J. S. W. Conjugated linoleic acid impairs endothelial function. **Arteriosclerosis, Thrombosis, and Vascular Biology**., v. 26, n. 2, p. 307-312, 2005.

THOM, E.; WADSTEIN, J.; GUDMUNDSEN, O. Conjugated linoleic acid reduces body fat in healthy exercising humans. **Journal of International Medical Research**., v. 29, n. 5, p. 392-396, 2001.

VIEIRA, C. P. *et al.* Kefir grains change fatty acid profile of milk during fermentation and storage. **PlosOne.**, v. 10, n. 10, 2015.

VIEIRA, C. P. *et al. Lactococcus lactis* ssp. *cremoris* MRS47, a potential probiotic strain isolated from kefir grains, increases cis-9, trans-11-CLA and PUFA contents in fermented milk. **Journal of Functional Foods**., v. 31, p. 172-178, 2017.

VIEIRA, C. P. **Bioactive compounds in kefir and their beneficial effects on human health. Kefir: Nutrition, Consumption and Health Benefits.** Nova York: Nova Science Publishers, v. 1, p. 1-131, 2020.

VILAMIDOU, H.; BASSAGANYA-RIERA. Modulation of inflammation and immunity by dietary conjugated linoleic acid. **Eupean Journal of Pharmacology**., v. 785, p. 87-89, 2015.

WANG, Y. M. *et al.* Isomer-specific anti-obese and hypolipidemic properties of conjugated linoleic acid in obese OLETF rats. **Bioscience, Biotechnology, and Biochemistry**., v.70, p. 355-362, 2006.

WANNAMETHEE, S. G. *et al.* Serum Conjugated Linoleic Acid and Risk of Incident Heart Failure in Older Men: The British Regional Heart Study. **Journal of the American Heart Association**., v. 7, n.1, 2018.

WEST, D. B. *et al.* Conjugated linoleic acid persistently increases total energy expenditure in AKR/J mice without increasing uncoupling protein gene expression. **Journal of Nutrition**., v. 130, n. 10, p. 2471-2477, 2000.

WEST, D. B. *et al.* Effects of conjugated linoleic acid on body fat and energy metabolism in the mouse. **American Journal of Physiology**., v. 275, n. 3, p. R667-R672, 1998.

WHIGHAM, L. D. 2007. Efficacy of conjugated linoleic acid for reducing fat mass: a meta-analysis in humans. **The American Journal of Clinical Nutrition**., v. 85, p. 1203-1211, 2007.

WOHLFERT, E. A. *et al.* Peroxisome proliferator-activated receptor gamma (PPARgamma) and immunore gulation: enhancement of regulatory T cells through PPARgamma-dependent and –independent mechanisms. **Journal of Immunology**., v. 178, p. 4129-4135, 2007.

WONG, J. Y.; KIM, Y.; PARK, Y. Isomer specificity of conjugated linoleic acid on suppression of osteosarcomas. **Journal of Nature and Science**., v. 1, n. 4, p. 67, 2015.

XIE, J.; ITZKOWITZ, S. H. Cancer ininflammatory bowel disease. **World Journal of Gastroenterology**., v. 14, p. 378-389, 2008.

XIE, W. *et al*. MicroRNA-27 Prevents Atherosclerosis by Suppressing Lipoprotein Lipase-Induced Lipid Accumulation and Inflammatory Response in Apolipoprotein E Knockout Mice. **PlosOne**., v. 11, n.6, p. e0157085, 2016.

YANG, B. *et al*. Synthesis of conjugated linoleic acid by the linoleate isomerase complex in food-derived lactobacilli. **Journal of Applied Microbiology**., v. 117, n, 2, p. 430-439, 2014.

YANG, J. *et al.* Effect of conjugated linoleic acid on blood pressure: a meta-analysis of randomized, double-blind placebo-controlled trials. **Lipids in Health and Disease**., v. 14, n. 11, p. 1-6, 2015.

YANG, M.; COOK, M. E. Dietary conjugated linoleic acid decreased cachexia, macrophage tumor necrosis factor-alpha production, and modifies splenocyte cytokines production. **Experimental Biology and Medicine**., v. 228, p. 51-58, 2003.

YEGANEH, A.; ZAHRADKA, P.; TAYLOR, C. G. Trans-10, cis-12 conjugated linoleic acid (t10-c12 CLA) treatment and caloric restriction differentially affect adipocyte cell turnover in obese and lean mice. **The Journal of Nutritional Biochemistry**., v. 49, p. 123-132, 2017.

YU, L.; ADAMS, D.; WATKINS, B. A. Comparison of commercial supplements containing conjugated linoleic acids. **Journal of Food Composition and Analysis**., v. 16, p. 419-428, 2003.

ZHAO, W. S. Conjugated linoleic acid supplementation enhances antihypertensive effect of ramipril in chinese patients with obesity-related hypertension. **American Journal of Hypertension**., v. 22, p. 680-686, 2009.

8 ATTACHMENT A – PRESENTED AND PUBLISHED SUMMARIES IN CONGRESS

Anti-neuroinflammatory and neuroprotective effects of CLA in LPS-activated microglial cells

Vieira[1], C.P.; Neto[2], V.V.de A.; Rosa[3], B.G.; Cardoso[4], L.M. da F.; Almeida[5], M.R.; Albuquerque[5], L.H.P.; Pinheiro[6], L.P; Silva[7], P.F.; Foguel[7], D.; Alves[4], L.A.; Romão[3], L.F.; Lima[3], F.R.S.; Mariante[6], R.M.; De Luca[8], P.M.; Antas[5], P.R.Z.; Conte-Júnior[1], C.A

[1]Laboratório de Análises Avançadas em Bioquímica e Biologia Molecular, Instituto de Química, Universidade Federal do Rio de Janeiro

[2]Laboratório de Bioquímica de Tripanosomatídeos, Instituto Oswaldo Cruz, Rio de Janeiro

[3]Laboratório de Biologia das Células Gliais, Instituto de Ciências Biomédicas, Universidade Federal do Rio de Janeiro

[4]Laboratório de Comunicação Celular, Instituto Oswaldo Cruz, Rio de Janeiro

[5]Laboratório de Imunologia Clínica, Instituto Oswaldo Cruz, Rio de Janeiro

[6]Laboratório de Biologia Estrutural, Instituto Oswaldo Cruz, Rio de Janeiro

[7]Laboratório de Agregação de Proteínas e Amiloidoses, Instituto de Bioquímica Médica, Universidade Federal do Rio de Janeiro

[8]Laboratório de Imunoparasitologia, Instituto Oswaldo Cruz, Rio de Janeiro

Keywords: conjugated linoleic acid; bioactive compound; neurological disorders

Abstract: Neuroinflammatory processes influence most neurological disorders, including Alzheimer disease and Parkinon´s disease. During their progression, microglia becomes neurotoxic through the production of nitric oxide (NO) and proinflammatory cytokines. Currently, pharmacological treatments fail to halt the progression of brain disorders and immunoactive treatments has been seen as promising. Conjugated linoleic acid (CLA) is a mixture of geometric and positional isomers of linoleic acid. Animal and human studies with CLA-rich diets have shown CLA as potent anti-inflammatory candidate. However, there are few reports on the CLA effect in central nervous system. This study investigated the anti-neuroinflammatory and neuroprotective effects of CLA in microglial cells activated by LPS (1 μg/mL). Cell viability was evaluated by MTT reduction, flow cytometry and live/dead assays. The NO secretion was measured by HPLC and Griess diazotization methods. Cytokines quantifications were determined using sandwich ELISA kits. Arginase activity was measured by spectrophotometric method. CLA (60 μM) reduced production of proinflammatory cytokine IL-1β, and at 100 μM increased the secretion of immunosuppressive cytokines IL-4 and IL-10. CLA had no effect to the levels of NO in BV2 cell-line. However, CLA at 100 μM reduced NO release (up to 26.72%) in n9 cell-line. For primary microglia, effects of NO reduction by CLA were more dramatic, with levels reduced (up to 53.65%) similar to control levels in all CLA concentrations tested. Treatments with CLA showed significant correlation between arginase activity and NO levels both for BV2 ($r = -0.820$; $p=0.008$) as for n9 ($r = -0.717$; $p=0.037$). This indicates that CLA primes microglia to drive into a M2 phenotype (neuroprotection). Consistently, co-treatment with CLA and LPS completely prevented neuronal death induced by the endotoxin: CLA increased neuron viability from 53.31% (LPS- induced neuron death) to 100.91%. Altogether, these results suggest that CLA has potential as anti-inflammatory and neuroprotective agents against microglia-mediated neuroinflammatory disorders.

Financing Bodies: CNPq; CAPES; FIOCRUZ and FAPERJ.